Inhibitory Effect of Genistein and Daidzein on Ovarian Cancer Cell Growth

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Abstract. *Background: Survival from ovarian cancer has not changed significantly in the past twenty years requiring development of additional treatment protocols. We studied the effect of genistein and daidzein on ovarian cancer cell growth. Materials and Methods: Five ovarian cancer cell lines from Stage IIIC disease were evaluated. Sulforhodamine B and colony formation assays were used to analyze growth inhibitory effects of genistein and daidzein alone and with cisplatin, paclitaxel or topotecan. Apoptosis induction was studied by determining caspase-3 activity. Results: Inhibition of growth (50-80%), colony formation and colony size was seen at 144 µm of genistein, 0-23% reduction was demonstrated at 9 µm. At 144 µm, the colony size was inhibited >75%; at 9 µm 4/5 cell lines had >50% reduction. Caspase-3 activity was induced (0.10 to 0.56 pmol/min/µg protein) with all concentrations of genistein. Cisplatin (2-50 µg/ml) and topotecan (0.5-50.0 µm) combined with genistein resulted in a mostly additive effect, paclitaxel (8-200 nM) was slightly less than additive. Conclusion: We demonstrate an inhibitory effect of genistein on ovarian cancer cell growth.*

Despite dedicated research and increased understanding of the molecular defects associated with ovarian cancer, survival after diagnosis has changed little. Ovarian cancer remains the most dreaded of the female genital tract cancers, without much to offer currently for early diagnosis or prevention. Recently, interest in specific foods for cancer chemoprevention has increased including retinoic acid, curcumin and the isoflavanoids (genistein, daidzein and biochanin A). The effects of these agents have been studied to various degrees in many different cancers, but their role in the prevention or treatment of female genital tract cancers has not been extensively reported.

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Genistein, a soy isoflavanoid, has been intensely studied in relation to breast cancer. Interest first arose upon discovery of the vast difference in breast cancer rates in Asia *versus* Western countries (1). Large dietary differences exist, especially in genistein consumption, as the average Asian intake is 20-80 mg/day whereas the average US intake is only 1-3 mg (2,3) The dietary and disease discrepancy prompted further study into the chemopreventive and potentially therapeutic properties of genistein.

Genistein has been found to inhibit cell proliferation, oncogenesis and clonogenic ability in animal and human cells (3-5). Several studies have been performed to evaluate the effects of genistein on breast cancer cells. Numerous cellular mechanisms are affected by genistein and include an up-regulation of Bax, and a down-regulation of Bcl-2 and p53 expression leading to induction of apoptosis (6). Another study examining the preventive aspects of genistein in breast cancer found a down-regulation of MMP-9, as well as an inhibition of angiogenesis (7). Decreased angiogenesis correlated with low levels of vascular endothelial growth factor and transforming growth factor-beta 1. An earlier study showed that the inhibition of breast cancer cells by genistein was independent of estrogen receptor status (8). Genistein has also been shown to inhibit tyrosine kinase activity as well as DNA topoisomerases I and II (9). These studies show that the mechanism of genistein's inhibition of cancer cell growth is multifactorial.

Study of genistein in genitourinary cancers has focused mainly on bladder cancer. A recent study measured urinary excretion of isoflavones and found them to correlate directly with intake (10). Results in bladder cancer confirm those of breast cancer, again showing induction of apoptosis and cell cycle arrest by soy isoflavones. A recent report provides data on the preventive effects of isoflavones, genistein and daidzein on estradiol-17α-related endometrial carcinogenesis in mice (11). Inhibition of one ovarian cancer cell line was reported with genistein by the same group of investigators in three different reports with varying outcomes (12-14). Inhibition of the growth of two cervical cancer lines was also reported recently, demonstrating differential effects resulting

in apoptosis and non-apoptosis related death by genistein only (15). Further evidence indicates that genistein causes a down-regulation of cdc2 kinase, and is the more potent of the isoflavones acting in both a cytostatic and cytotoxic manner, and that daidzein induces apoptosis (10).

Similar to breast cancer, differences also are seen in ovarian cancer rates among Asians and women of Western cultures suggesting that dietary differences may play a role as seen with breast cancer. Women of North American and European descent have the highest incidences of ovarian cancer while Japanese women have the lowest incidence (16). The knowledge of genistein's effects in genital cancers, however, is sparse. A study of the synergistic effects of quercetin and genistein in ovarian cancer did show greater growth inhibition when these agents were used together than alone, suggesting that genistein could have some role in treating or preventing ovarian cancer (12).

