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American Ginseng and Breast Cancer Therapeutic Agents Synergistically Inhibit MCF-7 Breast Cancer Cell Growth

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Background and Objectives: American ginseng (*Panax quinquefolius L.*) purportedly alleviates menopause symptoms because of putative estrogenicity.
Methods: Using a standardized American ginseng (AG) extract in MCF-7 breast cancer cells, the objectives were to evaluate the ability of AG to induce the estrogen- regulated gene pS2 by Northern blot analysis, determine the effect on cell growth using the MTT assay, and evaluate the cell cycle effects by flow cytometry.
Results: AG and estradiol equivalently induced RNA expression of pS2. AG, in contrast to estradiol, caused a dose-dependent decrease in cell proliferation ($P < 0.005$). AG had no adverse effect on the cell cycle while estradiol significantly increased the proliferative phase (percent S-phase) and decreased the resting phase (G_0–G_1 phase) ($P < 0.005$). Concurrent use of AG and breast cancer therapeutic agents resulted in a significant ($P < 0.005$) suppression of cell growth for most drugs evaluated.
Conclusions: In vitro use of AG and breast cancer therapeutics synergistically inhibited cancer cell growth.
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KEY WORDS: **phytoestrogens; cell proliferation; menopause**

INTRODUCTION

Alternative or complementary medicine is the term applied to a system of health care that exists for the most part outside of the mainstream of conventional medicine [1]. The use of alternative medicine plays a significant role in the US health care system [2]. It was estimated in 1990 that Americans made 425 million visits to alternative practitioners compared with 388 million visits to primary care practitioners [3]. The sale of herbal products was anticipated to reach $2 billion in 1996 with the expectation that 63% of the population would be using herbs as part of their daily routine within the next 5 years [4].

Cancer is a serious disease that has led to many affected individuals exploring the realm of alternative medicines. Surveys estimate that anywhere from 20% to 60% of cancer patients are using these therapies [2,5–7]. Cancer patients turn to alternative therapies for a number of reasons. These include a desire to increase personal control over their treatment, an attempt to discover treatments with less side effects, as a source of hope, and to enhance or complement conventional therapies [8–11]. Other reasons include a distrust of conventional medicine, lack of confidence due to ineffectiveness of some established therapies, or the perception that alternative practitioners are more caring and willing to deal with a patient's medical and personal problems. A common per-

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ception is that alternative therapies offer a nontoxic and more natural treatment at lower costs than conventional medicines.

American ginseng (AG; *Panax quinquefolius L.*) is a popular herbal remedy used for various medicinal benefits [12]. The AG species is closely related to the Asian ginseng species (*Panax* C.A. Meyer) [13]. Ginseng is called an adaptogen because its main effect is to bring balance to the body. It has been noted to have anti-cancer properties and a wide range of pharmacologic and therapeutic actions on the cardiovascular, immune, and central nervous systems [13–18]. Frequently, AG has been recommended to alleviate the symptoms of natural and chemically induced menopause as well as to promote feelings of well-being [19–21]. However, there are no scientific data that address issues regarding the efficacy of such therapy in alleviating the symptoms of menopause. In addition, little data exist that evaluate the interactions of herbal therapies and chemotherapeutic agents.

The purpose of this study is to investigate the in vitro biologic properties of AG using a standardized AG extract. This study evaluates the possible estrogenic responses of AG, the effect of AG on the cell cycle, and the interaction of AG and standard breast cancer therapeutic agents, including cytoxan, doxorubicin, tamoxifen, taxol, methotrexate, megace, and fluorouracil on cellular proliferation using an estrogen receptor (ER) positive breast cancer cell line model.

MATERIALS AND METHODS

Test Compounds

The standardized AG root extract, CNT2000™, was donated by Chai-Na-Ta Corp. (Langley, British Columbia). CNT2000™ is a trademark extract produced from AG root grown in Canada by a proprietary extraction process.

The breast cancer cell lines were treated with AG dissolved in phosphate-buffered saline (PBS). The cells for the RNA assays were treated with 10 ml of steroid-stripped medium (SSM) containing AG at a concentration of 50–2,000 μg/ml per 10^7 cells per flask for the RNA for a total dose of 0.50–20 mg/10^7 cells. The cells for the MTT (3-[4,5,-dimethyl thiazolyl-2]-2,5-diphenyl tetrazolium bromide) assays were treated with 2 ml of SSM containing AG at concentrations of 5–1,000 μg/ml per 10^5 cells per flask for a total dose of 1–200 mg/10^7 cells. The cells for the flow cytometry studies were treated with 5 ml of SSM containing AG at concentrations of 60–1,200 μg/ml per 3×10^6 cells per flask for a total dose of 1–20 mg/10^7 cells. Experiments evaluating the maximum tolerated dose were performed for each study.

The chemotherapeutic agents cytoxan, doxorubicin, fluorouracil, methotrexate (Sigma, St. Louis, MO) were dissolved in PBS and tested in concentrations ranging from 10^{-4} to 10^{-8} *M* in equivalent volumes. Taxol, megace, and tamoxifen (Sigma) were tested in a similar range of concentrations in equivalent volumes after they were dissolved in 100% ethanol, previously shown not to adversely affect RNA or MTT assays [10]. ICI-182,780 (10^{-6} *M*, Zeneca Pharmaceuticals, England) was used in the RNA studies as a complete estrogen antagonist control. Tamoxifen is a partial estrogen antagonist. Estradiol (E_2; Sigma) was used as the positive control at a concentration of 10^{-9} *M* after being dissolved in 100% ethanol. A PBS-treated negative control group was used for all experiments.

