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13. ABSTRACT *(Maximum 200 words)*

Epidemiological studies have suggested that phytochemicals, genistein and daidzein are the anti-cancer agents found in Asian diets contribute to the decreased breast cancer risk. In the current study, we investigated the molecular mechanism by which genistein inhibit cell growth and induce apoptosis. Our data show that genistein can induce G2/M cell cycle arrest, dose-dependent cell growth inhibition, down-regulation of Bcl-2 and cyclin B, and up-regulation of p16 and $p21^{WAF-1}$ at low concentration (15-30μM) in MCF-7 breast cancer cells. In contrast, the effect of genistein on MCF-10A normal breast epithelial cells was significantly different than MCF-7 or MCF-10AneoT cells. The cell cycle arrest, cell growth inhibitory effect, and up-regulation of p21 were much more pronounced in MCF-10AneoT compared to MCF-10A cells. We could not find any change on the levels of p53 or Bcl-2 in MCF-10A cells, whereas, MCF-10AneoT showed up-regulation of p53. We also observed down-regulation of cyclin B1 (CycB1) in both cell lines, similar to those found in MCF-7 breast cancer cells. In addition, we could not find any evidence of apoptosis in genistein treated MCF-10A and MCF-10AneoT cells compared to MCF-7 cells where apoptosis was observed. The significance of these results will further be explored by additional studies as planned in our proposal.

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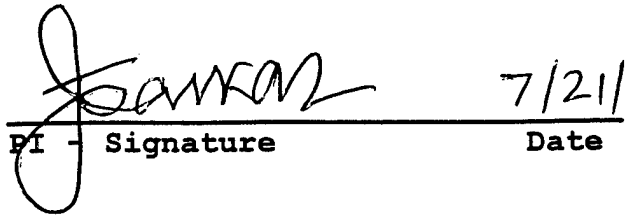

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INTRODUCTION:

The hypothesis to be tested in this proposal was that soy metabolites selectively eliminate aberrant breast epithelial cells (tumor cells) by altering the expression of specific cell cycle regulatory genes which, in turn, causes cell cycle arrest and induces apoptosis.

Tumors often occur as a result of uncontrolled and deregulated cell cycle caused by an imbalance of CKIs and CDKs. Current knowledge dictates that the deregulation of many classes of genes are involved in tumorigenesis, such as oncoproteins, tumor suppressors, signaling pathways, growth factors or cytokines, apoptosis inducers and potentiators (1). Protectors and promoters of apoptosis interact with each other, and the outcome of this interaction, apoptosis or survival, depends on the ratio of the death promoters to the death suppressors (2). These apoptosis regulators are part of and interact with the cell cycle machinery. Therefore, the purpose of this study is to explore the mechanism of the soy metabolites, genistein and daidzein, in breast epithelial cells by examining any changes in the levels of cell cycle regulatory proteins induced or inhibited by these metabolites. We will investigate whether the observed changes in specific gene expression are consistent with apoptosis in breast cancer cells. All results will be generated with MCF-7 breast cancer cells as well as with a series of non-transformed and non-tumorigenic (MCF-10A) and ras-transformed tumorigenic MCF-10AneoT breast epithelial cell lines (3-7), in addition to Bcl-2 transfected MCF-10A cells (8). The data generated through this proposal should provide a precise molecular picture of the apoptotic mechanism(s) leading to breast cancer cell death, as well as the molecular mechanism of the soy metabolites, genistein and daidzein. Since genistein and daidzein are structurally quite similar, yet differ in their known biological activities (such as differences in tyrosine kinase inhibitory activity and differences in cell cycle arrest), use of both compounds in our planned experiments together should yield information that will aid in the elucidation of their molecular mechanisms for the anti-proliferative and anti-tumor activities of isoflavones. **Thus, the purpose of this study is to elucidate the molecular mechanism(s) by which soy metabolites exert their biological effects in non-tumorigenic and tumorigenic breast epithelial cells.**

BODY:

The following section describes the progress to-date on our project. These results are comprehensive and provide experimental methods, results and discussion in relation to the statement of work as proposed.

