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BY

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ABSTRACT

Genistein and Mammary Tumorigenesis

BY

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Soybeans contain a high level of isoflavones including genistein, daidzein and glycetein. I have focused on genistein due to its estrogenic and antiproliferative activities which could affect mammary tumorigenesis. Dietary genistein (750 µg/g American Institute of Nutrition semi-purified diet, AIN-76A) increased uterine weight and uterine c-fos mRNA expression, increased plasma prolactin, and stimulated lobular/alveolar development of the mammary gland in ovariectomized Sprague Dawley rats. The plasma concentration of total (free + conjugated) genistein was 2.54 µM. Competitive binding studies indicated that genistein competes with estradiol for the estrogen receptor with an affinity approximately 1/100th that of estradiol. However, genistein (750 µg/g AIN-76A) did not antagonize the action of concurrently fed estradiol (1.0 µg/g AIN-76A) in ovariectomized rats or in intact peripubertal rats fed through 30-44 days of age.

Genistein exerts antiproliferative effects on both estrogen dependent and independent cell lines. I elected to study the effects of genistein on the growth of estrogen independent MDA-MB-231 human breast cancer cells. Genistein (20 μM) inhibited cell proliferation *in vitro* by 50%. The cell cycle was blocked at G_2/M when 40 μM or 80 μM genistein was added to the medium. To evaluate the effect of genistein *in vivo*, female athymic mice were inoculated with cells and fed genistein (750 μg/g AIN-93G). Genistein at this dose did not affect tumor growth. Genistein (3000 μg/g AIN-93G) was then fed to tumor bearing mice. This dose inhibited tumor growth when compared to untreated control mice; however, there was approximately a 10% reduction in food intake in the genistein group. Weight gain did not differ. Total plasma genistein concentration was 5.88 μM. The effect of dietary genistein on initial tumor development was studied by feeding mice 750 μg genistein/g AIN-93G before tumor cell inoculation. This dose of genistein did not inhibit tumor development or growth.

This research demonstrates that dietary genistein is estrogenic in estrogen responsive tissues in ovariectomized rats. Genistein inhibited cell growth in culture by blocking the cell cycle at G_2/M. In addition dietary genistein (3000 μg/g AIN-93G) inhibited MDA-MB-231 cell growth in athymic mice.

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INTRODUCTION

This dissertation presents data on the soy phytoestrogen genistein and its effect on estrogen responsive tissues in the female Sprague Dawley rat. Genistein's effect on estrogen independent MDA-MB-231 human breast cancer cell growth was studied in cell culture and in athymic female mice inoculated with MDA-MB-231 cells.

The impetus for these studies came from epidemiological data that point out a geographic variation in the site specific incidence of various cancers (Kodama et al. 1991, Staszewski et al. 1971, Wynder & Hirayama 1977). For example, the incidence of mammary cancer is much higher in US women than it is in Japanese women. It is also clear from the epidemiological data that, in immigrant populations, the site specific tumor incidence present in a home country will, over time, change to that of the host country (Staszewski et al. 1971). For example investigators have shown, that in each of two subsequent generations of Japanese immigrants to the US the incidence of mammary cancer in women has risen to levels approaching that in the US (Buell 1973). These data suggest that environmental factors, including diet, may be largely responsible for the change in tumor incidence patterns. Dietary habits of

immigrant populations also tend to change over time, eventually reflecting those in the host country. For example the dietary habits of Japanese migrants to Hawaii change from one in which vegetables (including soybeans) are primarily consumed with little meat, as in their home country, to one of less vegetable consumption, including soy, and more meat common in the US (Hankin et al. 1975). Thus the change in dietary habits that occurs concurrently with changes in site specific tumor incidence suggest that diet could play a major role in the etiology of this disease. A review of the literature covering the epidemiology and etiology of cancer and the role of the diet, especially soybeans and specifically the soy phytoestrogen genistein, in these processes is presented in Chapter 1 of the dissertation.

Genistein has estrogenic activity in uterine tissue and has been shown to antagonize estrogen in this tissue (Folman & Pope 1966); however, its affect upon the mammary gland and the hypothalamic/pituitary axis, with respect to plasma prolactin concentration, in adult rats is unknown. It is clear that estradiol is required for mammary gland development and is also required for estrogen dependent mammary tumorigenesis in many species. Therefore, compounds with estrogenic activity such as genistein may promote

mammary gland development and may also stimulate estrogen dependent mammary tumorigenesis. Our overall objective in the studies presented in Chapter 2 was to determine the in vivo activity of dietary genistein by analyzing its effect on estrogen responsive tissues in the rodent. It is our hypothesis that dietary genistein will exert estrogenic effects in the uterus, mammary gland and the hypothalamic/pituitary axis. Results from experiments conducted to ascertain the estrogenic activity of various doses of dietary genistein in estrogen responsive tissues in ovariectomized and intact female rats are presented in Chapter 2.

During the time the studies in chapter 2 were being conducted there were additional reports on genistein's ability to inhibit the in vitro proliferation of both estrogen dependent and independent cells. I decided to pursue the antiproliferative effects of genistein. Since the antiproliferative effects were observed in estrogen dependent and independent cells I decided to conduct our in vitro and in vivo studies using estrogen independent MDA-MB-231 human breast cancer cells. By using estrogen receptor negative cells the potential estrogenic activity of genistein in affecting estrogen dependent tumor formation and growth is eliminated. Cell culture studies were

conducted to determine a possible mechanism for genistein inhibiting tumor cell growth and animal studies were conducted to determine whether similar effects could be observed in animals. Our overall objective in these studies was to determine the effect of genistein on the growth of MDA-MB-231 cells *in vitro* and *in vivo*. It is our hypothesis genistein will inhibit the *in vitro* proliferation of MDA-MB-231 cells and the *in vivo* growth of MDA-MB-231 cell inoculated into the athymic mouse. Results from experiments conducted on genistein's effect on the proliferation and cell cycle of MDA-MB-231 cells *in vitro* and the growth of these cells in athymic mice is presented in Chapter 3.

A number of preliminary studies not included in chapters in the dissertation were conducted to assist in methods development and designing future experiments. Procedures and data from these studies are presented in the appendices.

Chapter 1

Literature Review

A. Cancer Epidemiology

Data from population studies show a wide geographical variation in the incidence of site specific tumors, suggesting that environmental factors play a major role in the etiology of this disease. Indeed there are certain cancers that are classified as 'Western cancers' due to their increased incidence in the Western world. For example, Kodama et al. (1991), found that bladder cancer is higher in western countries than in Japan. Furthermore, bladder cancer was positively related to the occurrence of other western cancers, notably colon and lung, and negatively related to the occurrence of non-western cancers such as stomach and esophageal. Studies of Japanese immigrants to the US have shown an increase in the incidence of bladder cancer and a decrease in the incidence of stomach cancer with both figures approaching the tumor incidence pattern found in US whites (Kodama et al. 1991). The incidence of stomach cancer is high in Poland whereas the incidence of intestinal and breast cancer is low. However the tumor incidence profile of Polish immigrants to Australia, where tumor incidence patterns are the inverse of

that in Poland, eventually changes to those of the host country, again implicating environmental causes (Staszewski et al. 1971, McMichael et al. 1980). Other studies have shown similar trends in immigrants to the US regarding cancer of the prostate, colon, breast, bladder and uterus (Wynder & Hirayama 1977).

1. Breast Cancer Incidence

Breast cancer is relatively uncommon in Japan whereas in the US it will afflict one in eight to ten women (Claus et al. 1991, Marshall 1993). Approximately 5-10% of breast cancer is thought to be due to inherited defective genes which predispose women to the disease (Claus et al. 1991). For example mutations in chromosome 17q, the locus of the BRCA1 gene which codes for a protein that functions as a tumor suppressor, are linked to early inherited breast cancer in families (Miki et al. 1994). The remaining 90% of breast cancer incidence is believed to be caused by environmental factors such as diet, lifestyle and exposure to environmental contaminants (Wright 1990, Lynch et al. 1992).