Cell Culture

The following cell lines were purchased from American Type Culture Collection (ATCC; Rockville, MD): MCF-7, an ER positive breast cancer cell line; T-47D, an estrogen- insensitive breast cancer cell line; and MDA-MB- 231, an estrogen and progesterone receptor negative breast cancer line [22]. The cells were grown in 75- cm^2 flasks to 70% confluence in Eagle's minimum essential medium (EMEM; BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals Norcross, Atlanta, GA), 4 m*M* L-glutamine (BioWhittaker), and 50 μg/ml gentamicin (Sigma). The cells were grown in SSM consisting of phenol red-free Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 5% charcoal/dextran-treated FBS (Hyclone Laboratories, Inc., Logan, VT), 4 m*M* L-glutamine, 50 μg/ml gentamicin, and 2 ng/ml insulin (Sigma) for hormonal withdrawal [23]. After 7 days of steroid withdrawal, 10^7 cells per flask were treated with AG, E_2, a chemotherapeutic or hormonal agent, or a combination of AG and a chemotherapeutic or hormonal agent at select doses.

Probes

The pS2 (a gift from Dr. Pierre Chambon, Strasbourg, France) and 36B4 cDNA (a gift from Dr. Sam Lee, Boston, MA) were labeled with ^{32}P alpha-dCTP by the random primer method (Oligolabeling Kit, Pharmacia Biotech, Piscataway, NJ) [24]. 36B4 was used as the single copy control gene.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from the cells after 48 h of treatment with either E_2, AG, tamoxifen, PBS or ICI-182,780 using the Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX). Northern blot analysis was performed using 20 μg of total RNA electrophoresed through a 1% formaldehyde agarose gel and then transferred to a nylon membrane (ICN Pharmaceuticals, Inc., Irvine, CA) [25]. The RNA was cross-linked to a nylon membrane using the UV stratalinker (Stratagene, La

Jolla, CA) and prehybridized at 42°C for 5 h. The RNA was hybridized with pS2 and 36B4 labeled with ^{32}P alpha-dCTP for 18 h. Unlabeled probe was removed with a series of washes of 0.2% SDS with 2 × SSC and 0.2% SDS with 1 × SSC at room temperature and washes at 65°C in 0.2% SDS with 0.5 × SSC. The blots were exposed to Kodak XAR-5 film at -80°C and autoradiographs were developed. The RNA content was quantified using laser densitometry and ImageQuant software (Molecular Dynamics 400-B 2D, Sunnyvale, CA) and normalized against 36B4.

Cell Proliferative Assays

The MTT assay was used to assess cell proliferation as previously described [26–28]. A few experiments were also performed by the ^{3}H thymidine incorporation and DNA quantification method for technical comparisons [29,30]. Cell viability studies were also performed using trypan blue. After 7 days of steroid withdrawal, the MCF-7 cells were stained with 0.4% trypan blue and counted using a hemocytometer. Cells were plated at 10^5 per well (24-well plates, Corning, Cambridge, MA) in a volume of 2 ml of medium. At 24 h after plating, the cells were treated for 48 h with individual doses of E_2 10^{-9} *M*; AG 5–1,000 μg/ml; tamoxifen 10^{-6} *M*; taxol 10^{-4} to 10^{-6} *M*; cytoxan 10^{-4} to 10^{-6} *M*; doxorubicin 10^{-4} to 10^{-6} *M*; fluorouracil 10^{-4} to 10^{-6} *M*; megace 10^{-4} to 10^{-6} *M*; and methotrexate 10^{-4} to 10^{-6} *M*. Combination studies were performed using selected doses of the therapeutic agents based on the initial dose response studies. After 48 h of treatment, the medium is removed and replaced with 100 μl of tetrazolium (MTT, 1 mg/ml, Sigma) in RPMI medium. The plates are incubated for 3 h at 37°C and centrifuged for 10 min at 400*g*. The medium is replaced with 100 μl of dimethylsulfoxide (DMSO). The multiwell plates are shaken on a plate shaker for 7 min and read on a microplate reader using a wavelength of 570 nm and a reference of 630 nm. Each experiment was performed in triplicate and repeated to confirm results.

Flow Cytometry

MCF-7 cells were grown in 25-cm^2 Corning flasks to 50% confluence (3×10^6 cells per flask) in EMEM supplemented with 10% FBS, 4 m*M* L-glutamine, and 50 μg/ml gentamicin. The cells were grown in SSM for 7 days. The cells were treated with either E_2 10^{-9} *M*; AG 60, 600, and 1,200 μg/ml; or tamoxifen 0.6×10^{-6} *M* for 48 h. Testing of higher doses of AG resulted in no viable cells for evaluation. Following treatment, the cells were collected into microcentrifugal tubes and washed with PBS twice. DNA content of the nuclei was determined by staining nuclear DNA with propidium iodide containing 50 μg/ml in PBS with 0.1% bovine serum albumin (BSA) and 0.1% Saponin. 10^6 cells were used for measuring the relative DNA content of nuclei using a fluorescence-activated cell sorter (FACS; Becton-Dickinson, Mountain View, CA). The proportion of nuclei in each phase of the cell cycle was determined using the MODFIT LT DNA analysis software (Becton-Dickinson) [31,32].

Statistical Analysis

Statistical analysis was performed by the SPSS software package using the one-way analysis of variance (ANOVA) test with Bonferroni post hoc multiple comparisons (SPSS INC., Chicago, IL).

RESULTS

RNA/Northern Blot Analysis

A 4.2 and 4.3-fold increase in pS2 RNA expression was identified in MCF-7 cells treated with E_2 and AG, respectively, compared with PBS-treated controls. Figure 1 shows the dose-related increase in RNA expression in MCF-7 cells treated with AG. A dose response curve is noted with increasing expression of RNA with increasing doses of AG. A concentration of 2,000 μg/ml (total dose 20 mg/10^7 cells) of AG is equivalent to a dose of 10^{-9} *M* E_2 for inducing pS2 expression. While both E_2 and AG increase the pS2 RNA expression in the MCF-7 ER positive cell line, no pS2 expression was detected in either T-47D or MDA-MB-231 ER negative cell lines or in the PBS-treated negative control groups.