(i). ***Data on MCF-7 breast cancer cell line:*** Our preliminary data demonstrated that genistein can inhibit proliferation of a human breast cancer cell line–MCF-7 in a dose dependent manner when exposed for four days in culture with different concentrations of genistein (presented as preliminary results in the application). Genistein concentrations of 5, 15, 25 and 50μM clearly demonstrated progressive growth inhibition and that at concentrations of 25 to 50μM was found to be optimal when cells were exposed for 3 days in culture. Similar results were also reported in the literature (9, 10), however, our data suggest an ID_{50} of about 30μM. We, therefore, decided to use 30μM of genistein for subsequent experiments. The evidence for genistein inducing apoptosis was based on the results of DNA laddering observed in MCF-7 cells treated with genistein, however, in contrast to our results, Pagliacci, et al. found no evidence of DNA laddering, even though they showed apoptosis (9). Genistein treatment of MCF-7 cells showed G2/M cell cycle arrest, as shown previously by other investigators. In addition, genistein treatment also altered the expression of several cell cycle regulatory proteins, such as down-regulation of Bcl-2 and cyclin B, and up-regulation of p16 and $p21^{WAF-1}$ (data not shown). Similar results were observed with 15μM of genistein, indicating that genistein at 15 to 30μM is sufficient to elicit biological and molecular responses in breast cancer cells. Similar results were

also obtained from daidzein–treated MCF-7 cells (data not shown). Our results support published observations and further extended to the molecular alterations observed in MCF-7 cells. These results clearly indicate the selective effect of genistein in the expression of cell cycle regulatory proteins at concentrations that are quite similar to the physiological concentration of genistein. It is important to note that the effective concentrations of genistein used by many investigators, including us, are different than those found in human samples *in vivo*. The reported *in vivo* concentrations of genistein in the plasma of humans consuming a soy rich diet varies considerably (276 nM to 4μM) and that humans are exposed chronically to genistein, whereas *in vitro* results are obtained with cells exposed to genistein for a short period of time. Therefore, it is not scientifically valid to correlate effective concentrations of genistein between *in vitro* and *in vivo*, even though these concentrations are not drastically different. In summary, our MCF-7 cell data showed that genistein can inhibit cell growth, cell cycle arrest, modulate genes and induce apoptosis. These results are different than those obtained from MCF 10A cells as detailed below.

Thus, at the molecular level, our preliminary data suggest that genistein may trigger or induce apoptosis via the regulation of cell cycle regulatory proteins in breast cancer cells. Bcl-2 expression suppresses apoptotic signals, thereby protecting cells from apoptosis. Expression of Bcl-2 is characteristic of rapidly dividing cells which are differentiating into mature components, while Bcl-2's down-regulation seems to occur during tissue differentiation and is coupled to terminal differentiation of epithelial tissues (8). In other words, Bcl-2 expression declines in cells as they mature or at stages where cells may be eliminated. The regulation of these genes are relevant to cancer since carcinogenesis can be viewed as a series of clonal selections and alterations in cell cycle regulatory molecules.

It is apparent that both estrogen and genistein may exert potent biological effect on Bcl–2 gene expression, cell proliferation and cell differentiation. *In vivo*, genistein's putative ability to down-regulate Bcl-2 may be enhanced by its negative effect on estradiol. Estradiol treatment of MCF-7 cells increases Bcl-2 mRNA and protects MCF-7 cells against apoptosis (11). Since genistein down-regulates estradiol, it helps keep Bcl-2 at the lowest level. However, our results suggest that in the absence of estrogen, genistein may have a direct effect on the apoptotic pathway by down-regulating Bcl–2 expression *in vivo* and that these results warrant further in-depth investigations.

In summary, we have generated substantial amount of data using MCF-7 cells. Similar experiments will be done using other breast cancer cell lines, and the results from these experiments will be complied for a manuscript within the next year.

