Estrogenic Effects of Genistein on the Growth of Estrogen Receptor-positive Human Breast Cancer (MCF-7) Cells in Vitro and in Vivo


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ABSTRACT

Genistein, found in soy products, is a phytochemical with several biological activities. In the current study, our research focused on the estrogenic and proliferation-inducing activity of genistein. We have demonstrated that genistein enhances the proliferation of estrogen-dependent human breast cancer (MCF-7) cells in vitro at concentrations as low as 0.1 nM, with a concentration of 100 nM achieving proliferative effects similar to those of 1 nM estradiol. Expression of the estrogen-responsive gene pS2 was also induced in MCF-7 cells in response to treatment with a concentration of genistein as low as 1 nM. At higher concentrations (above 20 nM), genistein inhibits MCF-7 cell growth. In vivo, we have shown that dietary treatment with genistein (750 ppm) for 5 days enhanced mammary gland growth in 28-day-old ovariectomized athymic mice, indicating that genistein acts as an estrogen in normal mammary tissue. To evaluate whether the estrogenic effects observed in vitro with MCF-7 cells could be reproduced in vivo, MCF-7 cells were implanted s.c. in ovariectomized athymic mice, and the growth of the estrogen-dependent tumors was measured weekly. Negative control animals received the American Institute of Nutrition (AIN)-93G diet, the positive control group received a new s.c. estradiol (2 mg) pellet plus the AIN-93G diet, and the third group received genistein at 750 ppm in the AIN-93G diet. Tumors were larger in the genistein (750 ppm)-treated group than they were in the negative control group, demonstrating that dietary genistein was able to enhance the growth of MCF-7 cell tumors in vivo. Increased uterine weights were also observed in the genistein-treated groups. In summary, genistein can act as an estrogen agonist in vivo and in vitro, resulting in the proliferation of cultured human breast cancer cells (MCF-7) and the induction of pS2 gene expression. Here we present new information that dietary genistein stimulates mammary gland growth and enhances the growth of MCF-7 cell tumors in ovariectomized athymic mice.

INTRODUCTION

The phytoestrogen genistein is present at high concentrations in plant foods such as soybeans. Phytoestrogens are known to reduce the risk of breast cancer (1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.1 Supported by National Cancer Institute Grant CA77355 (to W. G. H.).

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1 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; AIN, American Institute of Nutrition; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; MEM, minimum essential media; IMEM, improved MEM.

MATERIALS AND METHODS

In Vitro Studies

Cell Proliferation Study. MCF-7 cells were maintained in IMEM (Biofluids, Inc., Rockville, MD) containing 5% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were harvested and then plated at 1.5 X 10⁴ cells/well in 24-well tissue culture polystyrene plates in media containing 5% FBS-IMEM for 24 h. Media were removed, and the cells were washed with 150 mM PBS and then incubated with media containing 5% FBS-IMEM for 24 h. Media were replaced with the same media at an atmosphere of 5% CO₂. Cells were harvested and then plated at 1.5 X 10⁴ cells/well in 24-well tissue culture polystyrene plates in media containing 5% FBS-IMEM for 24 h. Media were removed, and the cells were washed with 150 mM PBS and then incubated with media containing 5% FBS-IMEM for 24 h. Media were replaced with the same media containing genistein, which was synthesized as described by Chang et al. (11). Concentrations of genistein used were 0.01, 0.1, 1.0, 10, 25, 50, and 100 nM. Proliferation was assessed by fluorometric analysis of DNA content daily for 96 h. Cells were lysed in situ with 10 min EDTA (pH 12.3) at 37°C for 30 min and neutralized with KH₂PO₄, and then fluorescent dye (Hoechst reagent 33258) was used to determine the DNA content. Fluorescence was measured by excitation at 350 nm and emission at 455 nm to determine the DNA content when compared with a standard of salmon sperm DNA (12, 13).