As mentioned in the preceding section site specific cancer incidence patterns can change as a population migrates from their home country to the host country: this

is also true for breast cancer. Early work (Haenszel & Kurihara 1968), using data from 1959-62 to study cancer incidence patterns in Japanese immigrants to the US, did not find a change in breast cancer yet did find increases in cancer of the colon, pancreas, lung, and ovary. Initially these data lead to speculation that genetic differences in these populations accounted for the difference in breast cancer incidence among these countries; however, later work (Buell 1973) using data from 1969-71 did show an increase in the incidence of breast cancer in female Japanese immigrants, albeit occurring at a slower rate than that for cancers of the colon and lung. There was an increase in breast cancer in first generation offspring (Issei) of Japanese immigrants to the US and a further increase approaching that in the US in the second generation (Nisei)(Buell 1973). The research by Buell, in which breast cancer incidence increased in a population upon migrating, discounted population based genetic differences as the basis for the difference in breast cancer incidence between Japanese and US women. This suggests other factors, such as changes in the environment, including diet, could be responsible.

2. Diet and Cancer

Dietary choices made throughout life can affect the well-being of the individual both in terms of their present and future health. Examples of the potential role of diet in carcinogenesis include: ingesting carcinogens or metabolic activation of dietary carcinogenic precursors, dietary manipulation of gut transit time thereby decreasing or increasing exposure, and ingesting compounds that interfere with tumorigenesis thereby inhibiting initiation, promotion or progression.

Doll & Peto (1981) suggest that the diet's role in tumorigenesis has been underestimated and that from 10-70% of cancers could be prevented by dietary intervention. Epidemiological studies have shown that the consumption of yellow and green vegetables is inversely related to the incidence of cancer (lklk et al. 1990). Furthermore these same studies have shown a positive correlation between meat and fat consumption and tumor incidence. Epidemiological studies which analyzed a countries breast cancer mortality rate and fat consumption have found that these two factors are positively correlated (Carroll 1975).

Studies have sought to determine a mechanism for the relationship between breast cancer and fat by analyzing the relationship between dietary fat and serum estrogen levels,

the rationale being increases in serum estrogen can lead to an increase in tumor formation in the estrogen responsive mammary gland. When fat intake in both premenopausal and postmenopausal women was decreased from ca. 40% of dietary calories to ca. 20%, serum estradiol concentration was reduced; however, the reduction occurred concurrently with significant reductions in body weight and caloric intake (Rose et al. 1987, Ingram et al. 1987, Prentice et al. 1990). Androgen aromatization to the estrogens can occur in adipose tissue thus weight loss (decrease in adipose tissue) could account for the decrease in estradiol and the role of dietary fat in this process would be secondary to the decrease in body weight brought about by caloric reduction. So the effect of fat in independently lowering serum estradiol, as opposed to weight loss or caloric reduction, is unclear.

Numerous animal studies have shown that as the percent of fat in the diet is increased the incidence of tumor formation also increases. However, by increasing the fat content of the diets in these experiments the diets were no longer isocaloric. This is because the body metabolizes dietary carbohydrate (CHO) and fat differently. For example, in de novo fat synthesis from CHO, CHO must be broken down to acetyl CoA and then the acetyl CoA is used

for fat synthesis (an energy requiring process). Dietary fat, on the other hand, is efficiently converted to body fat requiring less energy. So although the diets are isocaloric when determined using Atwater values they are not equivalent metabolically. It is therefore difficult to separate effects due to caloric intake, a reduction of which is known to inhibit tumor development, from fat intake (Welsch 1994).

Caloric reduction has been shown to alter the hormonal milieu in rodents and this could affect development of hormone responsive tumors (Boutwell et al. 1948). For example prolactin, estrogen and insulin are decreased in chronically, caloric restricted rodents; whereas, the glucocorticoids are higher (Pariza & Boutwell 1987). Estrogen and prolactin both enhance mammary tumorigenesis and decreasing their concentration, as a result of caloric reduction, could inhibit tumorigenesis.

Research in humans exploring the relationship between dietary fat intake and breast cancer has yielded conflicting results. An ongoing prospective study (Willett et al. 1992) involving over 80,000 nurses of which ~1500 had breast cancer, has not found an association between dietary fat intake and the incidence of breast cancer. Fat intake in this study varied from less than 29% to over 49% of calories. A prospective study by van den Brandt et al.

(1993) involving over 60,000 postmenopausal women of which 471 cases of breast cancer were available for analysis, has also failed to find an association between total dietary fat and breast cancer. However in this study saturated fat was weakly associated with breast cancer ($p = 0.052$). In this study fat intake varied from 32% to 46% of total calories. Many other studies have failed to show a link between the amount of fat consumed and breast cancer incidence in humans (Lee et al. 1991, van den Brandt et al. 1993, Graham 1982, Graham et al. 1991).

Contrary to these reports, a prospective study conducted by Howe (1991), involving over 55,000 women of which 519 breast cancer cases were available for analysis, did find a positive association between total fat intake and breast cancer. In this study saturated fat was not associated with breast cancer however monounsaturated fats were associated with breast cancer ($p = 0.04$). In this study relative risk for breast cancer from dietary fat was actually lower in the second quartile (0.73) and slightly lower in the third quartile (0.98) while in the fourth quartile the relative risk rose to 1.3.

There are a number of studies which suggest dietary fat is associated with breast cancer (Marshall et al. 1992, Howe et al. 1991) and a number of studies that have not found an

association. At present the role of fat in the development of breast cancer is not clear.

It is possible that as migrant populations adopt the dietary habits of their host country, this change in diet eventually gives rise to the site specific tumor incidence pattern seen in the host country. Whether the absence of protective compounds found in their native diet or the presence of harmful compounds in their new diet are responsible for the change in cancer incidence patterns is unknown. It is known however, that as immigrants adopt the new dietary habits of the host country they also give up those of their home country. These changes, with respect to the Japanese, are reflected in the consumption of more meat and fat, and less plant products including soy products (Nomura et al. 1978, Hankin et al. 1975).

Plants contain nutrients and non-nutrients which have demonstrated beneficial effects upon processes thought to be involved in tumorigenesis. For example carotenoids, a group of micronutrients found in vegetables and fruits (Khachik et al. 1995), have demonstrated anticarcinogenic activity in animal experiments (Nishino 1995). Monoterpenes (Gould 1995), found in plants, and polyphenols (Stoner & Mukhtar 1995), found in plants and various teas, have also been shown to inhibit tumorigenesis. Soybeans also contain a

number of compounds (discussed below) that have demonstrated anticarcinogenic abilities.