Figure 2 characterizes the results of the Northern blot analyses investigating the effects of ICI-182,780, a complete estrogen antagonist, on AG and E_2- induced pS2 expression. ICI-182,780 inhibits the increased pS2 RNA expression induced by both E_2 and AG. The inhibition of pS2 expression by ICI-182,780 is dose dependent with greater inhibition seen with the higher doses (10^{-6} vs. 10^{-8} *M*) of the estrogen antagonist.

MTT Assay

MCF-7 cells were treated with concentrations of AG ranging from 5 to 1,000 μg/ml (total dose equivalent of 1–200 mg/10^7 cells). Cell growth inhibition was identified in a dose-dependent fashion for AG-treated cells, with doses of 100 mg and above achieving significance at $P < 0.005$ (Fig. 3). Cell viability for AG doses of 1, 5, 10, 60, 80, and 100 mg was 91%, 91%, 90%, 90%, 88%, and 85%, respectively. E_2-treated cells exhibited a significant increase in cell proliferation in comparison to untreated cells ($P < 0.005$) and to all doses of AG- treated cells ($P = 0.001$ to $P < 0.005$). There was no difference in results when comparing the MTT assay with the ^{3}H thymidine incorporation technique (data not shown). When T-47D and MDA-MB-231 cells were treated with either E_2 or AG, there was no effect on cell growth in comparison to the PBS control groups.

Tamoxifen 10^{-5} *M* alone, but not tamoxifen 10^{-6} *M*, significantly inhibits cell growth ($P < 0.005$) in compari-

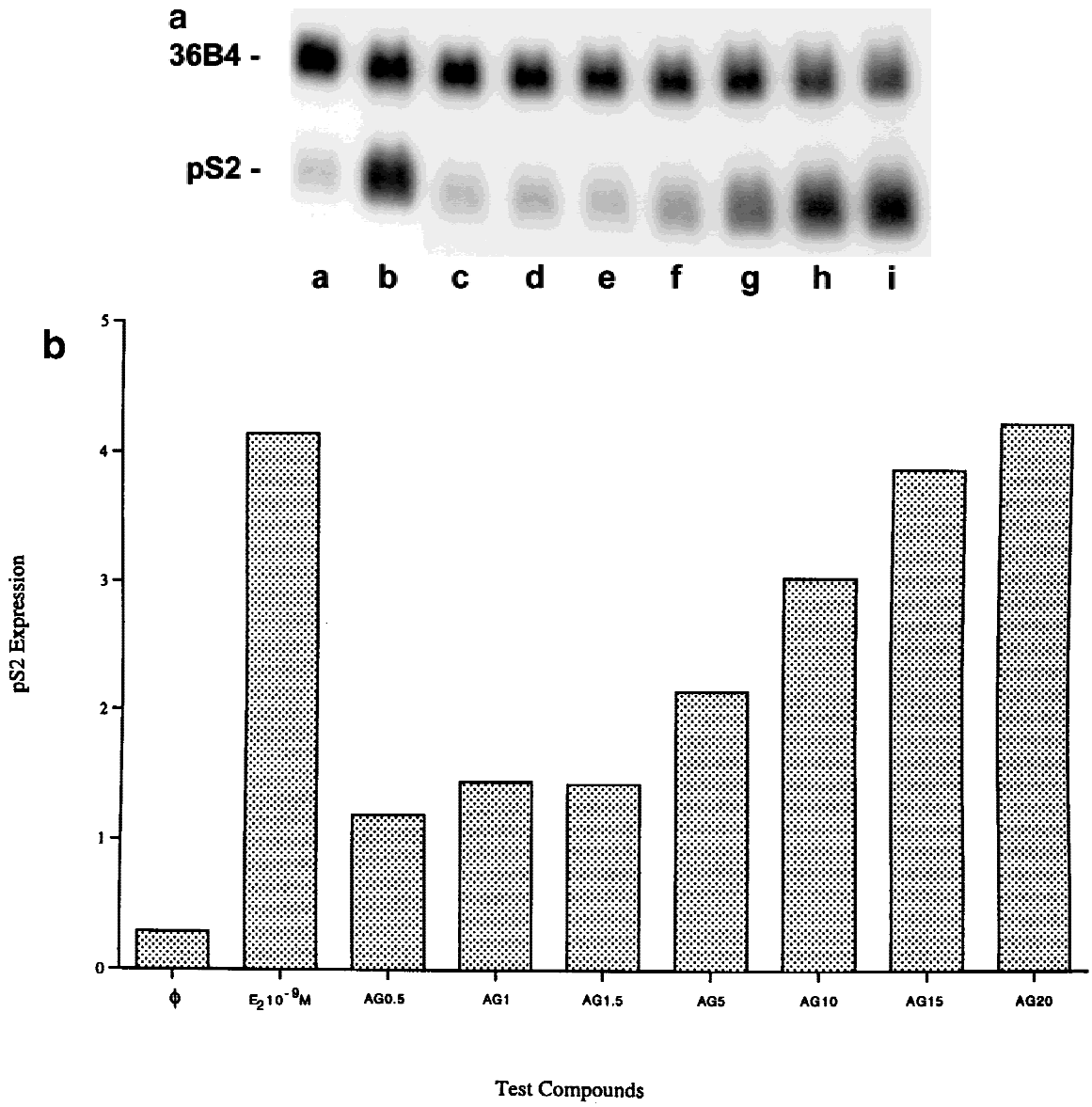

Fig. 1. pS2 RNA induction by AG and E_2. **a:** Results of the Northern blot demonstrating an increase in pS2 RNA expression in MCF-7 cells treated with PBS, E_2 10^{-9} *M*, AG at doses of 0.5, 1.0, 1.5, 5.0, 10, 15, and 20 mg **(lanes a–i)**. There is an equivalent increase in pS2 expression induced by E_2 and AG 20 mg/10^7 cells (4.2- and 4.3-fold increase, respectively). **b:** Bar graph representing the results of the Northern blot analysis, showing a dose-dependent increase in pS2 RNA expression with increasing doses of AG.

son to PBS controls (Table I). The combination of tamoxifen 10^{-6} *M* and AG 100 mg resulted in a synergistic effect on cell growth inhibition. A significant decrease in cell proliferation was found in the MCF-7 cells treated concurrently with both therapies in comparison to tamoxifen alone ($P < 0.005$) and AG 100 mg alone ($P < 0.005$).