(ii). ***Data on MCF-10A cell lines*:** In contrast to MCF-7 cells, the effect of genistein on MCF-10A (normal breast epithelial cells, see references, 36-40) was significantly different. Cell growth inhibition of MCF-10A (considered normal) and MCF-10AneoT (preneoplastic) is shown in figure-1, which clearly show a differential effect in these cells. The effect was much more pronounced in MCF-10AneoT compared to MCF-10A using low concentrations of genistein. However, at high concentration (>45μM), both cell lines showed similar growth inhibition. Treatment of MCF-10AneoT with genistein showed drastically different effects on cellular morphology compared to MCF-10A (figure 2). It is also important to note that the morphology of the MCF-10AneoT is different than that of MCF-10A without treatment. In addition, there was a significant difference in genistein's ability to arrest cells at G2/M between MCF-10AneoT and MCF-10A as shown in figure-3 (41% vs 17% of cells in G2/M respectively). These results suggest that the Ras-transformed MCF-10A is more susceptible to the overall growth arrest at lower concentration (<30μM) of genistein by inducing G2/M cell cycle arrest. At the molecular level, the up-regulation of the p21 gene was observed in both cell lines at 90μM, however the

level of induction was much more pronounced in MCF-10AneoT even at lower concentration (30μM) of genistein (figure-4). In addition, our results show that there was no effect on the levels of p53 in MCF-10A cells, whereas, MCF-10AneoT showed up-regulation of p53 (figure-5). It is interesting to note that the endogenous levels of p21 and p53 was drastically reduced in Ras-transformed MCF-10A cells. This reduction in the endogenous levels of both p53 and p21 may indicate transformed phenotype of MCF-10AneoT, however, the precise molecular mechanism remains to be elucidated. We also observed down-regulation of cyclin B1 (CycB1) as shown in figure-6 in both cell lines, similar to those found in MCF-7 breast cancer cells. The level of CycB1 in control culture for 72 hours time point was found to be lower compared to the 48 hours time point. This may represent experimental variations, which will be investigated thoroughly during our experiments as proposed in this application. It is important to note that there was no effect of genistein in the up-regulation of p16 in MCF-10A or MCF-10AneoT compared to MCF-7 breast cancer cells as shown previously. In addition, we could not find any evidence of apoptosis, such as DNA laddering or PARP degradation when MCF-10A and MCF-10AneoT cells were exposed to genistein compared to MCF-7 cells where both DNA laddering (data not shown) and PARP degradation were observed (fig-7) at 72 hours. Similarly, we could not find any alterations in Bcl-2 expression in genistein-treated MCF-10A or MCF-10AneoT cells (data not shown). However, these experiments will be repeated with longer period of exposure as planned in this proposal. In addition, further studies using MCF-10AneoT derived cell lines with increased tumorigenic potential will determine whether Bcl-2 and apoptosis plays any role in this model system.

The above results are not unexpected, since previous studies have also shown no effect of genistein on normal mouse, NIH3T3 cells, but showed a significant effect on Ras–transformed NIH3T3 cells. Our results also suggest that genistein can inhibit the growth of transformed cells (Ras-transformed–MCF-10AneoT) only at low concentrations and have no significant effect on the growth inhibition of normal epithelial cells (MCF-10A) and that the modulation of gene expressions are specific with transformed phenotype. However, we still do not know the consequence of these differential molecular alterations found in these cell lines, justifying the need for further in-depth investigations in order to determine the precise molecular mechanism of isoflavones in breast carcinogenesis. Overall, these results supports our hypotheses that the biological effects of genistein are, perhaps, tumor cell specific and that the tumor cells are selectively growth arrested and undergo apoptotic cell death when treated with a low concentration of genistein, whereas, normal or preneoplastic cells do not undergo apoptosis even though they are differentially growth arrested. These preliminary results strongly justify the use of MCF-10A model cell lines for our planned experiments as outlined in this revised proposal.

In summary, we have shown that genistein has no significant growth inhibitory activity at low concentration on normal breast epithelial cell line, MCF-10A, but has a significant effect on Ras–transformed MCF-10A cells (MCF-10AneoT). Both, MCF-10A and MCF-10AneoT showed up-regulation of p21, but the levels of induction was much more pronounced in Ras-transformed cells. In addition, we found down-regulation of CycB1in both cell lines, however, there was a differential effect at the levels of p53 induction only found in MCF-10AneoT cells. Further studies are planned using several transplant generations of MCF-10AneoT, such as MCF-10AneoTG1, TG2 and TG3 which show progressive growth of carcinoma in nude mice. Thus, these model cell lines should provide critical data correlating the growth inhibitory effects of genistein and daidzein with progression of normal epithelial cells to carcinoma and its association with the modulation in gene expression. These results should firmly establish the much needed information regarding the molecular mechanism of action of the soy metabolites, genistein and daidzein in the chemoprevention of breast cancer. In addition, this model should also provide an unique *in vivo* model to test the chemopreventive role of dietary soy in breast

cancer development and progression in future studies.