Cell Culture and Treatment for Expression of pS2 mRNA. MCF-7 cells were cultured in 100 X 20-mm polystyrene tissue culture plates with the
growth conditions and growth media described above. One week before the initiation of experimental treatments, cells were switched to medium supplemented with 5% charcoal-dextran-stripped FBS. Due to its potential estrogenic effect, phenol red-free media were used 24 h before treatments, and the cells were switched to medium supplemented with phenol red-free IMEM containing penicillin (100 units/ml), streptomycin (100 µg/ml), and 5% charcoal-dextran-stripped FBS. Twenty-four h after initial plating of the MCF-7 cells, estradiol or genistein was added. The final concentrations of estradiol in the culture media were 200 pm and 1 nm, and the final concentrations of genistein were 1.0 and 10 nm and 1 µM. Both estradiol and genistein were dissolved in DMSO; the final concentration of DMSO in the medium was 0.1%. The growth medium was changed every 36 h after initial plating, and fresh estradiol and genistein were added with each media change.

RNA Isolation. RNA was isolated 72 h after the addition of estradiol or genistein using the method of Chomczynski et al. (14) as modified by Xie and Rothblum (15). Ten µg of RNA were used for Northern blot analysis.

Probe for pS2. A plasmid containing a 559-bp cDNA fragment encoding full-length pS2 (16) was obtained from the American Type Culture Collection. PstI restriction endonuclease digestion of the plasmid (16) produced DNA fragments of 4.4, 0.32, and 0.24 kb. These fragments were isolated from the agarose gel slice using methods adapted from Moore et al. (17). Twenty-five ng of pS2 cDNA (0.32 and 0.24 kb) and 50 µCi of [α-32P]dCTP were used to label the DNA by the random primed labeling method using a commercial kit and following the manufacturer’s instructions (Life Technologies, Inc., Gaithersburg, MD).

Northern Blot Hybridization and Detection. For detection of pS2 expression, 10 µg of total RNA were separated on 1.2% formaldehyde agarose gels and transferred to a Hybond-N Nylon membrane (Amersham, Arlington Heights, IL). The RNA was cross-linked onto the membrane using UV light for 3 min by a 25-W transilluminator (Hoefer Scientific Instruments, San Francisco, CA; wavelength, 312 nm). The membrane was then hybridized with the 32P-labeled DNA probe, and hybridizing DNA molecules were detected by performing autoradiography. Northern blots were also probed for glyceraldehyde-3-phosphate-dehydrogenase cDNA (Clontech Laboratories, Inc., Palo Alto, CA) as a housekeeping gene to confirm equal loading of RNA among treatment and control groups.

In Vivo Studies

Diet Selection. Diet selection was based on recommendations of the AIN (18). The AIN-93G semipurified diet has been established as meeting all of the nutritional requirements of mice. One concern for the use of a commercial diet is the presence of phytoestrogens from plant sources such as soybean meal. Protein in the AIN-93G diet is derived from casein; thus, the potential interference of phytoestrogens in soy-based protein supplements is eliminated.

Protein Pellet Preparation. Each pellet of estradiol contained 2 mg of 17β-estradiol mixed with 18 mg of cholesterol as a carrier. The 20-mg mixture containing estradiol and cholesterol was placed into a pellet mold and pressed into a compact. It was approximately 4.5 mm in diameter and approximately 2.5 mm in depth. These pellets then were placed s.c. in the interscapular region of mice as described previously (19).

Analysis of Uterine Weight and Mammary Gland Growth in Ovariectomized Athymic Female Mice Fed Genistein. Athymic nude mice (Harland, Indianapolis, IN) were ovariectomized at 21 days of age. Dietary treatments were initiated at 28 days of age for a period of 5 days. Mice were fed the AIN-93G semipurified diet containing estradiol at 1 ppm or genistein at 750 ppm. Control animals received only the AIN-93G diet. Mice were killed, and the uteri were removed for assessment of wet weights and mammary gland growth. Mammary glands were removed and fixed and stained as whole mounts (20). Mammary gland growth was assessed by determining the size and number of end buds and the extent of ductal development as viewed under a dissecting scope (21).

Analysis of Tumor Growth in Athymic Nude Mice Fed Genistein. Female 21-week-old athymic nude mice (Harland) were allowed to acclimate to 1 week. Mice were ovariectomized at 28 days of age and then allowed to recover for 7 days before any treatment. Estradien-dependent human breast cancer (MCF-7) cells were grown to confluence and collected using trypsin-EDTA. Cells were counted using a hemocytometer (Fisher Scientific, Pittsburgh, PA) and diluted in culture media to a concentration of 1 × 106 cells/ml.