Epidemiological studies have found that concurrent changes in tumor incidence and dietary habits accompany a change in geographical location. These observations implicate environmental factors, including diet, in the etiology of breast cancer. This is the premiss for the proposed research: are nutrients consumed in an immigrants home country responsible for the lower incidence of breast cancer and upon migrating, when dietary habits of the home country are no longer followed, the lack of these nutrients responsible for an increase in breast cancer? For example does the consumption of soy products in the traditional Japanese diet offer some protection against breast cancer and when consumption of these products decrease the protective effect is lost?

a. Soybeans and Cancer

As immigrant populations assimilate into the host country they eventually adopt dietary practices of the host country while at the same time forgo the traditional dietary habits of their home country (Hankin et al. 1975). With regard to the Japanese this is especially true for soy products. Soy products constitute an integral part of the

traditional Japanese diet (Wynder & Hirayama 1977, Hankin et al. 1975) with 1-2 servings consumed per day. Research has shown that the consumption of soy inhibits tumorigenesis in rodent models. For example, Barnes et al. (1990) found that powdered soybean chips and autoclaved powdered soybean chips, a process that destroys much of the protease inhibitors (Dipietro & Liener 1989), both led to a decrease in mammary tumor formation in rats subjected to 7,12-dimethylbenz[a]anthracene (DMBA) and nitroso-methyl urea (NMU) chemical carcinogens. Hawrylewicz et al. (1991) showed a similar decrease in tumor incidence when rats were fed a diet containing 19% soy protein isolate. Baggott et al. (1990) also showed similar results in rats treated with DMBA and then fed an AIN-76A diet supplemented with miso (soy soup) at 25%. Troll et al. (1980) has shown that mammary tumor incidence in irradiated rats was reduced by feeding a 50% soybean diet; however, in this study food intake was also decreased.

Soybeans and soy products contain compounds such as protease inhibitors, phytic acid and isoflavones which are thought to inhibit tumorigenesis (Barnes et al. 1990, Kennedy & Manzone 1995). The majority of research conducted to date has concentrated on the Bowman-Birk protease inhibitor, and the isoflavone genistein.

1. Protease Inhibitors

Protease inhibitors are thought to inhibit carcinogenesis by binding to and inhibiting the activity of proteases involved in cell growth (negative transcription factors), secreted by tumor cells, or by decreasing protein absorption by inhibiting intestinal proteases; however, the exact mechanism is unknown (reviewed in Kennedy 1995). Natural protease inhibitors, including the soybean trypsin inhibitor (SBTI), inhibited transformation of irradiated C3H/10T1/2 mouse embryo fibroblast cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Kennedy & Little 1981). The authors speculated that the protease inhibitors inhibited the action of TPA induced proteases involved in carcinogenesis. Cell culture experiments have shown that the soybean derived Bowman Birk protease inhibitor (BBI), an 8 kd peptide, will inhibit cytosolic proteases involved in cell growth (Billings & Habres 1992). BBI has also been shown to inhibit transformation of C3H/10T1/2 cells <u>in vitro</u> when administered immediately after carcinogen exposure (St. Clair & St. Clair 1991), or after radiation (Kennedy 1985); however, it is ineffective when given during or days after carcinogen exposure. Furthermore, modified BBI, no longer able to inhibit trypsin activity, was just as effective in inhibiting radiation induced transformation however when BBI

was modified to no longer inhibit chymotrypsin it lost the ability to inhibit transformation (Yavelow et al. 1985). This suggests that BBI inhibits proteases similar to chymotrypsin. BBI also inhibits the formation of a number of different tumors in laboratory animals treated with a variety of chemical carcinogens (Troll & Kennedy 1989, reviewed in Kennedy 1995). For example, dietary BBI (0.1% of the diet) inhibited dimethyhydrazine (DMH) induced colon cancer in mice whereas autoclaved BBI (a process that destroys BBI) did not suggesting that the BBI was responsible (Billings et al. 1990). Research has shown cytoplasmic localization of dietary derived BBI in intestinal cells leading to speculation that it may be able to inhibit tumorigenesis at sites other than the gastrointestinal tract (Billings et al. 1991, Dipietro & Liener 1989). In fact studies have shown BBI to inhibit tumor formation in the liver and lung suggesting systemic effects (Kennedy 1995). Research by Hirayama (1982) on the relationship between gastric cancer and the consumption of soybean paste soup, has shown significant reductions in gastric cancer in those who consume daily amounts of the soup, a traditional component of the Japanese diet. The authors speculated protease inhibitors could be responsible for the effect. Barnes et al. (1990) found that powdered

soybean chips and autoclaved-powdered soybean chips (a process that destroys much of the protease inhibitors) (Dipietro & Liener 1989), both lead to a decrease in mammary tumor formation in rats subjected to DMBA or NMU chemical carcinogens, suggesting compounds other than the protease inhibitors (isoflavones) are responsible for the decrease in tumor incidence. However, Kennedy (1995) points out that the autoclaved soy chips still had a sufficient amount of BBI to inhibit tumorigenesis. The research to date suggests soybean derived protease inhibitors may inhibit tumorigenesis in <u>in vitro</u> and <u>in vivo</u> models; however their effect upon human carcinogenesis is unknown.

2. Phytic Acid

Phytic acid or inositol hexaphosphate (IP_6) is a natural antioxidant found in fiber rich foods, including soybeans, and is suspected of contributing to the apparent decrease in colon carcinogenesis in populations consuming high fiber diets (Alabaster et al. 1996). It is present in soybeans at up to 1.4% on a dry weight basis (Graf & Eaton 1990). IP_6 is absorbed quickly and distributed to various organs within one hour after ingestion (reviewed in Shamsuddin 1995). Its mode of action is unknown. One hypothesis suggests IP_6 inhibits oxidative damage (DNA damage) induced by iron by binding iron and keeping it in

the ferric state (Graf & Eaton 1990). Another hypothesis suggests IP_6 functions in signal transduction pathways by interacting with the intracellular inositol phosphate-3 (IP_3) pool and altering signal transduction (Shamsuddin 1995). IP_6 has been shown to inhibit <u>in vitro</u> cell growth and azoxymethane induced colon cancer in rats (Shamsuddin 1995). In addition IP_6 fed to mice on high-fat-high-calcium or high-fat-high-iron diets reduced the labeling index (indicator of cell proliferation) in the colon and breast to that of the low-fat group (Thompson & Zhang 1991). It was suggested that the calcium and iron were bound by IP_6 and that this inhibited tumor growth. This study illustrates an important feature of IP_6, its ability to bind divalent cations. Although there have been very few published studies on the role of soy-derived phytic acid in carcinogenesis, phytates have been studied for their ability to bind divalent cations. Binding of divalent cations, particularly calcium and iron, could inhibit the absorption of these minerals and have a negative impact on nutrition.

3. Isoflavones

Soybeans contain many aromatic compounds, flavones and isoflavones, distributed in varying concentrations throughout the plant (Graham 1990). The quantity of these compounds varies depending upon growing conditions,

location, and variety of soybean (Eldridge & Kwolek 1983). These are thought to protect the plant from infection, regulate cellular processes, and also aid in the symbiotic process of nitrogen fixation (Sadowsky et al. 1991, Parniske et al. 1991). Genistin and its aglycone genistein account for the majority of the isoflavones found in soybeans with ~2-3 mg/g (Eldridge & Kwolek 1983). The isoflavones remain intact, to a large degree, upon processing of soybean into soy products such as flour, tofu, soymilk and miso (Murphy & Wang 1993). Substantial quantities of these isoflavones have been identified in the urine of primates (Adlercruetz et al. 1986) and humans (Adlercruetz et al. 1991, Axelson et al. 1984) consuming soy products. Of the isoflavones most research has focused on genistein which has demonstrated the most promise in inhibiting tumorigenesis.

B. Genistein

Genistein is an isoflavone with a molecular weight of 270.2 and a melting point of 297±8^{0}C (The Merck Index 1989). It is insoluble in water and oil but freely soluble in alcohol. Genistein and its glucose conjugate genistin are found in many plants, particularly soybeans where they are found at concentrations of 1-3 mg/g (Eldridge & Kwolek 1983). Genistein has demonstrated estrogenic effects

(Carter et al. 1955), antioxidant activity (Wei et al. 1995), topoisomerase II inhibition (Markovits et al. 1989), and tyrosine kinase inhibition (Akiyama et al. 1987).