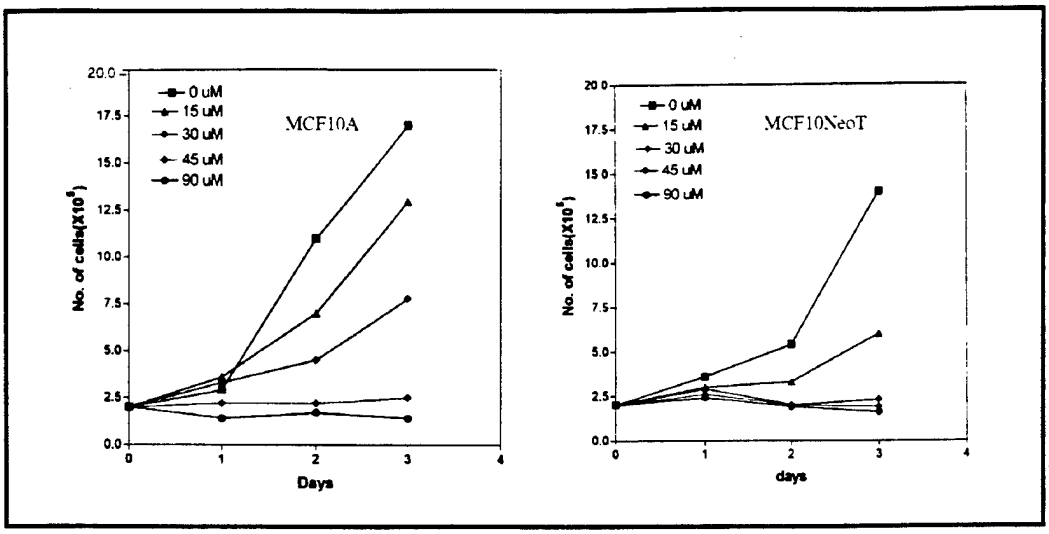


Figure-1: Cell growth inhibition by genistein. Cells were seeded in 24 well culture plates 24 hours prior to the addition of genistein. The cells were harvested by trypsinization and the viable cells were counted using as described under experimental design and methods.