Megace alone significantly ($P < 0.005$) inhibited cell growth at a dose of 10^{-4} *M*, but not at 10^{-5} or 10^{-6} *M* compared with PBS controls (M ± SD: 0.69 ± 0.18; 0.91 ± 0.21; 0.96 ± 0.24; and 0.96 ± 0.17, respectively). The concurrent use of AG 60 and 100 mg to cells treated with megace 10^{-5} *M* significantly inhibited cell growth (0.70 ± 0.17; 0.21 ± 0.19; $P = 0.003$, $P < 0.005$, respectively) compared with PBS controls. The combination of treatments also decreased cell growth ($P < 0.005$ for both doses) to megace alone. The combination, however, did not decrease cell growth in comparison to either dose of AG alone.

No single dose of cytoxan resulted in a significant decrease in cell growth in comparison to untreated controls (Table II). The addition of 60 and 100 mg of AG to cytoxan doses of 10^{-4} to 10^{-6} *M* resulted in a significant decrease in cell growth ($P < 0.005$) in comparison to the negative control group and to cytoxan alone ($P < 0.005$). A significant decrease in cell growth in the concurrently

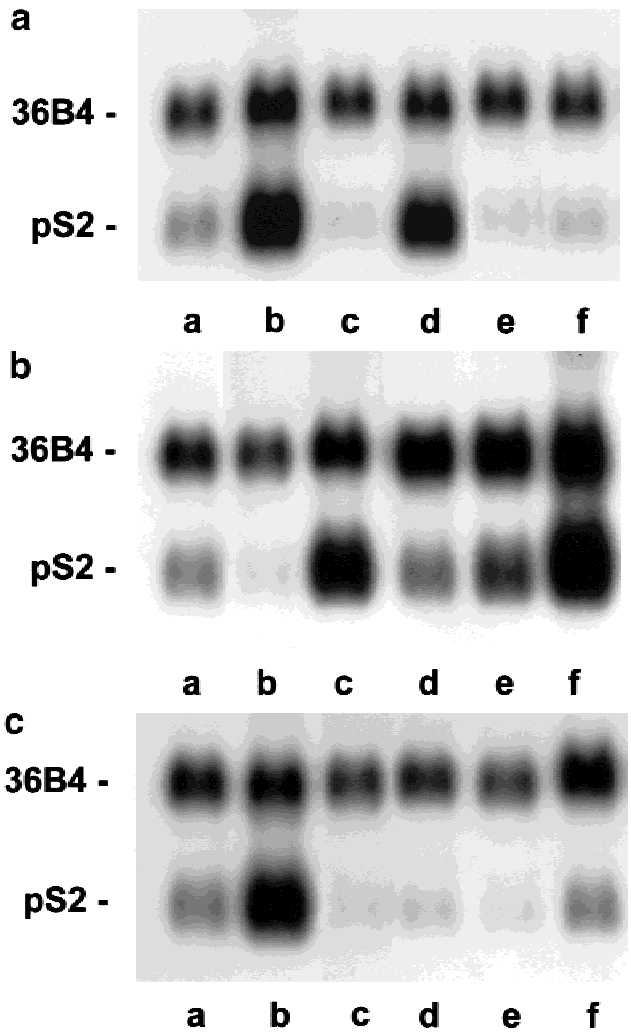

Fig. 2. The effect of the estrogen antagonist ICI-182,780 on pS2 RNA expression induced by AG and E_2. **a:** Results of the Northern blot analysis where MCF-7 cells were treated with PBS, E_2 (10^{-9} *M*), ICI-182,780 (10^{-6} *M*), a complete estrogen antagonist, and AG 20 mg **(lanes a–d)**. An increase in pS2 expression is found with E_2 and AG-treated cells (3.3- and 3.4-fold, respectively) in comparison to the PBS control group. No increase in pS2 expression is identified in the ICI-182,780-treated group. When MCF-7 cells are treated with ICI-182,780 and AG or E_2, the AG and E_2 **(lanes e, f)** induced pS2 expression is suppressed. **b:** Dose response results of the Northern blot analysis following the treatment of MCF-7 cells with PBS, AG 20 mg, and ICI-182,780 (10^{-6} *M*), a complete estrogen antagonist **(lanes a–c)**. There is a 3.4-fold increase in pS2 RNA expression induced by AG and no increase as expected with ICI-182,780 or PBS. When AG is combined with ICI-182,780 at concentrations of 10^{-6}, 10^{-7}, and 10^{-8} *M* **(d–f)**, the AG induced pS2 expression is suppressed at all dose levels (0.4, 0.3, and 0.9, respectively). **c:** Dose response results of the Northern blot analysis following the treatment of MCF-7 cells with PBS, E_2 (10^{-9} *M*), and ICI-182,780 (10^{-6} *M*), a complete estrogen antagonist **(lanes a–c)**. There is a 3.3-fold increase in pS2 RNA expression induced by E_2 in comparison tho the PBS control group and no increase as expected in the ICI-182,780-treated groups. When E_2 is combined with ICI-182,780 at concentrations of 10^{-6}, 10^{-7}, and 10^{-8} *M* **(lanes d–f)**, the E_2-induced pS2 expression is suppressed in a dose-related response (0.9, 1.3, and 3.3, respectively). Treatment of MCF-7 cells with E_2 and ICI-182,780 10^{-8} *M* did not suppress the E_2-induced pS2 RNA expression.

treated groups in comparison to AG alone was also identified.