1. Estrogenic Activity of Genistein

Phytoestrogens are a group of compounds found in plants that are capable of producing estrogenic effects. Some of the isoflavones in soybeans, genistein in particular, are also referred to as phytoestrogens due to their ability to mimic the effects produced by estrogen, albeit requiring much greater concentrations (Carter et al. 1955). Estrogens and estrogenic compounds such as the phytoestrogens elicit their effects through binding to and transforming estrogen receptors (ER) to a DNA binding form which binds to estrogen response elements leading to transcription of the estrogen responsive gene (Hirst et al. 1992, Reese & Katzenellenbogen 1991). Early assays assessed the estrogenic potency of compounds by monitoring the increase in mouse uterine weight upon feeding or injecting the compound (Farmakalidis et al. 1985, Folman & Pope 1966). Current assays utilize molecular approaches to assess estrogenic activity of a compound. For example, competitive binding studies quantitate the affinity of compounds, relative to estradiol, to the estrogen receptor. Genistein's affinity for the estrogen receptor is

reported to range from 1/50 to 1/1000 that of estradiol depending upon the species (Verdeal et al. 1980, Martin et al. 1978, Shutt & Cox 1972). Other assays assess the estrogenic effects of compounds by measuring their ability to increase proliferation of estrogen responsive cells (Makela et al. 1994), increase chloramphenicol acetyl transferase (CAT) expression of estrogen receptor-CAT constructs (Miksicek 1994), or expression of estrogen regulated proteins (Mayr et al. 1992).

a. Uterotrophic Effects of Genistein

The consumption of certain plants and plant products can result in impaired reproductive function in livestock. Over five decades ago a syndrome (clover disease), with effects ranging from temporary to permanent infertility, was described in sheep foraging upon subterranean clover in western Australia (Bennets et al. 1946). Additional studies have documented impaired reproductive function in a number of species (reviewed in Price & Fenwick 1985) including desert quail feeding upon desert brush (Leopold et al. 1976). Furthermore, a decrease in reproductive performance was observed in female rats fed either a soy-based diet or a diet supplemented with genistin (Carter et al. 1955), and in male mice fed genistin (Matrone et al. 1955). All of these effects were attributed to the phytoestrogen content of the

diets.

Phytoestrogens include the isoflavones, lignans, and other non-steroidal chemicals found in plants and plant products. These compounds can bind to the estrogen receptor and are thought to elicit their estrogenic effects through mechanisms similar to that of estradiol.

It was later discovered that genistein was responsible for the impaired reproductive performance seen in sheep ingesting subterranean clover (Bradbury & White 1951). Genistein is an isoflavone (4',5,7-trihydroxyisoflavone), which has estrogenic activity (Folman & Pope 1966), and is present in various plants including legumes (Naim 1974). Since the initial discovery of its estrogenic activity there has been a number of studies in which the effects of soy and genistein upon the uterus of mice and rats were evaluated (Farmakalidis et al. 1985, Carter 1953): all of these have demonstrated estrogenic effects except the work of Farmakalidis & Murphy (1984). In their study the potent estrogen agonist diethylstilbestrol also did not promote uterotrophic effects in this strain of mouse (CD-1).

b. Mammatrophic Effects of Genistein

Mammary development in the rat is controlled by estrogen, progesterone, growth hormone and prolactin (reviewed in Topper & Freeman 1980). Estrogen acts directly

at the mammary gland by inducing gene transcription and the subsequent synthesis of many proteins, including the progesterone receptor (Horwitz & McGuire 1978). Estrogen also acts indirectly through the induced synthesis and release of prolactin from the anterior pituitary gland which then elicits its mitogenic effects on the mammary gland (Jones & Naftolin 1990). Prolactin synthesis in the anterior pituitary gland is under tonic inhibition by dopamine produced in the hypothalamus (Jones & Naftolin 1990). Estrogen is thought to decrease the activity of tyrosine hydroxylase, thereby decreasing the concentration of dopamine in the hypothalamus and pituitary gland (Jones & Naftolin 1990) resulting in increased plasma prolactin. Removal of endogenous estrogen will result in regression of the mammary gland, particularly the lobulo-alveolar structures.

The estrogenic activity of genistein could affect the mammary gland in a manner similar to that of estradiol. There have not been any reports on the estrogenic effects of dietary genistein upon the mammary gland. However, there are recent data that show that genistein, when subcutaneously injected on days 23, 25, 27, and 29 postpartum into immature rats, will promote mammary gland development (Brown & Lamartiniere 1995). In this study

genistein increased cellular proliferation in the terminal endbuds, increased the number of Type I lobules (differentiated endbuds) and increased mammary gland size.

c. Antiestrogenic Effect of Genistein

The estrogenic effects of genistein upon the uterus are well known. However, genistein also has demonstrated antiestrogenic activity. When genistein was coadministered with estrone, genistein caused a suppression in the uterine weight increase seen with estrone alone, suggesting antiestrogenic effects (Folman & Pope 1966). These contradictory effects are not unusual for a compound. The chemical primarily used in the treatment of estrogen receptor positive breast cancer, tamoxifen, which is believed to compete with estrogen for the estrogen receptor, also has estrogenic properties when given to ovariectomized rats (Nicholson et al. 1988, Galman et al. 1990, Powers et al. 1989, Martinez-Campos 1986). Like tamoxifen, the estrogenic or antiestrogenic activity of genistein is dependent upon conditions in which it is used. For example, tamoxifen exerts uterotrophic effects in ovariectomized rats; however, in intact rodents tamoxifen does not appear to exert estrogenic effects but to the contrary can have antiestrogenic activity providing the dose is high enough. Genistein may also function in a similar manner.

2. Antioxidant Activity of Genistein

Metabolic reactions often produce reactive oxidized chemicals that can damage cell membranes and cause mutations in DNA. Genistein functions as an antioxidant and may potentially inhibit free radical formation thereby reducing cellular damage. For example, genistein suppressed H_2O_2 formation by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) in HL-60 leukemia cells (Wei et al. 1993). In these studies topical genistein also inhibited TPA induced H_2O_2 formation in mouse skin. Wei (1996) found that genistein inhibits oxidation of guanine residues in DNA. In this system 8-hydroxy-2'-deoxyguanosine formation (oxidized guanine) induced by UV light was inhibited when genistein was present in the reaction mixture. These experiments provide evidence that genistein may be able to inhibit oxygen radical formation and thereby decrease DNA damage. Genistein has also been shown to inhibit DNA adduct formation in mice treated with the mammary specific carcinogen DMBA (Giri & Lu 1995).

3. Genistein Inhibits Topoisomerase II

Topoisomerase II (Topo II) activity is required during replication of DNA. Topo II catalyzes double strand breaks in the DNA allowing the DNA to unwind to facilitate replication of the genome. Genistein (36-100 μM) inhibits

DNA topoisomerase II decantenation activity and stimulates Topo II:DNA complex dependent strand breaks (Yamashita et al. 1991, Markovits et al. 1989, Constantinou et al. 1990, Kondo et al. 1991, Okura et al. 1988). Genistein is thought to act in stabilizing the Topo II:DNA complex causing strand breakage. It does not intercalate, rather it is thought to interfere with the binding of ATP to Topo II. Topo II inhibitors are known to cause cells to differentiate (Constantinou et al. 1990). This has been observed in various cell lines and is confirmed by morphological changes occurring in the cell along with a change in proteins synthesized to those of a differentiated cell. Cell differentiation has also been demonstrated in cells treated with genistein. Whether cell differentiation is due to Topo II inhibition or tyrosine kinase inhibition is not clear. Some researchers have suggested that inhibition of tyrosine kinase activity was responsible for inducing cell differentiation (Rocchi et al. 1995, Watanabe et al. 1991, Honma et al. 1991). In the study by Honma (1991) genistein induced differentiation of cells transfected with v-abl (Honma et al. 1991). V-abl is thought to block differentiation of these cells. It was thought that genistein (18 μM) caused cell differentiation by blocking phosphorylation of v-abl on tyrosine residues thereby

inhibiting v-abl's kinase activity. The later studies did not look at Topo II activity nor did the earlier studies look at tyrosine kinase activity thus it is possible that genistein inhibited both activities leading to cell differentiation.