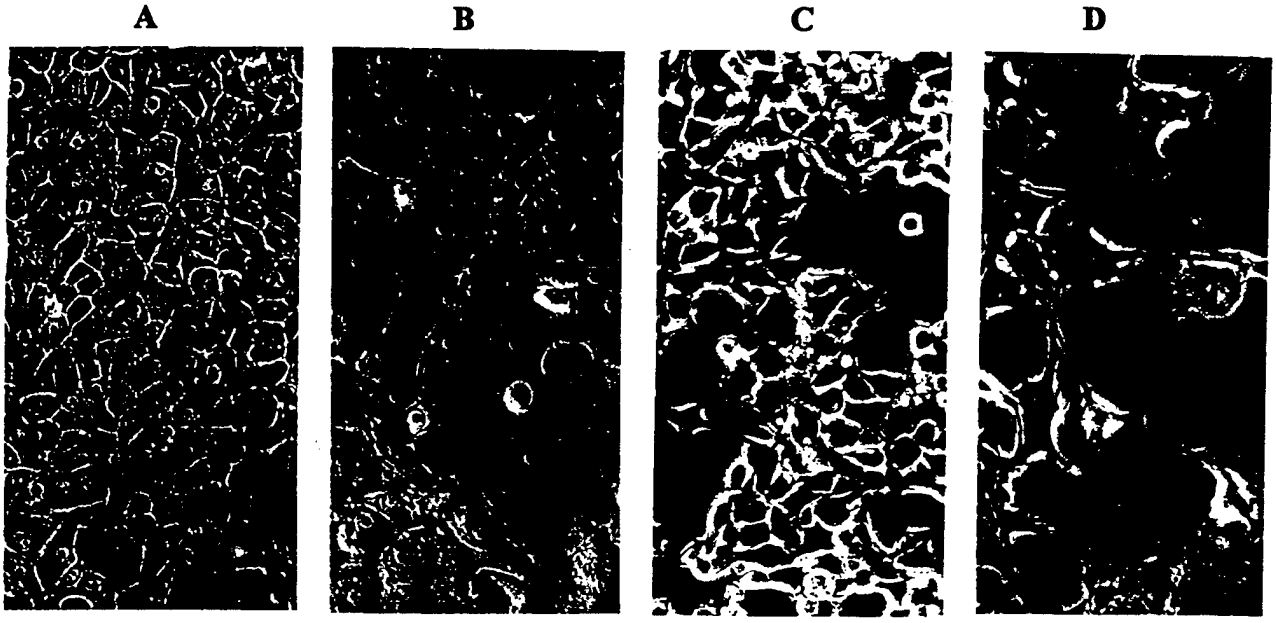


Figure-2: Photomicrographs showing cellular morphology of untreated (A and C) and 48 hours of 30μM genistein-treated (B and D) MCF-10A and MCF-10AneoT cells respectively.

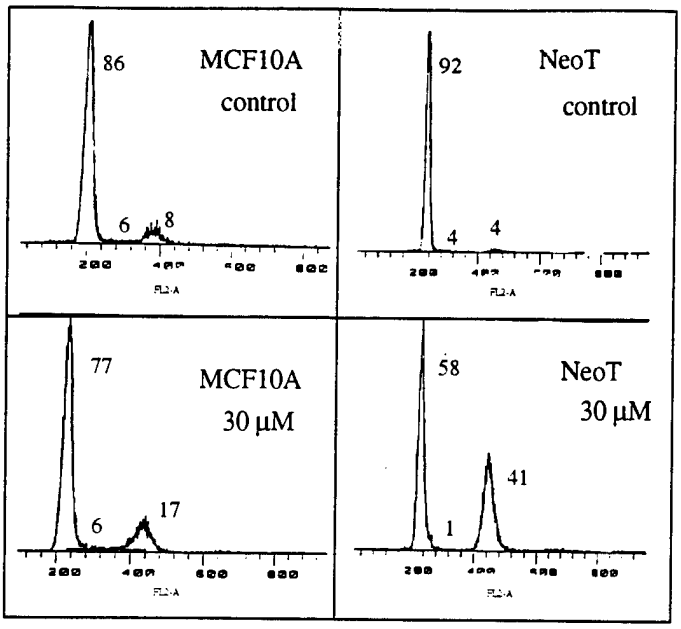


Figure-3: Propidium iodide stained flow cytometric analysis of untreated and 48 hours of 30µM genistein-treated cells showing significant and differential G2/M arrest in MCF-10AneoT compared to MCF-10A cells.