In this report, we demonstrate the growth inhibitory effect of genistein and daidzein on five recently-established ovarian cancer cell lines, as well as parameters involved in this inhibition. We also provide data on the action of genistein combined with the first-line chemotherapeutic agents, taxol, topotecan and cisplatin used in ovarian cancer treatment.

Materials and Methods

Cell culture. Cell lines were established from either the ascites or tumor samples of patients with ovarian cancer (Stage IIIC) at the time of initial diagnosis. In the case of ascites, the samples were spun down and cleared of red blood cells with Ficoll-paque gradient centrifugation. The cultures were established as monolayers and designated as cell lines after 30 passages. If cultures were contaminated with fibroblasts, they were discarded. Tumor samples were mechanically disrupted and cultured. Same passage conditions were applied to tumor-derived cultures. Cells were cultured in RPMI supplemented with 10% FBS at 37˚C in a CO_2 incubator. Charcoal/dextran-treated FBS was used in experiments with genistein and daidzein. All cell lines were shown to be estrogen receptor-positive by Western blot analysis.

Assay for colony forming ability. Ovarian cancer cell lines were plated at 3×10^2 /60-mm dishes. They were treated with various concentrations (9-144 µM) of genistein and daidzein. Cultures were incubated for 12 days. Tissue culture plates were fixed and stained with methylene blue/ethyl alcohol. Colonies (> 30 cells) were counted and diameters of a minimum of 50 colonies were determined.

Sulforhodamine B assay. All cytotoxicity experiments were performed twice in triplicate samples. Genistein and daidzein were made 7.4 mM in DMSO. Stock solutions of cisplatin (CDDP), 1 mg/ml in water; paclitaxel, 10 mM in DMSO (Sigma, St. Louis, MO, USA); and topotecan (kindly provided by Smith Kline Beecham Co.) 8 mM in saline were used in the experiments. Cell preparations from cultures in log-phase growth were removed from

the culture flask by trypsinization. Cells were added (5×10^3 - 1.0 $\times 10^4$ cells/well) to 96-well tissue culture plates in 100 µl of media and cultured overnight. One hundred microliters of various concentrations of genistein or daidzein were administered in complete media. Recovery experiments were designed to study the effect of incubation time on the growth inhibitory effect seen with genistein and daidzein. In these experiments, the cells were divided into four groups and incubated for a total of 72 hours, with the first group getting genistein or daidzein in the first 24 hours only followed by incubation in regular media, the second group for the first 48 hours, and the third group for the entire duration of the study. The fourth group consisted of the untreated controls.

Concentrations of the chemotherapeutic agents were 2 to 50 µg/ml for Cisplatin, 8 to 200 nM for paclitaxel and 0.5 to 50 µm for topotecan. Cisplatin, paclitaxel and topotecan treatments were for 2 hours. The supernatant was then aspirated and the cells were refed with complete media with or without genistein. The assay was terminated after 96 hours. Quantitation was performed by the sulforhodamine B cytotoxicity assay and read at 540 nm (17). Results are presented as expected (E)=%cytoxicity of genistein+%cytoxicity of CDDP, paclitaxel or topotecan, and actual (A)=% cytotoxicity observed with genistein+CDDP (or paclitaxel or topotecan).

Quantitation of caspase-3 activity. In order to assay for the induction of apoptosis, caspase-3 activity was measured by a kit designed to quantitate cell-associated enzyme induction (Biomol, Plymouth Meeting, PA, USA). Ovarian tumor cells in log-phase of growth were treated with genistein for 24 hours. Cells were lysed in 50mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA. The assay buffer consisted of 50 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol. The substrate was Ac-DEVD-pDNA, and the inhibitor was Ac-DEVD-CHO. Protein concentrations were determined by the BioRad assay. The assay was run in duplicate with appropriate controls including human recombinant caspase-3 as a positive control. Absorbance was read at 405 nm. Specific activity determinations are reported as pmol/min/µg protein.

Statistical analysis. Each data point was based on at least three independent experiments that were performed in triplicate (colony assays, caspase-3 determination, colony size) or quadruplicate (SRB assays). Statistical analyses were performed by Student's *t*-test.