4. Genistein Inhibits Tyrosine Kinases

Protein kinase activity is essential in all cells. The activity of enzymes involved in signal transduction pathways are controlled by phosphorylating serine, threonine and tyrosine residues in the protein. Genistein does not inhibit serine or threonine kinases however genistein is a non-specific inhibitor of tyrosine kinases (Akiyama et al. 1987). In vitro studies have shown that genistein inhibits a number of tyrosine kinases with IC_{50}'s ranging from 1 µM to >100 µM (Akiyama et al. 1987, Geissler et al. 1990, Huang et al. 1992). Protein tyrosine phosphorylation occurs during mitogenic activation of cell membranous receptors and the subsequent activation of cytosolic kinases involved in the regulation of proliferation and control of the cell cycle in a variety of cell types. As a tyrosine kinase inhibitor genistein may interfere with the phosphorylation of tyrosine residues on various proteins, particularly those that function in signal transduction pathways, leading to changes in the activity of these proteins. Inhibition of

tyrosine kinase activity would result in changes in the regulation of cell proliferation and may play an important role in the prevention and treatment of cancer (Akiyama et al. 1987, Nakafutu et al. 1992). For example, genistein was conjugated to an antibody developed against the CD19 antigen found on leukemia cells. Treatment of mice with 25 µg of the immunocomplex eliminated 99.999% of the leukemia cells whereas the antibody by itself, 50 µg, had no effect (Uckun et al. 1995). It is thought the immunocomplex targeted genistein to the CD19 receptor causing inhibition of src tyrosine kinase activity associated with and induced through the CD19 receptor, thereby leading to death of the leukemia cells. Inhibition of tyrosine kinases may therefore have profound effects upon the proliferation of tumor cells.

C. Breast Cancer Etiology

Normal functioning cells are usually in a quiescent state of the cell cycle called gap zero (G_0). Stimulation of the cell causes entry into gap 1 (G_1) phase where the necessary proteins required in the replication of DNA (S phase) are synthesized. In late G_1 there is a checkpoint, between G_1 and S, called the restriction point where the cell decides whether conditions are appropriate to enter S phase and replicate the DNA. If conditions are not

satisfactory the cell will arrest in G_1 or S phase until conditions are corrected and then proceed. During S phase and into gap 2 (G_2) phase the cell monitors the progress of DNA synthesis and if all is well will enter into mitosis (M) phase where the cell divides. Cell division will not occur until the genome has been properly replicated and the necessary machinery required for mitosis is present. If this is not the case then the cell will arrest at G_2 or M phase.

Tumorigenesis occurs through stages in which a normal cell is transformed into an immortal cell. These stages are initiation in which the DNA is mutated, promotion in which the growth of the mutated cell is favored, and progression in which the cells acquire the ability for anchorage independent replication and growth. The following section briefly describes the role that genistein could play in initiation and progression while genistein's role in promotion is covered in much more detail. This is because the tumor model employed in my studies uses cells that are already tumorigenic and these cells do not progress to metastasis.

1. Genistein and Initiation:

Initiation can be defined as a mutation in the genome that when not corrected leads to a change in function or activity of the gene product that will confer a growth advantage to the cell. Initiation can result from autosomal or somatic mutations. Autosomal (inherited) mutations are responsible for a small number of cancers and at the present time cannot be prevented; however, detection and interventions can be implemented to delay or prevent the onset of tumorigenesis (Henderson 1993, Henderson et al. 1993). For example women with a family history of breast cancer due to the inherited defective BRCA1 gene may undergo mastectomy to prevent the development of breast cancer (Roberts 1993). Somatic DNA mutations, on the other hand, can be prevented given the agent responsible for the insult can be identified and avoided in the future. This implies somatic mutations occur from exposure to an environmental mutagen, an initiator. This is true in a large number of cancers; however, inherent errors in the replication of DNA account for some. During the normal cell cycle, errors in DNA replication with or without external stimulus, commonly occur but are usually corrected before or during the DNA synthesis phase (S-phase) of the cell cycle (Al-Khodairy & Carr 1992, Rowley et al. 1992). However, at times mutations

are not corrected and if these mutations occur in a gene whose product is responsible for regulation of cell proliferation, uncontrolled cellular proliferation may occur resulting in tumor formation. For example a mutation could result in the synthesis of a dysfunctional protein whose normal function is regulation of the cell cycle. The presence of this protein could disrupt timing of the normal cell cycle, decreasing the time allowed for DNA repair, resulting in the incorporation of a number of additional mutations in the genome. It is thought that the initial mutation infers a growth advantage to the cell, perhaps less responsive to negative growth factors, although at this point rarely is the cell transformed to a tumorigenic cell. However, promotion of this growth advantage causes instability in the genome making the cell prone to additional mutations which can eventually lead to a tumorigenic cell (Kaufmann & Kaufman 1993). For example mutations in the tumor suppressor gene p53 eliminate a checkpoint in the cell cycle. Normally p53 is induced following DNA damage which causes cell cycle arrest at G_1/S or G_2. Cell cycle arrest provides the cell with an opportunity to correct the damage. By eliminating this checkpoint cells are likely to replicate the mutated DNA potentially contributing to greater instability (chromosomal

rearrangement, gene amplification) in the genome (Hartwell 1992).

Cell specific mitogens are initially required to stimulate (promote) normal cells from quiescent G_0 phase to enter and progress through G_1 phase, afterwards they are not required for progression of the cell cycle through S, G_2 and M phases. Mutations (initiation events) occurring in genes that encode proteins involved in normal cell growth could result in the synthesis of a dysfunctional protein that could cause uncontrolled cell growth. For example a point mutation in the gene encoding the proto oncogene $p21^{ras}$ (a cytosolic tyrosine kinase) causes the protein to become oncogenic (constitutively active). $p21^{ras}$ is involved in signal transduction pathways (described below) that eventually culminate in cell proliferation. Tyrosine kinase activity is required for cell growth, thus a cell with mutated $p21^{ras}$ may acquire a growth advantage due to constitutively active tyrosine kinase activity. Studies have found that $p21^{ras}$ is mutated in rats treated with the mammary carcinogen DMBA (Zarbl et al. 1985). Genistein's ability to inhibit DNA adduct formation in mice treated with DMBA (Giri & Lu 1995) suggests genistein could prevent DNA damage and therefore possibly prevent the initial stages of tumorigenesis. Mutated $p21^{ras}$ is found in the MDA-MB-231

human breast cancer cell line (Kozma et al. 1987). Mutations in $p21^{ras}$ are uncommon in human breast cancers however mutations in the tumor suppressors p53 and Rb tumor suppressor proteins are common. For example, analyzes of breast tumor tissue have found that p53 is mutated in 61% and Rb in 35% of the samples (Yokota & Sugimura 1993). Whether genistein is capable of preventing mutations in these and other proteins is unknown.

2. Genistein and Promotion:

Initiated cells are still dependent on growth factors or other stimuli for proliferation, or require these factors to promote proliferation of the initiated cell. Promotion of genetically unstable initiated cells can lead to additional mutations in negative growth regulator (tumor suppressor) or positive growth regulator (proto oncogenes) genes and result in a tumorigenic cell that is no longer dependent upon growth factors or promoters for proliferation. For example, estradiol is thought to be required initially in mammary tumorigenesis and these tumors have a better prognosis; however, over time these cancers progress to an estrogen independent state associated with a very poor prognosis. The mechanisms responsible for a tumor progressing to hormone independence are not clear but could involve synthesis of a dysfunctional protein in the

signaling pathway of estradiol allowing growth independent of estradiol.