Figure-4

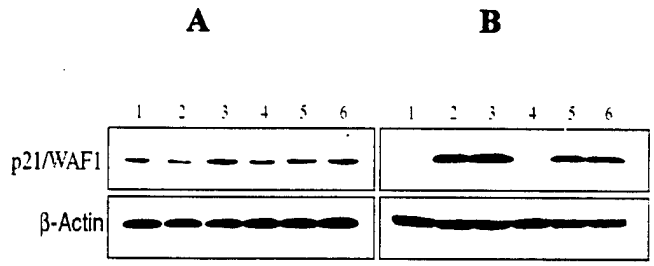


Figure-5

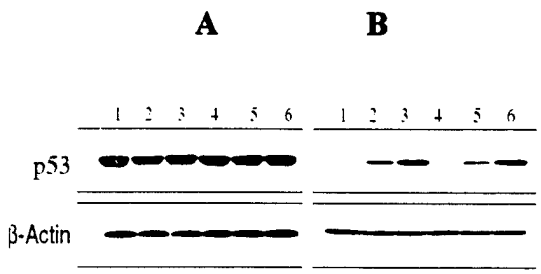


Figure-6

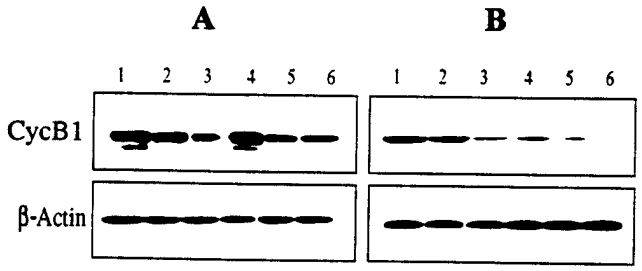
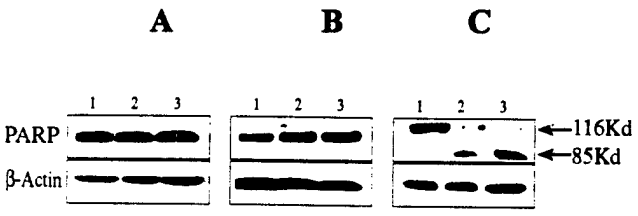


Figure-7



Legend for figures 4 (p21), 5 (p53), 6 (CycB1) and 7 (PARP): Western blot analysis of cell extracts form untreated (lanes 1 and 4), 30µM (lanes 2 and 5) and 90µM (lanes 3 and 6) genistein treated MCF-10A (panel A) and MCF-10AneoT (panel B) for 48 hours (lanes 1, 2 and 3) and 72 hours (lanes 4, 5 and 6) respectively. Figure-7 show the degradation of PARP in MCF-7 cells at both 30µM (lane-2) and 90µM (lane-3) at 72 hours (panel C), whereas no degradation was observed in MCF-10A (panel A) or MCF-10AneoT (panel B).

Experimental Details:

Many of the following experimental procedures have already been applied to generate the data as presented in this annual report. In addition, many experiments are currently been conducted as detailed below:

Cell growth and cell cycle analysis: The effects of genistein and daidzein on the proliferation of breast cancer cells and MCF-10A and MCF-10AneoT cell series, including several transplant generations are generated by constructing growth curves in the presence and absence of these isoflavones in the culture media at different concentrations and exposed for different time points (days in culture) following the standard cell counting method. **Isoflavones are dissolved in 0.1M sodium carbonate at 1mM stock and are used for all experiments. Control culture include a similar concentration of sodium carbonate for all experiments.**

From these growth curves, an approximate ID_{50} are determined for each compound with each cell line. These experiments will be repeated with other breast cancer cell lines as indicated under statement of work. Similarly the ID_{50} will also be determined for combinations (specific aim-2c). MCF-10A and various Ras-transformed MCF-10A clones with varied tumorigenic potential, and a stably **Bcl-2 transfected MCF-10A** (overexpresses Bcl-2 which inhibits apoptosis initiated by multiple routes) will be used for the above mentioned experiments in our subsequent experiments. Growth curves will be constructed using all cell lines with and without the treatment of genistein and daidzein.

All the cell lines with and without genistein and daidzein treatment will also be subjected to routine flow cytometric analysis using FACS-Scan (Beckton/Dickinson) automated flow cytometer. The distribution of cells in G0/G1, S and G2/M will be calculated and the percent cells in each phase of the cell cycle will be determined to compare untreated control to both genistein and daidzein treated cells as demonstrated in this report.

Flow cytommetry: Cells are seeded at a density of 5×10^5 per well in 6 well dishes. After 24 hours, the cells are treated with or without 50 μM genistein or daidzein and harvested at various time periods by trypsinization. The cells are centrifuged at 2000 rpm for 5 min, washed in PBS, and resuspended in cold 70% ethanol. The cells are then subjected to flow cytometric analysis on FACStar Plus (Becton Dickinson) after propidium iodide labeling.

Western blotting: Control as well as increasing doses of genistein or daidzein treated cells are harvested by scraping the cells from cultured dishes using a cell scraper and collected by centrifugation. Whole-cell extracts are then prepared by lysing the cells using 4% SDS-Gel sample buffer at a volume of 1×10^6 cells/μl. Protein concentration is measured using protein assay reagents (Pierce, IL). Cell extracts are boiled for 10 minutes and chilled on ice, subjected to 14% SDS-PAGE analysis, and electrophoretically transferred to nitrocellulose membrane. Each membrane is incubated with either Cyclin A, Cyclin B, CDK1, CDK2 antibodies (Neomarkers) or $p21^{WAF1}$, beta-actin (used as a control for protein loading) antibodies obtained from Oncogene Science (Cambridge, MA) and other primary antibodies. The membranes are washed and incubated with secondary antibody conjugated with peroxidase and the signal is detected using chemiluminescent detection system (Pierce, IL).