Results

The effect of genistein on various parameters of ovarian tumor cell growth was studied. Sulforhodamine B assay was used to quantitate overall growth of ovarian cancer cells following 96 hours in the presence of genistein. A dose-dependent inhibition of cell growth was seen in all cell lines (Figure 1). Low doses of genistein (9 and 18 µM) resulted in 0-23 and 0-42% inhibition respectively. At 36 µM, the range of growth inhibition was 0-63%. At higher doses of genistein (72 µM), 6-64% and at the highest dose tested (144 µM) 50-80% growth inhibition was demonstrated. UL-8 cell line was the most resistant cell line with significant growth inhibition only at the highest doses of genistein. The most sensitive line
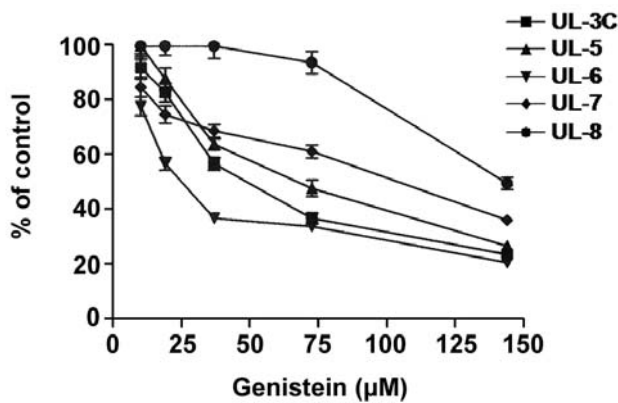
Figure 1. *Effect of genistein on ovarian tumor cell growth as determined by the SRB assay. Cell growth was quantiated following 4 days of incubation with genistein with the five ovarian cancer cell lines. Data are presented percent growth (± SEM) compared to untreated controls.*
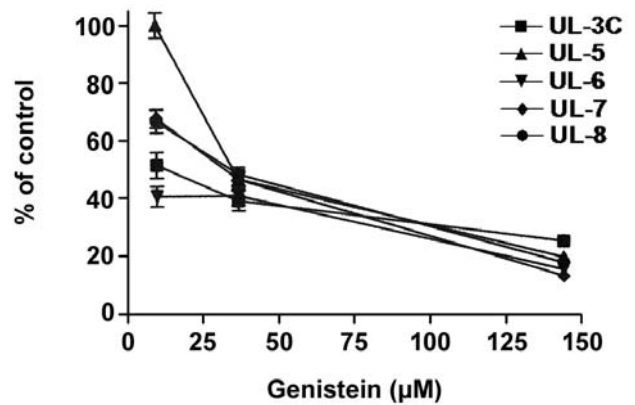

Figure 3. *Effect of genistein on colony size of ovarian cancer cells. Colony sizes were determined (50 colonies) with each treatment and cell line. Mean values were determined and normalized to untreated controls and results are presented with standard error of the mean.*
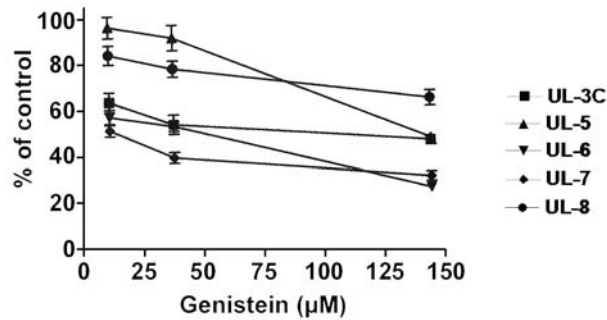

Figure 2. *Inhibition of colony formation by genistein. Ovarian cancer cells (3 X 10²) were grown in the continuous presence of genistein for 10-12 days. Colonies with a minimum of 30 cells were counted. Results are presented as percent colony counts compared to untreated controls (± SEM).*
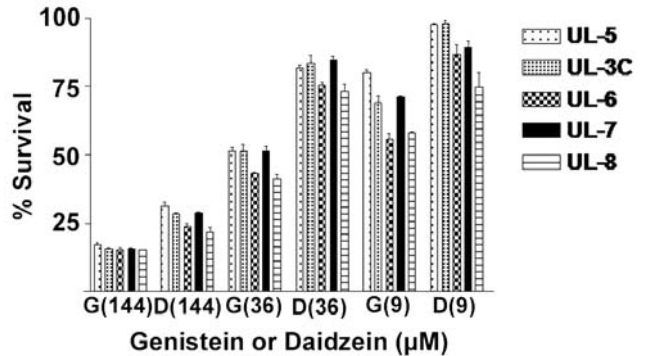

Figure 4. *Effect of daidzein on ovarian tumor cell growth. SRB assay was used to determine the inhibitory effect. Results are presented as % of control (untreated) and also include data from genistein for comparison.*

was UL-6 with significant inhibition at all concentrations. UL-3C, 5 and 7 had slightly different dose-response curves with inhibition at concentrations ranging from 18-144 μM.