An aliquot of cell suspension (100 µl) was injected into four sites in the flank of 35-day-old female athymic mice. MCF-7 cells will not produce tumors in ovariectomized athymic nude mice unless the mice are supplemented with estrogen. Estradiol was administered in pellets containing 2 mg of estradiol (19, 22) implanted s.c. in the interscapular region of each mouse. Tumors were allowed to develop for 5–6 weeks and were measured weekly, and the cross-sectional areas were determined using the formula described previously (22). When tumors reached an average cross-sectional area of 35–40 mm², animals were divided into three treatment groups (four to six animals/group), with each group normalized for tumor number, tumor size, and animal number. Estradiol pellets were removed from all animals. Positive controls were reimplanted with a fresh estradiol pellet, negative control mice received the AIN-93G diet, and dietary treatments with 750 ppm genistein were initiated. Tumor areas were measured weekly for the duration of the study. At the completion of the study, mice were killed, and the uteri were removed for the assessment of wet weights. Blood was collected from the animals by cardiac puncture for the analysis of genistein content.

RESULTS

In Vivo Studies

Effect of Genistein on MCF-7 Cell Proliferation. To determine the minimum concentration of genistein that will stimulate or inhibit MCF-7 cell growth, we conducted a cell proliferation dose-response study. MCF-7 cells were monitored in response to estradiol (1 nM) and various concentrations of genistein ranging from 0.01–100 µM (Fig. 1). Data are expressed as a percentage of the control cell cultures. These levels were chosen because the genistein blood levels reported in animals and humans consuming diets high in genistein (such as soy-containing diets) have blood concentrations ranging from 0.1–6 µM (26). Estradiol (1 nM) stimulated cell proliferation 2.4-fold over that of the control MCF-7 cells. Genistein increased cell growth in a dose-dependent manner in the range of 0.01–1 µM. Maximal growth inhibition occurred at 100 µM (Fig. 1). Data are expressed as a percentage of the control cell cultures. These levels were chosen because the genistein blood levels reported in animals and humans consuming diets high in genistein (such as soy-containing diets) have blood concentrations ranging from 0.1–6 µM (26). Estradiol (1 nM) stimulated cell proliferation 2.4-fold over that of the control MCF-7 cells. Genistein increased cell growth in a dose-dependent manner in the range of 0.01–1 µM. Maximal growth inhibition occurred at 100 µM (Fig. 1).

Effect of Genistein on Estrogen-dependent pS2 mRNA Expression in MCF-7 Cells. To evaluate the potential of genistein to enhance the expression of an estrogen-responsive gene, we conducted
Northern blot analysis using RNA isolated from genistein-treated MCF-7 cells and probing with a human pS2 cDNA probe (16). The cell proliferation studies (Fig. 1) suggested that genistein acts via the estrogen receptor to enhance cell proliferation at low concentrations. Estradiol at concentrations of 0.2 nM and genistein at concentrations of 1–10 μM were observed to induce pS2 gene expression (Fig. 2). Additionally, we have observed an increase in pS2 mRNA expression by genistein at concentrations up to 50 μM (data not shown). These data indicate that genistein can act as a weak estrogen agonist in vitro as measured by estrogen-dependent (pS2) gene expression.

**In Vivo Studies**

**Effect of Genistein on Uterine Weight and Mammary Gland and End Bud Development in Ovariectomized Athymic Female Mice.** Mammary gland whole mounts were obtained from ovariectomized mice consuming either estradiol (1 ppm) or genistein (750 ppm) in the AIN-93G diet or from controls fed the AIN-93G diet only. Mammary gland growth occurs via ductal elongation with the end buds as the major growth points (26). Increases in the number and size of the end buds were observed in animals consuming estradiol and genistein (Fig. 3). The average end bud number was 2.9 ± 0.85 in the ovariectomized control group, 9.1 ± 0.67 in the estradiol group, and 6.4 ± 0.89 in the genistein-treated groups (Fig. 4). Mammary gland growth was significantly greater (P < 0.05) in mice fed genistein or estradiol when compared with that of ovariectomized controls. Genistein- or estradiol-treated animals were not significantly different from each other (P > 0.05). The uterine weight of the genistein treatment group was also increased in comparison with that of the control group (data not shown). These data suggest that dietary genistein has the potential to stimulate estrogenic responses in vivo.