No growth inhibition was found with fluorouracil 10^{-7} to 10^{-4} *M* treatment of MCF-7 cells in comparison to PBS controls (0.96 ± 0.27; 0.87 ± 0.28; 0.83 ± 0.30; 0.79 ± 0.29; 0.88 ± 0.17, respectively). The combination of fluorouracil 10^{-7} *M* and AG 100 mg resulted in a significant inhibition of cell growth (0.30 ± .003) in comparison to PBS controls ($P = 0.002$) and fluorouracil 10^{-7} *M* ($P < 0.005$). The combination of fluorouracil 10^{-6} *M* and AG 60 and 100 mg (0.41 ± 0.22; 0.29 ± 0.16, respectively) resulted in a significant decrease in cell growth in comparison to PBS controls for both combinations ($P < 0.005$) and to fluorouracil alone ($P = 0.001$, $P < 0.005$, respectively). There was no significant difference for combination therapy when compared to AG alone. Similar results were seen for the other doses of fluorouracil in combination with AG. The only combination dose that significantly decreased cell growth in comparison to AG 100 mg was fluorouracil 10^{-5} *M* and AG 100 mg (0.23 ± 0.14, $P < 0.005$).

There was a significant decrease in cell growth for doxorubicin at 10^{-6}, 10^{-5}, and 10^{-4} *M* in comparison to untreated controls ($P < 0.005$ all doses). Table III demonstrates that the addition of various doses of AG to all molar concentrations of doxorubicin resulted in decreased cell growth in comparison to the untreated negative control group. For all molar concentrations, except 10^{-4} *M*, the addition of select doses of AG to doxorubicin also resulted in a significant decrease in cell growth in comparison to doxorubicin alone.

Table IV shows that treatment of MCF-7 cells with taxol $10^{-}4$ *M* resulted in a significant decrease in cell growth in comparison to the PBS negative control group ($P < 0.005$). When AG was added to taxol 10^{-5} and 10^{-4} *M* at doses of 60, 80, and 100 mg, there was a significant decrease in cell growth in each concurrently treated group in comparison to untreated controls, taxol alone, and AG alone.

Only 10^{-5} *M* of methotrexate caused a significant decrease in cell growth in comparison to PBS controls (0.64 ± 0.009; 0.79 ± 0.13, respectively). Methotrexate 10^{-7}, 10^{-6}, and 10^{-4} *M* did not significantly inhibit MCF-7 cell growth (0.85 ± 0.005; 0.76 ± 0.006; 0.68 ± 0.009, respectively). The combination of AG 60 mg and methotrexate 10^{-5} *M* significantly inhibited cell growth (0.61 ± 0.008) in comparison to PBS ($P < 0.005$) and methotrexate 10^{-5} *M* alone ($P = 0.009$) but not to AG 60 mg alone. The combination of AG 100 mg and methotrexate 10^{-4} *M* (0.36 ± 0.007) significantly inhibited cell growth in comparison to PBS controls ($P < 0.005$), methotrexate 10^{-4} *M* alone ($P < 0.005$), and AG 100 mg ($P < 0.005$).

In all of these studies, E_2 was used as a positive control and significantly increased ($P < 0.005$) MCF-7 breast cancer cell proliferation in each experiment.

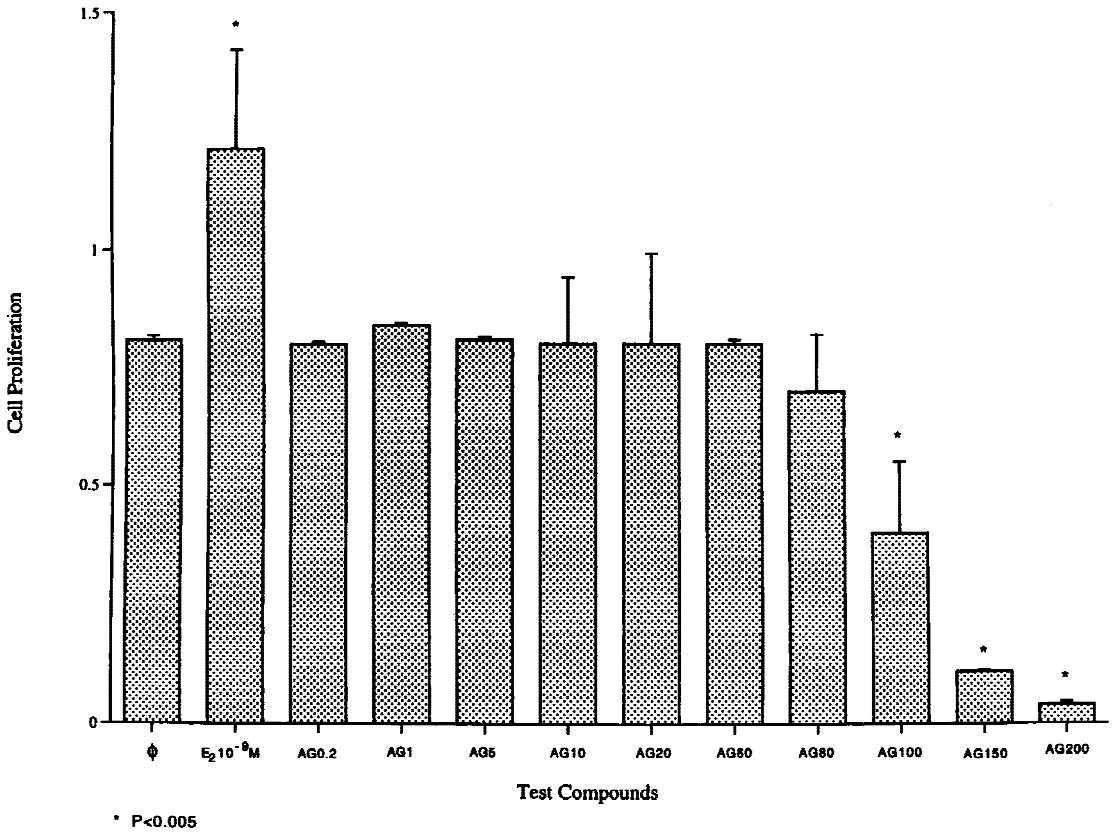

Fig. 3. Treatment of MCF-7 cells with AG results in cell growth inhibition. Results of the MTT assay treating MCF-7 cells with PBS, E_2 (10^{-9} *M*), and AG 0.2, 1, 5, 10, 60, 80, 100, and 200 mg are shown. There is a significant ($P < 0.005$) increase in cell proliferation in MCF-7 cells treated with E_2. There is a significant decrease in cell growth ($P < 0.005$) when MCF-7 cells were treated with AG 100, 150, and 200 mg.