We also determined the inhibitory effect of genistein on the colony forming ability of ovarian tumor cells (Figure 2). UL-6 and UL-7 cells were most sensitive to reduction in colony forming ability, with UL-8 again being the most resistant cell line. Reduction of colony forming ability of UL-8 cells was seen even at 9 μM, in contrast to growth inhibition experiments. For all cell lines, LD_{50} values were 27-148 μM for growth inhibition, and 15-150 μM for inhibition of colony formation. We also observed that colony size was significantly affected in all cell lines (Figure 3). At 36 μM and higher concentration of genistein, colony size was reduced to similar levels in all cell lines. Colony size was affected most consistently in all cell lines with genistein.

Growth inhibitory effect of daidzein in a 3-day assay on ovarian cancer cells was also studied at similar concentrations as genistein. There was significantly less growth inhibition by
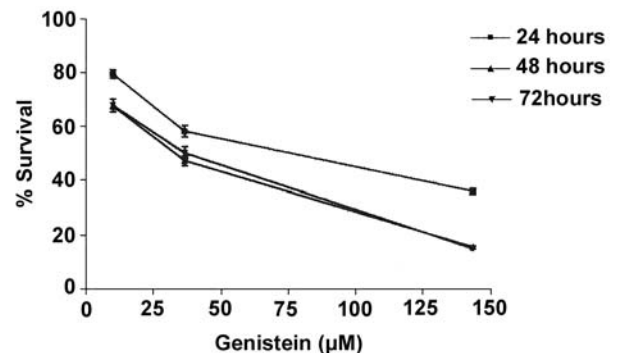

Figure 5. *Recovery of ovarian tumor cells from the inhibitory effect of genistein and daidzein. Cells were cultured for 24, 48 and 72 hours. Data are expressed as % survival ± SEM.*

daidzein than genistein in all cell lines. (Figure 4) Of interest, UL-6 was the most sensitive cell line and UL-8 was the most resistant to daidzein, similar to the effect of genistein.
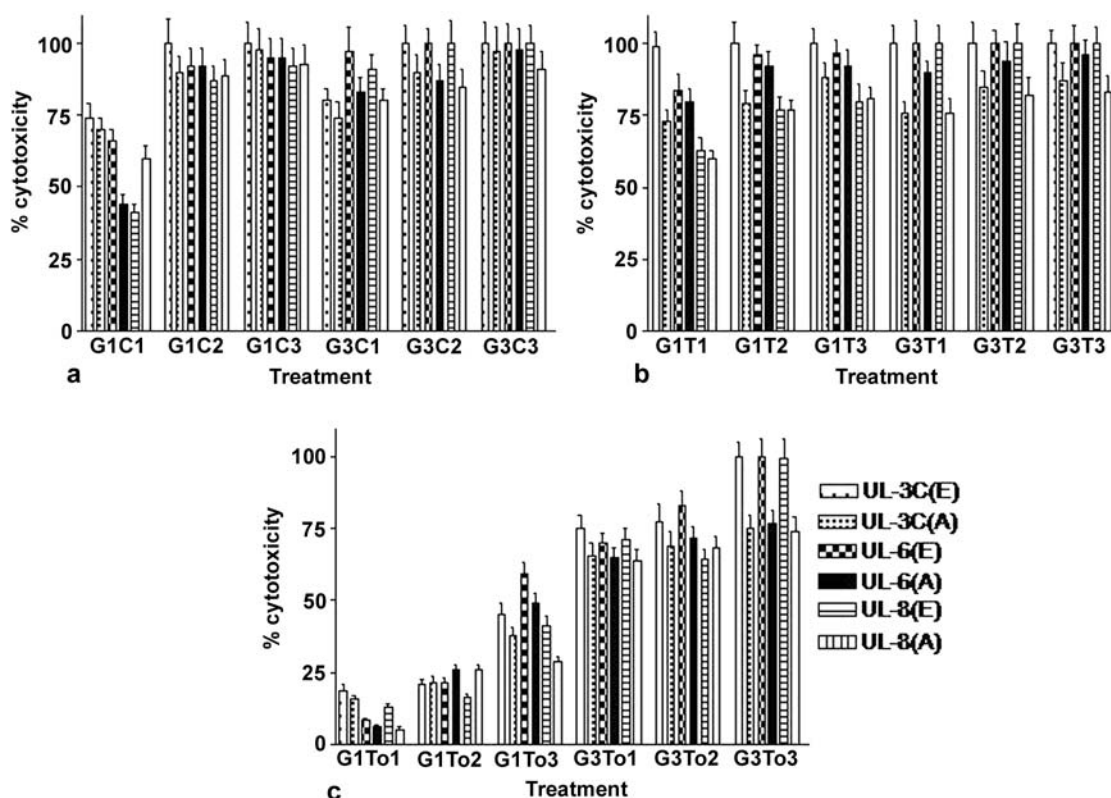
Figure 6. *Combined cytotoxic effect of genistein [G] with (a)cisplatin [C]; (b) paclitaxel [T]; and (c) topotecan [To] on ovarian tumor cells. After a two-hour treatment with the designated chemotherapeutic agent, cells were cultured with genistein or control media for 96 hours.*