**Effect of Genistein on Tumor Growth in Athymic Nude Mice.** Because genistein stimulated both mammary gland and uterine growth, it was of interest to determine the effects of an estradiol (2 mg) pellet or dietary genistein (750 ppm) on estrogen-dependent tumor growth. Ovariectomized athymic mice implanted with MCF-7 cell tumors were divided into three treatment groups after tumors reached an average cross-sectional area of approximately 55 mm².

The three treatment groups were: (a) negative controls (without estradiol pellets or genistein treatments); (b) positive controls (estradiol pellets); and (c) dietary genistein (750 ppm) treatment groups. Tumor growth was monitored weekly. Tumors in the positive control group grew rapidly, and after an additional 2 weeks on estrogen treatment, the average tumor cross-sectional area had reached 120 mm² (Fig. 5). After 2 weeks of estradiol treatment, the average tumor weight was 750 mg, and the total tumor weight/mouse was approximately 3 g (approximately 10% of the body weight). Therefore, mice were killed after 2 additional weeks of estrogen treatment due to the size of the tumors. After the estrogen pellets were removed in the negative control group, tumor growth stopped, and tumor size was maintained at an average cross-sectional area of approximately 58 mm² for an additional 12 weeks study (Fig. 5). In the dietary genistein treatment group, after the estrogen pellets were removed and the mice were switched to 750-ppm genistein diets, tumor growth increased. The average tumor cross-sectional area in the dietary genistein treatment group increased from 55 to 120 mm² after 12 additional weeks on genistein treatment. These MCF-7 cell tumors were similar in size to that of tumors after 2 weeks on estradiol treatment. These results indicated that dietary genistein acts as an estrogen agonist in vivo to stimulate the growth of estrogen-dependent MCF-7 tumor cells implanted into athymic mice.

**Plasma Genistein Concentration Analysis in Athymic Nude Mice Fed Genistein.** Plasma genistein levels were measured by HPLC. The concentration of free genistein in plasma was 0.24 ± 0.08 μM. The concentration of total genistein including free and conjugated forms in plasma was 2.1 ± 0.14 μM. These concentrations are within...
Genistein elicits a concentration-dependent dual threshold effect with regard to growth stimulation or inhibition on cultured estrogen-dependent human breast cancer (MCF-7) cells in vitro. At low concentrations of genistein in dextran-charcoal-stripped FBS, we observed a dose-dependent increase in MCF-7 cell proliferation from 0.01 to 1 μM (Fig. 1). Maximal growth stimulation was observed at 1 μM, and this level of growth was sustained up to 10 μM. At higher concentrations, we observed a dose-dependent inhibition in cell growth when concentrations were 25–100 μM. These results are consistent with those reported by Martin et al. (4), in which growth stimulation in MCF-7 cells was observed at a concentration of 200 nM, and Wang et al. (5), in which growth stimulation in MCF-7 cells was observed in a concentration-dependent manner between 10 nM and 1 μM. Wang et al. (5) also observed growth inhibition at concentrations higher than 10 μM. These results suggest that there are at least two dose-dependent mechanisms by which genistein can alter cell proliferation. One mechanism stimulates proliferation at low concentrations of genistein (10 nM to 1 μM) and is likely to be mediated via the estrogen receptor. The other mechanism, which is antiproliferative, is active at high concentrations of genistein (25–100 μM) and is likely to be mediated via anti-tyrosine phosphorylation and inhibition of cell cycle progression (6). The threshold for stimulation of proliferation