TABLE I. MTT Assay: Treatment of MCF-7 Cells with AG and Tamoxifen (Tam)

Treatment	M ± SD	*n*	% Cell viability	*P* value[a]		
				PBS	10^{-x} *M*	AG
PBS	0.82 ± 0.11	12	90	—	—	—
E_2	1.11 ± 0.18	12	93	<0.005	—	—
Tam 10^{-6} *M*	0.82 ± 0.10	12	87	NS	—	—
Tam 10^{-5} *M*	0.06 ± 0.005	3	10	<0.005	—	—
AG 60 mg	0.83 ± 0.16	9	90	NS	—	—
AG 80 mg	0.74 ± 0.11	9	85	NS	—	—
AG 100 mg	0.60 ± 0.13	9	80	0.002	—	—
Tam 10^{-6} *M*/60 mg	0.76 ± 0.11	9	75	NS	NS	NS
Tam 10^{-6} *M*/80 mg	0.65 ± 0.006	9	78	0.067	NS	NS
Tam 10^{-6} *M*/100 mg	0.54 ± 0.003	9	75	<0.005	<0.005	<0.005

[a]*P* values are compared to the PBS negative control groups, the molar concentration of the agent used alone, and to the specific dose of AG tested for that group. NS = not significant.

Flow Cytometry

Representative graphs of the flow cytometry studies are shown in Figure 4. Treatment of MCF-7 cells with E_2 resulted in a significant increase in the proliferative phase (percent S-phase, $P < 0.005$) and a significant decrease in the resting phase (G_0–G_1 phase, $P < 0.005$) of the cell cycle in comparison to PBS-treated negative controls. None of the AG doses tested caused a significant increase in percent S-phase or a decrease in the G_0–G_1 phase. Tamoxifen treatment also had no effect on percent S-phase or G_0–G_1 phase in comparison to the PBS control group. None of the treated groups had a significant effect on the GM phase of the cell cycle. The M ± SD for the percent S-phase for the PBS, E_2, AG 1.0, 10, and 20 mg, and tamoxifen-treated groups were 7.57 ± 1.3; 22.27 ± 3.1; 7.61 ± 1.9; 10.14 ± 3.4; 9.50 ± 4.0; and 9.63 ± 0.9, respectively. The M ± SD for the G_0–G_1 phase for the

TABLE II. MTT Assay: Treatment of MCF-7 Cells with AG and Cytoxan (Cyt)

Treatment	M ± SD	*n*	% Cell viability	*P* value[a]		
				PBS	10^{-x} *M*	AG
PBS	0.84 ± 0.11	24	91	—	—	—
E_2	1.31 ± 0.21	24	92	<0.005	—	—
AG 60 mg	0.79 ± 0.006	18	90	NS	—	—
AG 100 mg	0.41 ± 0.007	18	78	<0.005	—	—
Cyt 10^{-6} *M*	0.77 ± 0.15	15	89	NS	—	—
Cyt 10^{-5} *M*	0.78 ± 0.16	24	85	NS	—	—
Cyt 10^{-4} *M*	0.79 ± 0.25	15	82	NS	—	—
Cyt 10^{-6} *M*/60 mg	0.58 ± 0.12	6	84	NS	NS	NS
Cyt 10^{-6} *M*/100 mg	0.54 ± 0.17	6	74	0.007	NS	NS
Cyt 10^{-5} *M*/60 mg	0.58 ± 0.18	15	84	<0.005	0.017	0.009
Cyt 10^{-5} *M*/100 mg	0.36 ± 0.004	6	50	<0.005	<0.005	<0.005
Cyt 10^{-4} *M*/60 mg	0.36 ± 0.007	6	83	<0.005	<0.005	<0.005
Cyt 10^{-4} *M*/100 mg	0.34 ± 0.005	6	65	<0.005	0.004	NS

[a]*P* values are compared to the PBS negative control groups, the molar concentration of the agent used alone, and to the specific dose of Ag tested for that group. NS = not significant.

TABLE III. MTT Assay: Treatment of MCF-7 Cells with AG and Doxorubicin (Dox)

Treatment	M ± SD	*n*	% Cell viability	*P* value[a]		
				PBS	10^{-x} *M*	AG
PBS	0.90 ± 0.14	27	91	—	—	—
E_2	1.30 ± 0.23	27	94	<0.005	—	—
Dox 10^{-7} *M*	0.92 ± 0.15	12	nd	NS	—	—
Dox 10^{-6} *M*	0.62 ± 0.16	18	92	<0.005	—	—
Dox 10^{-5} *M*	0.38 ± 0.14	21	90	<0.005	—	—
Dox 10^{-4} *M*	0.28 ± 0.01	9	30	<0.005	—	—
AG 60 mg	0.75 ± 0.18	15	91	NS	—	—
AG 100 mg	0.35 ± 0.20	15	87	<0.005	—	—
Dox 10^{-7} *M*/60 mg	0.95 ± 0.01	6	nd	NS	NS	NS
Dox 10^{-7} *M*/100 mg	0.43 ± 0.36	6	nd	0.045	<0.005	NS
Dox 10^{-6} *M*/60 mg	0.28 ± 0.006	9	92	<0.005	<0.005	<0.005
Dox 10^{-6} *M*/100 mg	0.12 ± 0.005	9	88	<0.005	<0.005	0.12
Dox 10^{-5} *M*/60 mg	0.13 ± 0.006	12	87	<0.005	<0.005	<0.005
Dox 10^{-5} *M*/100 mg	0.06 ± 0.002	12	80	<0.005	<0.005	<0.005
Dox 10^{-4} *M*/60 mg	0.11 ± 0.002	3	66	<0.005	NS	<0.005
Dox 10^{-4} *M*/100 mg	0.06 ± 0.006	3	10	<0.005	NS	<0.005

[a]*P* values are compared to the PBS negative control groups, the molar concentration of the agent used alone, and to the specific dose of AG tested for that group. NS = not significant.