The cytostatic and cytotoxic effects of genistein and daidzein were studied by determining the recovery of tumor cells from growth inhibition. Cell growth was quantitated after either 72 hours of incubation with genistein, 48 hours incubation followed by 24 hours in regular media, or 24 hours in genistein followed by 48 in regular media. Data from UL-3C cell line are presented, since data obtained from other cell lines were similar. Our results demonstrate that tumor cells recover from some of the inhibitory effect (cytostatic) of genistein after 24 hours of incubation, followed by growth in regular media (Figure 5). However, longer incubation periods of 48 and 72 hours result in the same amount of inhibition with no significant reversal of inhibitory activity. More recovery could potentially be observed if experiments included shorter periods of time of incubation with genistein. The effect of daidzein was also studied in the recovery experiments. In this case, no recovery was observed with the shortest incubation period (24 hours) with daidzein in any of the cell lines (Figure 5). These results suggest that the effect of daidzein is not reversible, which would be compatible with its mode of action as an inducer of apoptosis. Our results suggest that genistein can be both cytostatic and cytotoxic depending on

the time of exposure. The heterogeneous nature of the cells potentially contributes to the differential response seen with genistein.

The effect of genistein alone and in conjunction with the most commonly used agents to treat ovarian cancer, paclitaxel, cisplatin and topotecan, was studied. Data obtained from all five cell lines were similar; for simplicity three of the cell lines are presented, representing the most, least and average sensitivities to genistein (Figure 6). Overall, an additive effect was seen with all chemotherapeutic agents. Specifically, with cisplatin and genistein, there was no difference at 9 μM with UL-3C cells, however slightly less cytotoxicity was observed with genistein in UL-6 cells, slightly more with UL-8 cells. Remaining concentrations did not demonstrate significant differences. Similarly paclitaxel and genistein resulted in a slightly less than additive effect for UL-3C, UL-6 and UL-8 cells. For topotecan and genistein, an additive effect was seen in all combinations except in the highest concentrations where in none of the cell lines expected was maximum cell kill achieved.

Genistein's mechanism of growth inhibition and cytotoxicity in ovarian cancer cell lines was evaluated. As prior studies in breast cancer cells had shown the inhibitory

Table I. *Demonstration of caspase-3 induction following treatment of ovarian tumor cells with various concentrations of genistein for 24 hours. Results are expressed as pmol/min/μg protein ±SEM.*

Gen (μM)	UL-3C	UL-5	UL-6	UL-7	UL-8
144	0.3831±0.0241	0.2502±0.0130	0.3147±0.0210	0.5561±0.0180	0.2795±0.0140
9	0.3608±0.0230	0.2436±0.0110	0.2016±0.0080	0.0959±0.0040	0.1957±0.0080
Control	0.1674±0.0054	0.0888±0.0030	0.1759±0.0060	0.0808±0.0030	0.0000±0.0000

effect may be mediated by apoptosis, the caspase-3 activity was measured in the ovarian cancer cell lines after exposure to genistein. All concentrations of genistein were shown to induce caspase 3 activity, although no correlation was seen between the level of enzyme induction and inhibition with genistein or the concentration (Table I).