Estradiol functions as a mitogen in part by binding to the ER and inducing expression of several genes including the progesterone receptor (Horwitz & McGuire 1978) and transforming growth factor alpha (TGF-α)(Saeki et al. 1991). TGF-α binds to the epidermal growth factor receptor (EGFR) resulting in autophosphorylation of the EGFR on tyrosine residues which then serve as substrate for the binding of cytosolic proteins containing the src homology domain 2 (SH2) (Sierke & Koland 1993, Koch et al. 1991, Russell et al. 1992). This serves to localize the targets of EGFR to the EGFR where they are then activated by phosphorylation on tyrosine residues and eventually results in cell division (Zhu et al. 1992, Feng et al. 1993, Gale et al. 1993, Zhu et al. 1993). $p21^{ras}$, a proto-oncogene, is one protein that interacts with the EGFR (Gale et al. 1993, Li et al. 1993). Activated $p21^{ras}$ phosphorylates raf, a cytosolic kinase, on a tyrosine residue and activates it. Raf then phosphorylates and activates cytosolic mitogen activated protein kinases (MAPKK) which then phosphorylate and activate MAPK's $p42^{MAPK}$ and $p44^{MAPK}$ (Kyriakis et al. 1992). Nuclear $p42/44^{MAPK}$ phosphorylates a number of nuclear proteins including the transcription factors c-myc and c-jun (Pulverer et al.

1991). This causes the formation of active transcription complexes that initiate transcription of early genes whose products are necessary for cell cycle progression. One of the early genes required for entry into and progression through G_1 is cyclin D1 (Sherr 1993). Cyclins bind to and activate constitutively expressed kinases (cdk's). Cyclin D1 binds to cdk4 and cdk6 and, as the levels of cyclin D increase, activates the kinase activity of the cyclin D:cdk4 complex. One of the key proteins phosphorylated by cyclin D:cdk4 is the key negative growth regulator protein retinoblastoma (Rb). Rb, in the hypophoshorylated state, binds to the transcription factor E2F and prevents E2F from initiating transcription of early genes required for G_1 (reviewed in Weinberg 1995). Hyperphosphorylation of Rb by cyclin D:cdk4 causes dissociation of E2F and allows E2F to initiate transcription of genes required for G_1 to S transition. After the cells have passed through G_1 phase they will normally complete mitosis.

Mitosis occurs concurrently with the activity of M-phase promoting factor (MPF), a serine/threonine kinase, suggesting MPF is required for mitosis. MPF is composed of cyclin B and $p34^{cdc2}$. In G_2 cyclin B synthesis increases and forms a complex with the dephosphorylated form of $p34^{cdc2}$. The activity of the cyclin B:$p34^{cdc2}$ complex is then

controlled by phosphorylation and dephosphorylation of cdk $p34^{cdc2}$, the catalytic unit (King et al. 1994). If the intracellular conditions required for mitosis (intact duplicated DNA, cytoskeletal structure, proper cell mass) are met then mitosis will proceed.

The controlled activity of tyrosine kinases involved in signal transduction pathways is essential for normal cell proliferation. Uncontrolled activity of certain tyrosine kinases can produce a cell that is unresponsive to negative growth factors that normally suppress cell growth. In fact tyrosine kinase activity is increased in human breast cancer (Hennipman et al. 1989, Bolla et al. 1993, Lower et al. 1993). In addition many of the oncogenes identified thus far are tyrosine kinases (Smith et al. 1993). As a tyrosine kinase inhibitor genistein could potentially affect a number of tyrosine kinases involved in signal transduction pathways. For example, amplified or mutated $p185^{neu}$, an EGFR like membrane bound receptor with tyrosine kinase activity (Stern et al. 1986, Wildenhain et al. 1990), is present in 20% of human breast cancers (Borg et al. 1991). $p185^{neu}$ functions in a manner similar to that of the EGFR (discussed above) in that it activates $p21^{ras}$ (Ben-Levy et al. 1994) and initiates a signal transduction pathway involving raf and the MAPKK cascade, eventually resulting in cell

proliferation. Whether genistein can inhibit $p185^{neu}$ as it does the EGFR (1 μM) (Akiyama et al. 1987) is unknown; however, as a tyrosine kinase inhibitor genistein could play an important role in inhibiting the promotion of initiated cells. Genistein has also been shown to inhibit GDP GTP exchange on $p21^{ras}$ by inhibiting EGFR phosphorylation. Inhibition of EGFR induced responses can occur at the receptor or downstream of the receptor. For example the activation and activity of MAPK in stimulated human neutrophils is inhibited by 36 μM genistein (Torres et al. 1993). Genistein competes with ATP for the ATP binding site in tyrosine kinases (Akiyama et al. 1987). The ATP binding site domain is well conserved among tyrosine kinases thus genistein has the potential to inhibit many other unidentified tyrosine kinases, potentially functioning in control of cell growth, in addition to those described above (Hanks et al. 1988). Genistein, in addition to inhibiting the tyrosine kinase activity of the EGFR, inhibits the oncogenes v-abl (39 μM)(Geissler et al. 1990), $pp60^{v\text{-}src}$ (26 μM), and $pp110^{gag\text{-}fes}$ (24 μM). Genistein also inhibits fibroblast growth factor (FGF) and insulin induced $p21^{ras}$:GTP complex formation in rat pheochromocytoma cells (PC-12)(Nakafutu et al. 1992), and erythropoietin induced $p21^{ras}$:GTP formation in human erythroleukemia cells (Torti et

al. 1992).

In addition to genistein's ability to inhibit tyrosine kinase activity genistein also inhibits Topo II activity (discussed above). Many cancer chemotherapeutic drugs act by inhibiting topoisomerase II (Topo II) activity (Corbett et al. 1993). These drugs are thought to act by stabilizing the Topo II:DNA complex, by inhibiting Topo II's catalytic activity, leading to strand breakage and cell cycle arrest at G_2/M (Ishida et al. 1991). Genistein's inhibition of Topo II activity could therefore also inhibit promotion of initiated cells.

3. Genistein and Progression:

The third stage of carcinogenesis is progression. After the cells have been initiated and the growth of initiated cells promoted, the tumorous cells can progress to an invasive and sometimes metastatic state. These cells are characterized by a number of mutations in addition to changes in cellular activities, in particular the extent of vascularization (Marx 1993). The density of vessels in the tumor is highly predictive of the potential for metastasis. A vasculature must be established in order for a solid tumor mass to grow beyond 2-3 mm^2 in diameter (Folkman 1989). Genistein has been shown to inhibit angiogenesis <u>in vitro</u> (150 μM) and has also been shown to inhibit endothelial cell

proliferation (5 μM), thus genistein could prevent progression by inhibiting angiogenesis (Fotsis et al. 1993). In addition, genistein (1 μM) has been shown to inhibit the *in vitro* invasion of a highly metastatic murine mammary tumor cell line (Scholar & Toews 1994).