TABLE IV. MTT Assay: Treatment of MCF-7 Cells with AG and Taxol (Tax)

Treatment	M ± SD	*n*	% Cell viability	*P* value[a]		
				PBS	10^{-x} *M*	AG
PBS	0.84 ± 0.004	15	91	—	—	—
E_2	1.16 ± 0.006	15	93	<0.005	—	—
Tax 10^{-5} *M*	0.74 ± 0.007	15	90	NS	—	—
Tax 10^{-4} *M*	0.60 ± 0.17	9	90	<0.005	—	—
AG 60 mg	0.80 ± 0.007	9	89	NS	—	—
AG 80 mg	0.77 ± 0.005	9	89	NS	—	—
AG 100 mg	0.48 ± 0.13	9	89	<0.005	—	—
Tax 10^{-5} *M*/60 mg	0.53 ± 0.10	9	83	<0.005	<0.005	<0.005
Tax 10^{-5} *M*/80 mg	0.51 ± 0.003	6	83	<0.005	0.04	0.05
Tax 10^{-5} *M*/100 mg	0.19 ± 0.004	9	83	<0.005	<0.005	<0.005
Tax 10^{-4} *M*/60 mg	0.44 ± 0.003	6	88	<0.005	<0.005	<0.005
Tax 10^{-4} *M*/80 mg	0.23 ± 0.002	6	75	<0.005	<0.005	<0.005
Tax 10^{-4} *M*/100 mg	0.33 ± 0.02	6	71	<0.005	<0.005	<0.005

[a]*P* values are compared to the PBS negative control groups, the molar concentration of the agent used alone, and to the specific dose of AG tested for that group. NS = not significant.

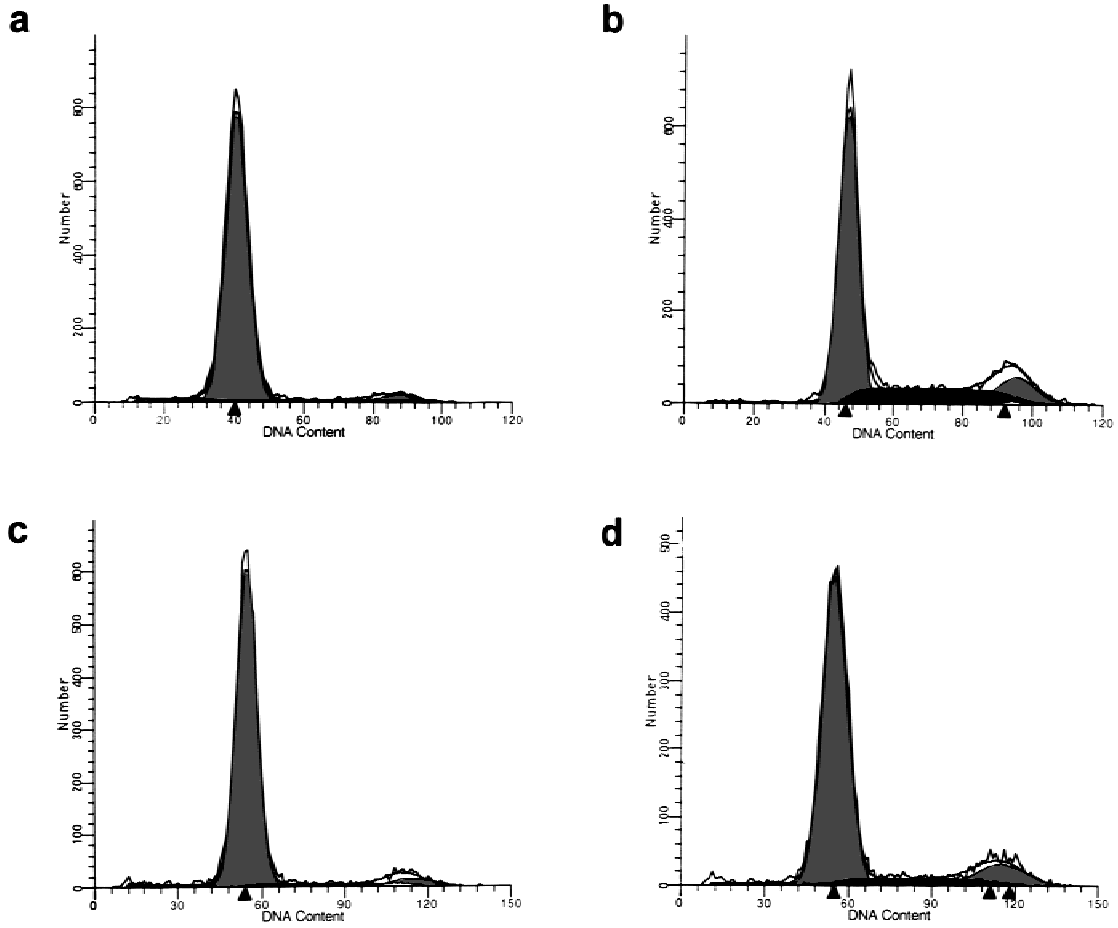

Fig. 4. The effect of AG on the MCF-7 cell cycle. **a–d:** Representative flow cytometry graph results of MCF-7 cells treated with PBS, E_2 (10^{-9} *M*), AG (20 mg), and tamoxifen (10^{-6} *M*) (N = 6). E_2-treated cells exhibited a significant increase in the proliferative percent S-phase ($P < 0.005$) and a significant decrease in the G_0–G_1 resting phase ($P < 0.005$) of the cell cycle in comparison to the PBS-treated control group. AG and tamoxifen-treated cells exhibited no significant change in either the proliferative or resting phases of the cell cycle.

same groups were 85.06 ± 3.8; 65.05 ± 5.6; 84.38 ± 4.5; 78.98 ± 5.0; 78.99 ± 6.0; and 78.79 ± 8.3, respectively.