Determination of apoptotic cell death.

PARP Assay: Briefly, cells are washed twice with ice-cold PBS and harvested by scraping the cells with a rubber policeman in ice-cold PBS. Whole cell extraction buffer (WCE buffer,

beta-glycerophosphate, 5mM MgCl2, 1mM Na3VO4, 1mMDTT and double distilled water containing protease inhibitors leupeptin, pepstain, aprotinin and PMSF) are added to the cell pellet and collected in an eppendorf tube. Samples are allowed to sit on ice for 30 min. The tubes are centrifuged at 4°C for 5 min. After spinning, supernatant are transferred to a new tube, the protein concentration are quantitated and the lysates are stored at -80°C. Proteins are then be subjected to Western blot analysis using 10% SDS-PAGE and transferred to nitrocellulose membranes. Targets are detected using ECL Western detection system, with mouse monoclonal anti-PARP as primary and HRP-conjugated Goat anti-mouse as secondary antibodies.

DNA laddering assay: Treated and untreated cultured cells are harvested and centrifuged at 2000 rpm for 5 minutes. The cells are re-suspended in lysis buffer (0.2% Triton X-100, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and centrifuged for 15 minutes at 13,800 x g. RNAse (0.5 mg/ml) is added to the supernatant followed by 20% SDS, 20 mg/ml proteinase K and 5 M NaCl. The DNA is extracted with an equal volume of phenol and then an equal volume of phenol/chloroform/isoamyl-alcohol (50:49:1 v/v). The DNA is precipitated with 100% cold ethanol and resuspended in TE buffer. The DNA is electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and detected by UV transilluminator and photographed.

TUNEL assay: Control and 30 μM genistein or daidzein treated cells are harvested at 72 and 96 hours by trypsinization and collected by centrifugation. Cells are washed twice in PBS/1% BSA and prepared for TUNEL assay per manufacturer's protocol (Boehringer Mannheim, CA). Briefly, cells are fixed for 30 min. in 4% paraformaldehyde, washed twice in PBS and then permeabilized in 0.1% Triton-X and 0.1% sodium citrate. Cells are labeled with terminal deoxynucleotidyl transferase (TdT) for 60 min. at 37°C in a dark humidified incubator. The samples are washed twice, resuspended in 500 μl of PBS and then analyzed on a FACscan (Becton Dickinson, CA). Data on 10,000 cells is acquired and processed using LYSII software (Becton Dickinson, CA).

Staining with 7 amino-actinomycin D (7-AAD): Control and 30 μM genistein treated cells are harvested at 72 and 96 hours. Cells are trypsinized, centrifuged and resuspended at 1 x 10^6 cells/ml. 7-AAD dissolved in acetone and diluted in PBS to 200 ng/ml is used. 100 μl of the working solution is added to each sample and incubated at 4°C in the dark for 20 min. The cells are pelleted by centrifugation and re-suspended in 500 μl of PBS. Cells boiled for 5 min. are used as a positive control and unstained cells are used as a negative control. Samples are analyzed on a FACscan (Becton Dickinson, CA). Data on 20,000 cells is acquired and processed using LYSII Software (Beckton, Dickinson, CA).

Conclusions:

The data generated during the first year is very significant in order to understand the molecular mechanism of action of genistein in MCF-7 and MCF-10A cells. First year was also very critical for the standardization of many techniques as indicated under experimental details. Since all of the necessary reagents and techniques are at hand, all the subsequent experiments will progress very rapidly. Collectively, it appears that genistein is rather selective under low concentration to cause the demise of cancer cells and that this data is very important in order to justify further chemoprevention studies using soy isoflavones. Our next several experiments using different breast cancer cells will also determine whether the effects of genistein is universal in all breast cancer cells. These results should firmly establish the much needed information regarding the molecular mechanism of action of the soy metabolites, genistein and daidzein in the chemoprevention of breast cancer.

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