D. Antiproliferative Effects of Genistein:

If genistein is capable of inhibiting tumor cell growth *in vitro* then it may be able to inhibit tumor cell growth *in vivo*. Genistein has been shown to inhibit proliferation of both estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-468) human breast cancer cells *in vitro* (Peterson & Barnes 1991). Recent studies have also shown genistein will inhibit the growth of estrogen dependent MCF-7 adriamycin resistant cells and MDA-MB-231 estrogen independent human breast cancer cells with an IC_{50} of 7.74 μM and 15.02 μM respectively (Monti & Sinha 1994). Furthermore, genistein inhibited epidermal growth factor (EGF) stimulated AGS human gastric cancer cells with an IC_{50} of 7 μM (Piontek et al. 1993). Genistein has been shown to reversibly arrest cell cycle progression of human gastric cancer (HGC-27) cells at G_2/M (Matsukawa et al. 1993). Genistein also arrests the cell cycle at G_2/M in Jurkat T-leukemia cells while at higher doses blocked cell cycle progression through S phase

(Spinozzi et al. 1994). In this study genistein also induced apoptotic cell death. The phosphorylation of tyrosine residues in key cell cycle regulatory proteins control the progression of the cell through the cell cycle. The inhibition of tyrosine kinases by genistein may therefore be responsible for the observed effects on cell proliferation and the cell cycle. For example inhibiting tyrosine kinases involved in early G_1 or S phase could arrest the cells in G_1 and inhibiting the activity of cyclin B:$p34^{cdc2}$ could arrest the cells in G_2/M.

It is well established that genistein will inhibit proliferation of initiated tumor cells *in vitro*; however, the effect of genistein upon the growth of tumor cells *in vivo* is unknown. For example, can genistein be consumed in sufficient amounts to produce plasma genistein concentrations similar to those found to inhibit signal transduction pathways *ex vivo* and *in vitro* or cell proliferation *in vitro*?

E. Human Consumption of Genistein:

During the last 5 years there have been a number of studies in which the absorption, plasma concentration and excretion of soybean isoflavones has been analyzed in humans. These studies suggest that dietary genistein is

absorbed intact and that it may be possible for genistin, the glycoside of genistein, to be hydrolyzed in the gastrointestinal system to the aglycone genistein and then absorbed. Glucoronic and sulfated genistein conjugates have been found in the urine of primates and humans consuming soy products. Furthermore, genistein and genistein conjugates have been found in the plasma of humans. Today it is clear that genistein is absorbed intact and is therefore potentially capable of exerting biological effects in humans, provided adequate concentrations necessary to elicit biological effects are achieved. Clearly genistein concentrations necessary to exert biological effects in rodents have been produced through dietary intake however in these studies the plasma isoflavone concentrations responsible for these effects were not reported.

Plasma total genistein concentrations of approximately 1.0 μM have been reported in women consuming soy products (~16 oz. soymilk/day) (Xu et al. 1994). Women consuming larger amounts of soymilk powder containing 226 mg genistein were found to have plasma total genistein concentrations of up to 6 μM (Xu et al. 1995). Thus, it is possible to achieve relatively high plasma concentrations of total genistein in humans similar to those shown to have biological effects in rodents. However, data on the effects

of human consumption of soy have been conflicting. For example, a study measuring the number of hot flushes in postmenopausal women receiving either dietary soy or wheat flour found both were effective in reducing hot flashes; however, they were not significantly different from each other (Murkies et al. 1995). This suggests the soybean isoflavones in themselves had little effect in this model. Additional work by Baird et al. (1995) also failed to show any difference in estrogenic effects in postmenopausal women consuming either texturized vegetable protein or soybeans, each containing 40.3 mg of genistein per day. However, Cassidy et al. (1994) found a significant change in the length of the follicular phase of the menstrual cycle in women consuming soy protein containing 19.85 mg genistein per day. Furthermore, Wilcox et al. (1990) saw an increase in vaginal maturity in postmenopausal women consuming a combination of soy flour, red clover sprouts and linseed, all known to contain phytoestrogens although they were not measured in this study. These studies, although inconsistent, suggest that dietary consumption of soy products may produce estrogenic effects in humans. There have not been any studies in which the effect of dietary genistein on the *in vivo* promotion of chemically induced mammary tumors or human breast cancer cells has been

explored.

F. Justification of Models:

1. Rat Model:

The female rat has been used for decades to study the effects of various compounds on a number of different systems. For example the uterus of the immature rat has been used as an indicator to measure the estrogenic potency of a number of compounds (Kitts et al. 1980, reviewed by Adams 1989). The rat mammary gland has been used as a model in which to study development and identify the hormones and growth factors involved in this process (reviewed in Topper & Freeman 1980). Furthermore the rat serves as a model to study mammary carcinogenesis induced by the carcinogens 7,12-dimethylbenz(a)anthracene (DMBA) and nitrosomethylurea (NMU) (McCormick et al. 1981, Sukumar et al. 1983, Thompson & Adlakha 1991, Welsch 1985). The Sprague Dawley female rat was selected for these studies because it is a well characterized laboratory model.

2. Athymic Mouse Model:

The athymic mouse serves as a model for the inoculation of human breast cancer cells or tissue and subsequent development of estrogen dependent and independent tumors (Soule & McGrath 1980, Osborne et al. 1985, McManus & Welsch

1981, Brunner et al. 1989, Robinson & Jordan 1989, Marics et al. 1989). Due to genistein's estrogenic activity the estrogen independent MDA-MB-231 cell line was used in these studies. By using estrogen independent cells the potential confounding effects due to genistein's estrogenic activity are avoided. The athymic mouse model will enable analysis of the effect of dietary genistein in the establishment and development of estrogen independent human derived mammary tumors in vivo. Furthermore this model will allow assessment of the effect of genistein upon hormone independent tumors which are currently unresponsive to endocrine therapy. Finally correlations between in vivo and in vitro experimental data might be possible by using the same cell line in both systems.

G. Summary

There are geographical variations in the site specific incidence of various cancers including breast cancer in women. Epidemiological studies have shown that the incidence of breast cancer in a population will change depending on where that population lives. Studies have also shown that a population will adopt the dietary habits of the host country upon assimilating into that culture. This is true of the Japanese where the incidence of breast cancer is

relatively low in Japan; however, upon migrating to the US their incidence of breast cancer approaches that in the US which is relatively high. This suggests environmental factors, particularly diet, play a major role in the onset of this disease. Immigrant populations have shown a progressive adoption of the host country's dietary practices while at the same time forgoing traditional habits. In Japan soy products constitute an integral part of the traditional diet with 1-2 servings consumed per day. Japanese immigrants to the US consume less vegetable products, including soy, and more meat and fat. The role of dietary fat, independent of calories, in tumorigenesis is not clear, particularly in free living humans. Given the scarcity of data to support the 'fat' effect, independent of calories, we looked elsewhere for other factors that may be responsible for the change in mammary tumor incidence in Japanese immigrants. In doing so consumption of soy products emerged as a possibility. As stated earlier soy consumption decreases as immigrants assimilate into the US culture. Are there factors in soy that could be potentially anticarcinogenic? There are a number of compounds in soy that have exhibited anticarcinogenic properties in <u>in vitro</u> systems. One of the most promising is genistein. For example genistein inhibits a number of tyrosine kinases

involved in signal transduction. Furthermore, tyrosine kinase activity is higher in breast cancer. To explore the relationship between diet and cancer this research sought to look at the role of genistein in tumorigenesis.

The athymic mouse tumor model has superior utility over other models in that human breast cancer cells can be cultured directly in the mouse. Tumors arising from the inoculation of human cells retain characteristics of the human tumor. This facilitates a better analysis on the efficacy of treatment modalities upon an *in vivo* human tumor. Furthermore, comparisons between experiments conducted in cell culture and those in an *in vivo* system are better made by utilizing the same cell line. These comparisons could provide important information in deciding dosages potentially necessary to produce effects similar to those seen *in vitro*.

Chapter II

Dietary Genistein Exerts Estrogenic Effects Upon the Uterus, Mammary Gland and the Hypothalamic/Pituitary Axis in Rats.