DISCUSSION

Phytoestrogens are a group of naturally occurring plant compounds that have been reported to possess weak estrogenic activity and include genistein, daidzein, and coumesterol among others [33]. Most phytoestrogens commonly consumed in the diet interact with intracellular ER and function as estrogen agonists at physiologically relevant doses [34,35]. Estrogen-like responses have been reported for these phenolic compounds found in fruits and vegetables. Several lignans, flavonoids,and isoflavonoids have been shown to increase RNA expression of pS2, an estrogen-regulated gene [36].

A survey assessing the frequency of use of alternative therapies in postmenopausal (PMW) women with and without a history of breast cancer has shown that 12% of the PMW without a history of breast cancer and 23% of PMW with a history of breast cancer used alternative medicines [37]. The frequency, prevalence, and severity of menopausal symptoms were similar for both groups. Ginseng was the most frequently used herbal therapy.

Because of the frequent use of AG by breast cancer patients and menopausal women without a history of breast cancer and the lack of data regarding safety and therapeutic issues, we evaluated the possible estrogenic effects of AG by investigating its ability to induce pS2. We have previously reported that an AG root extract prepared by our laboratory could induce the RNA and protein expression of pS2 and the protein expression of the progesterone receptor [38,39], suggesting estrogenic in vitro activity. These earlier studies also suggested that AG may function via the ER as the increase in pS2 was partially inhibited by tamoxifen, a partial estrogen antagonist. While the extract was controlled for concentration and species of ginseng, the ginsenoside profile was not determined.

This study was performed with a standardized AG root extract with known concentrations of the ginsenosides

and other components which comprise the ginseng extract. Ginsenosides are the most studied active components of ginseng, which vary among the species and may be responsible for the differences in clinical properties between AG and Asian ginseng [14,16]. AG contains higher concentrations of the ginsenoside RB1. It is hypothesized that the Rb1 content is related to the 'cooling' properties of AG. The ginsenoside content also varies with the part of the plant used for the preparation as well as the age of the root [14].

This study also confirmed the results of our initial investigations [38,39]. In addition, this standardized ginseng extract has also been found to exhibit a paradoxical effect on estrogen- sensitive breast cancer cell lines. It has been shown to induce the expression of pS2 at a level comparable to E_2 while at that same dose AG does not increase the proliferative phase (percent S-phase) of the cell cycle that was identified with E_2. E_2 has been previously reported to increase the percent S-phase of the cell cycle and to decrease the G_0 phase in MCF-7 cells [32,40]. Those results are confirmed in this study. At the doses tested, AG demonstrates a dose response effect on cell growth with no adverse effect on cell growth at the lower doses tested and a significant decrease in cell proliferation at the higher doses. It had been expected that AG would have caused an increase in cell growth parallel to E_2. Cell viability studies documented that the decrease in cell growth was not secondary to the concentration of AG being too toxic. We had also performed several of the initial cell growth studies using the thymidine incorporation method in comparison to the MTT assay. There was no difference in the results when the two methods were compared. MTT assays have been used extensively for evaluating the effect of growth factors, hormones, and drugs on growth and survival of tumor cells in culture [41].

The increase in RNA pS2 expression induced by AG and E_2 can be inhibited by ICI-182,780, a complete estrogen antagonist. This response was seen in a dose-related fashion for both. This would indirectly suggest that the AG may function via the ER, although other pathways have been hypothesized to play a role in pS2 induction [36]. Neither AG or E_2 induced pS2 expression in estrogen-insensitive cell lines. It is hypothesized that the mechanism of action of AG on breast cancer cells may be in part via the ER and in part by an as of yet undiscovered pathway.

Genistein, the most important phytoestrogen in soy, inhibits the proliferative activity in MCF-7, T-47D, and MDA-468 breast cancer cell lines [42]. However, a biphasic pattern had been reported demonstrating an increase in cell growth at concentrations of 10^{-8} to 10^{-6} *M* and an inhibitory effect at 10^{-5} *M* [43]. The growth inhibitory effect of genistein results from the summation of cytostatic and apoptotic effects with an arrest in the G_2/M phase of the cell cycle [41]. The combination of genistein and doxorubicin in cell proliferation studies has been reported to result in a synergistic inhibitory effect in both estrogen-sensitive and insensitive breast cancer cell lines [44].

The possible interaction of herbal therapies with standard chemotherapeutic and hormonal agents is an important issue for cancer patients who elect to use both concurrently. In this study, the concurrent treatment of MCF-7 cells with AG and the most frequently used chemotherapeutic and hormonal agents did not result in any adverse interactions. In comparison to the chemotherapeutic and hormonal agents alone, the addition of AG resulted in a further suppression in cell proliferation in comparison to either one alone. This suggests that a possible synergistic effect may exist between AG and the breast cancer therapeutic agents investigated in this study.

This is the first in vitro study to evaluate the efficacy and safety of the concurrent use of AG and breast cancer treatment drugs. This study suggests that the use of AG does not interfere with the action of standard breast cancer therapeutic agents in ER positive breast cancer cells. This is particularly important for the women using tamoxifen and megace for a prolonged duration who wish to use AG to reduce postmenopausal symptoms.

Further investigations are warranted to evaluate possible synergistic in vivo effects of the combination of AG and cancer therapeutics. Clinical studies are also warranted to determine if indeed, herbal remedies such as AG do possess beneficial estrogenic properties.

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