DISCUSSION

Genistein elicits a concentration-dependent dual threshold effect with regard to growth stimulation or inhibition on cultured estrogen-dependent human breast cancer (MCF-7) cells in vitro. At low concentrations of genistein in dextran-charcoal-stripped FBS, we observed a dose-dependent increase in MCF-7 cell proliferation from 0.01 to 1 μM (Fig. 1). Maximal growth stimulation was observed at 1 μM, and this level of growth was sustained up to 10 μM. At higher concentrations, we observed a dose-dependent inhibition in cell growth when concentrations were 25–100 μM. These results are consistent with those reported by Martin et al. (4), in which growth stimulation in MCF-7 cells was observed at a concentration of 200 nM, and Wang et al. (5), in which growth stimulation in MCF-7 cells was observed in a concentration-dependent manner between 10 nM and 1 μM. Wang et al. (5) also observed growth inhibition at concentrations higher than 10 μM. These results suggest that there are at least two dose-dependent mechanisms by which genistein can alter cell proliferation. One mechanism stimulates proliferation at low concentrations of genistein (10 nM to 1 μM) and is likely to be mediated via the estrogen receptor. The other mechanism, which is antiproliferative, is active at high concentrations of genistein (25–100 μM) and is likely to be mediated via anti-tyrosine phosphorylation and inhibition of cell cycle progression (6). The threshold for stimulation of proliferation

Fig. 3. Mammary whole gland mounts from ovariectomized athymic mice fed various dietary treatments. Mice were ovariectomized on day 28, and treatments began on day 35. Mice were fed (A) control (AIN 93-G), (B) estradiol (1 ppm)- or (C) genistein (750 ppm)-containing diets. Mice were killed on day 40, and mammary glands were removed for whole gland mount preparation as described in "Materials and Methods." Note the increase in the size and number of end buds indicated by arrows. Numerical data are presented in Fig. 4.

Fig. 4. Effect of genistein on end bud development in ovariectomized athymic female mice. Mice were ovariectomized on day 28, and treatments were initiated on day 35. Mice were fed control (AIN 93-G), genistein (750 ppm)- or estradiol (1 ppm)-containing diets. Mice were killed on day 40, and mammary glands were removed for whole gland mount preparation as described in "Materials and Methods." Each bar represents the mean ± SE of four mice/experimental group. *, P = 0.05 that estrogen- and genistein-treated groups had higher numbers of end buds than did controls, as determined by ANOVA.

Fig. 5. The effect of estrogen pellet (2 mg) and dietary genistein (750 ppm) on MCF-7 tumor growth in athymic nude mice. MCF-7 human breast cancer cells were injected s.c. into four sites on the flanks of mice at 1 × 10⁶ cells/sites. After tumors had formed, the mice were grouped to equalize the tumor area, and dietary treatment was initiated. Experimental groups included negative control AIN-93G (5 mice; n = 15 tumors), positive control implanted with a 2-mg estrogen pellet (5 mice; n = 17), and AIN-93G + 750 ppm genistein (5 mice; n = 17 tumors). Data are expressed as changes in tumor areas for each week of measurement. The treatment by week interaction is statistically significant (P < 0.0001). Treatment means for each week were compared using the least significant difference method.
was in the concentration range required to stimulate expression of pS2 mRNA (Fig. 2). Previous studies have determined that tamoxifen inhibits the induction of pS2 gene expression (5). Additionally, in our studies, pS2 gene expression was enhanced at concentrations up to 50 μM. However, cell proliferation was inhibited at concentrations above 25 μM. These observations suggest that the estrogen receptor-mediated mechanism is active at the higher concentrations; however, the growth-inhibitory mechanism associated with genistein overrides the growth-stimulatory effects from genistein.

Genistein binds to the estrogen receptor with an affinity approximately 100-fold less than that of estradiol (5, 23). To further confirm whether genistein is acting via an estrogen receptor mechanism, we evaluated the expression of the pS2 gene in cultured MCF-7 cells. The human pS2 gene was initially characterized as a gene whose expression is specifically controlled by estrogen in the breast cancer cell line MCF-7 (27). The increase in pS2 mRNA after the addition of estradiol to the culture medium is a primary transcriptional event, suggesting that control of pS2 gene promoter activity by the estrogen receptor is mediated by a cis-acting estrogen-responsive element that could be located in the 5'-flanking region of the pS2 gene (16). The results on pS2 stimulation by genistein (Fig. 2) are consistent with the results observed on cell proliferation in that 1 μM (Fig. 1) was effective at increasing proliferation and pS2 gene expression. Additionally, Wang et al. (5) reported that tamoxifen inhibits genistein-stimulated pS2 gene expression in MCF-7 cells. These data further support that genistein can act via an estrogen receptor-mediated mechanism.