Discussion

It has been reported that genistein inhibits cell growth of a wide range of cultured cancer cells including breast, lung and prostate cancers, leukemia and lymphoma (18-21). Numerous studies on the growth inhibitory effects of genistein and breast cancer have been performed and multiple mechanisms of inhibition have been elucidated (19,21). These effects have been shown to be estrogen receptor independent. We, therefore, decided to study the effects of isoflavanoids on ovarian cancer cells, as treatment is generally not related to estrogen receptor status, and therapy, to date, has minimally altered survival. As previously shown in many studies of breast cancer, lung cancer, prostate cancer and bladder cancer, we demonstrated that the isoflavanoids, genistein and daidzein, have growth inhibitory effects on ovarian cancer cells. Genistein has both cytostatic and cytotoxic effects on cell growth that are dose-dependent. We have demonstrated a time-dependence as well, with prolonged exposure leading to cytotoxicity. Our study confirms findings in other tumor types that daidzein is cytotoxic, inducing apoptosis. In our study, this induction was independent of duration of exposure with the shortest incubation time being 24 hours.

Genistein combined with chemotherapeutic agents used to treat ovarian cancer demonstrated additive effects in cisplatin and topotecan treated cells. A synergistic or additive effect with genistein would potentially allow for decreased dosing of these drugs that, by themselves or in combination with each other, result in severe toxicities. Previously, genistein has been shown to stimulate cisplatin accumulation by modulating the passive permeability of the plasma membrane in 2008 ovarian carcinoma cell line (22).

We demonstrated slightly less, in some cases significant, toxicity when paclitaxel was combined with genistein. A previous report had demonstrated that genistein at 30 μM markedly inhibited paclitaxel-induced toxicity and synergy with quercetin was observed (12). Later, the same group reported, using the same cell line, the IC_{50} to be 18 μM (44% less than their initial report) and 4 μM in clonogenic assay, while it was 32±2.5 μM for OVCAR-5 cells in growth inhibition and 5±0.5 μM in clonogenic assays DNA fragmentation and apoptosis of 697/neo cells. Even though these investigators report that the modulations of paclitaxel-induced DNA fragmentation and apoptosis by genistein occurred without significant effects on paclitaxel-mediated mitotic arrest of 697/neo cells (23). While we did not study the mechanism involved in this observation, inhibition of apoptosis may be the cause of our findings. There was substantial cytoxicity in all cell lines with the paclitaxel concentrations tested. It might be possible that lower doses of paclitaxel would have shown some effect if genistein were added, and this deserves further study. While interaction of genistein with the agents used in this work has not been reported before, the interactions reported with quercetin and tiazofurin were done in a different treatment protocol. Other investigators have demonstrated synergy with tiazofurin (13). While the exact mechanism is not clear, they demonstrated inhibition of PIP kinase with genistein for the synergy seen with tiazofurin with genistein and quercetin with genistein (14). Thus, additional treatment schedules may yield different data that requires further analysis.

Topotecan is a newer chemotherapeutic agent recently being used as first-line treatment for ovarian cancer. Its mechanism of action of inhibiting topoisomerase is identical to one the many mechanisms shown for genistein. Although, usually when choosing chemotherapy regimens, drugs of different mechanisms are used to increase cell kill at different cell cycle points, our study shows an additive effect of genistein with topotecan at all concentrations. This may be related to genistein's many other mechanisms of growth inhibition as well as its effect on the inhibition of DNA topoisomerase. A potential drug

regimen with decreased topotecan dosing and side-effects, but similar if not improved efficacy could result from this combination.

We demonstrate that genistein affects ovarian cancer cell proliferation and may have some role in treatment. With regards to research on breast cancer prevention, soy diets have shown a change in the urinary excretion of estrogen metabolites. High isoflavanoid consumption was associated with excretion of greater amounts of 2-hydroxyesterone, a metabolite that inhibits cell growth and may have a role in breast cancer prevention (24). Minimal research has been done on dietary measures to reduce risk of ovarian cancer. All aspects of diet in an Iowa study were evaluated and only high intake levels of green leafy vegetables were associated with a significantly decreased relative risk (25). Soy protein intake was not evaluated. Our study confirms the cytostatic as well as cytotoxic properties of genistein that may be useful in treatment protocols. This finding coupled with the decreased rates of ovarian cancer among Asian populations suggests a chemoprotective role for the isoflavanoids similar to breast cancer, however, further research needs to be performed in this area.

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