Here, we report that dietary genistein (750 ppm) fed to 28-day-old athymic ovariectomized mice will stimulate mammary gland growth (Figs. 3 and 4). This is the first report in which dietary genistein has been shown to enhance mammary gland growth in vivo. Reports (9, 28) in which high doses of genistein were administered s.c. to rats demonstrate that genistein stimulates mammary gland differentiation, promoting lobular-alveolar development in immature Sprague-Dawley rats. No studies have been reported in which the effect of genistein was evaluated for its potential to stimulate the growth of estrogen-dependent tumors in vivo. The studies presented here provide compelling evidence that MCF-7 estrogen-dependent tumors can be stimulated to grow in ovariectomized athymic host mice supplemented with dietary genistein (750 ppm). It is important to point out that both estrogen and genistein have been shown to be chemopreventive in the carcinogen-induced rat DMBA animal model (9, 28, 29). Neonatal administration of estrogen to rats has been shown to reduce incidences of DMBA-induced mammary tumors (29). In the studies with genistein, three s.c. injections of genistein were given to young rats, which reduced the number of mammary tumors initiated by DMBA. Naganawa et al. suggest that genistein acts as an estrogen agonist to stimulate mammary gland differentiation. This enhanced mammary gland differentiation may be the mechanism responsible for the chemopreventive effects of genistein in the rat DMBA mammary carcinogenesis model. Thus, there seems to be a paradox with regard to the action of genistein on tumor growth in rodent models. Our results in the ovariectomized athymic mouse suggest that genistein stimulates mouse mammary gland growth without maturation, which is different from the effect of genistein in the immature rat, in which genistein stimulates prolactin secretion (23) and ultimately results in lobular-alveolar development and mammary gland maturation (26). Genistein may act similar to estradiol, because estradiol is known to act to reduce tumor incidence by chemoprevention (30), whereas there are numerous reports (10) that indicate that estrogens act as tumor promoters. Thus, whether genistein acts as a chemopreventative agent or as an agent to stimulate tumor growth will likely depend on the timing of genistein administration.

The present study focused on the estrogenic effect of genistein in vitro and in vivo systems. Genistein binds to the estrogen receptor with an affinity approximately 100-fold lower than that of estradiol, resulting in enhanced proliferation activity. In cultured cells, genistein stimulates MCF-7 cell growth at concentrations as low as 100 nM, achieving similar effects to that of estradiol at 1 nM (Fig. 1). We observed a 240% increase in growth when treated with 100 nM genistein compared with that of the control. Expression of the estrogen-responsive gene pS2 was also induced in response to treatment with genistein at concentrations higher than 100 nM (Fig. 2). In vivo, using ovariectomized athymic nude mice implanted with estrogen-responsive MCF-7 cells, we have demonstrated that treatment with 750 ppm dietary genistein was able to stimulate the growth of these estrogen-responsive MCF-7 cell tumors in the absence of estrogen (Fig. 5). Plasma genistein was 2.1 μM, of which 0.24 μM present was in the free form. These concentrations are above the concentration required for MCF-7 cell growth stimulation observed in vitro. One can predict from the in vitro cell growth studies that the dosage of dietary genistein used in these studies will produce a blood concentration of genistein that will stimulate the growth of implanted MCF-7 cell tumors. Thus, these results are consistent with in vitro cell culture studies. Even if the conjugated form is biologically active, these levels are below the high concentrations (above 25 μM) required in vitro to produce a dose-dependent decrease in MCF-7 cell growth. It is unlikely that the concentration required to inhibit MCF-7 cell growth can be achieved in vivo.

In summary, genistein can act as an estrogen agonist resulting in the proliferation of human breast cancer cells (MCF-7) in vitro and enhanced growth of MCF-7 cell tumors implanted into ovariectomized athymic nude mice in vivo. Thus, there is the potential for dietary genistein to stimulate the growth of estrogen-dependent tumors in humans with low circulating endogenous estrogen levels, such as those found in postmenopausal women.

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REFERENCES

GENISTEIN STIMULATES TUMOR CELL GROWTH IN VIVO

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