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A. ABSTRACT

These studies were undertaken to assess the estrogenic and antiestrogenic effects of dietary genistein. To determine estrogenic effects, genistein was mixed into a modified AIN-76 or AIN-93G semi-purified diet at 0 (negative control), 150, 375 or 750 µg/g and 17,ß-estradiol at 1.0 µg/g and fed to ovariectomized 70 day old Sprague-Dawley rats. Estrogenic potency was determined by analyzing uterine weight, mammary gland development, plasma prolactin and expression of uterine c-fos. Dietary genistein (375 µg/g and 750 µg/g) increased uterine wet and dry weights ($p<0.05$). Mammary gland regression following ovariectomy was significantly inhibited by dietary genistein at 750 µg/g ($p<0.05$). Plasma prolactin was significantly greater in ovariectomized rats fed genistein (750 µg/g) compared to comparable rats not receiving genistein. Genistein demonstrated a relative binding affinity to the estrogen receptor $(ER)^3$ to be approximately 0.01 that of estradiol.

Genistein (750 µg/g) induced the uterine expression of c-fos. To evaluate potential antiestrogenic effects genistein and estradiol were mixed into the modified AIN diets at the doses noted above and fed to ovariectomized rats. Dietary genistein (375 or 750 µg/g) did not inhibit the effects of estradiol on uterine weight, mammary gland development or plasma prolactin. Serum concentration of total genistein (conjugated plus free) in rats fed 750 µg/g was 2.2 µmol/L and 0.4 µmol/L free genistein. Administration of dietary genistein at 750 µg/g can exert estrogenic effects in the uterus, mammary gland and hypothalamic/pituitary axis. Dietary genistein (750 µg/g) did not antagonize the action of estradiol in estradiol-supplemented ovariectomized rats or in intact rats.

B. Introduction

Phytoestrogens include the isoflavones, lignans, and other non-steroidal chemicals found in plants and plant products. These compounds can bind to the estrogen receptor and are thought to elicit their estrogenic effects through mechanisms similar to that of estradiol.

The consumption of certain plants and plant products can result in impaired reproductive function in livestock. Over five decades ago clover disease, a syndrome with

effects ranging from temporary to permanent infertility, was described in sheep foraging upon subterranean clover in western Australia (Bennets 1946). Additional studies have documented impaired reproductive function in a number of species (reviewed in Price & Fenwick 1985) including desert quail feeding upon desert brush (Leopold et al. 1976). Furthermore, a decrease in reproductive performance was observed in female rats fed either a soy-based diet or a diet supplemented with genistin at 2 g/kg diet(Carter et al. 1955), and in male mice fed genistin at 36 mg (mouse/d) (Matrone et al. 1955). All of these effects were attributed to the phytoestrogen content of the diets.

It was later discovered that genistein was responsible for the impaired reproductive performance seen in sheep ingesting subterranean clover (Bradbury and White 1951). Genistein is an isoflavone (4',5,7-trihydroxyisoflavone), which has estrogenic activity (Folman & Pope 1966), present in various plants including soybeans (Naim et al. 1974). Since the initial discovery of its estrogenic activity, there have been a number of studies in which the effects of soy and genistein upon the uterus of mice and rats were evaluated (Carter et al. 1953): all of these have demonstrated estrogenic effects except the work of Farmakalidis & Murphy (1984). In their study the potent

estrogen agonist diethylstilbestrol also did not promote uterotrophic effects in this strain of mouse (CD-1).

The estrogenic effects of genistein in the uterus are well documented. However, few experiments have been conducted to assess estrogenic effects in other tissues and to our knowledge, none have examined the effects of dietary genistein on the mammary gland or the hypothalamic/pituitary axis. These studies were undertaken to provide additional insight on the actions of dietary genistein by analyzing the effects upon the uterus, pituitary gland and mammary gland. In addition, the plasma concentration of genistein responsible for these estrogenic effects was also determined.

C. MATERIALS AND METHODS

1. Chemicals: Genistein was synthesized by the demethylation of biochanin A or from organic precursors as described by Chang, et al. (1994). In both processes chemical identity was assessed by NMR and purity assessed at greater than 98%. ^{3}H-17,β-estradiol (3.59 TBq/mmol) and ^{32}P-deoxycytidine 5'-triphosphate, tetra(triethylammonium) salt (111 TBq/mmol) were purchased from Dupont New England Nuclear (Boston, MA.). All other chemicals, unless otherwise specified, were purchased from Sigma, St. Louis, Mo.

2. Animals: All rats were maintained according to guidelines in the Guide for the Use and Care of Laboratory Animals (NRC 1985). Intact and ovariectomized 60 d old Sprague Dawley rats (Harlan, Indianapolis, IN) weighing from 200 to 225 g were used in experiments 1 and 2. In experiment 3, intact 30 d old rats weighing from 80 to 90 g were used. Upon receipt, rats were weighed and sorted to equalize animal weights within each treatment group. Unless otherwise noted, rats were acclimated to the animal care facility and diet for 7 d, prior to initiating the studies. Rats were maintained in the animal care facility with temperature 22±2°C , relative humidity (40-70%), and a 12 h light:dark cycle. Rats were housed in polycarbonate cages (3 rats/cage) with aspen woodchip bedding and were allowed unrestricted access to food and water.

3. Diets: Animals were fed the American Institute of Nutrition-93G (AIN-93G) or modified AIN-76 diets prepared in our facilities. For the AIN-76, diet cornstarch was substituted for sucrose to lower the sucrose concentration from 50g/100g to 10g/100g of the diet. Semi-purified diets are required because the potential presence of genistein and other phytoestrogens in the soy portion of commercial nonpurified diets could confound the experimental results.

Genistein and 17,ß-estradiol were mixed into the AIN-76 or AIN-93G diets at the concentrations specified in experiments 1, 2 and 3.

4. Experimental Design:

Experiment 1: Estrogenic Effects of Dietary Genistein and Estradiol. Forty-two rats were ovariectomized at 56 d of age and dietary treatments were begun at 70 d of age. The ovariectomized rats (six per treatment) were given free access to food and water for a period of five d. Dietary treatments consisted of genistein at 150, 375 and 750 µg/g or estradiol at 0.5, 1.0 and 1.5 µg/g. Eight intact and six ovariectomized 70 d old rats were fed a modified AIN-76 diet and served as positive and negative controls, respectively. Estrogenic activity was assessed by uterine wet and dry weights.

Experiment 2: Anti-estrogenic Activity of Dietary Genistein. Forty-six rats were ovariectomized at 56 d of age and fed the modified AIN-76 diet until 70 d of age at which time dietary treatments were began. Rats, six per group, were given free access to food and water for a period of 21 d. Dietary treatments included estradiol at 1.0 µg/g, genistein at 750 µg/g, genistein at 150, 375 and 750 µg/g plus estradiol at 1.0 µg/g and an untreated control group. Ten ovariectomized rats were killed at the start of the

experiment to obtain baseline values for uterine weight and mammary gland development. Estrogenic activity was assessed by comparing uterine weight, mammary gland development, plasma prolactin and uterine expression of an estrogen responsive gene, c-fos, to those of ovariectomized, untreated controls. Anti-estrogenic activity of genistein was assessed by comparing the uterine weight, mammary development and plasma prolactin of rats consuming diets with combinations of genistein and estradiol to the rats fed the diet containing 1.0 µg/g estradiol.

Experiment 3: Effects of Dietary Genistein On the Development of the Mammary Gland and Uterus of Intact Rats. Thirty-four, 30 d old rats were fed the AIN-93G diet for one day and then treatments began on the second day. Rats, eight per group, were given free access to feed and water for a period of 14 d. Dietary treatments consisted of genistein at 375 and 750 µg/g. Eight rats fed the AIN-93G diet without genistein served as positive controls. Ten rats were killed at the start of the experiment to establish baseline values for uterine weight and mammary gland development.

5. Analysis of uteri: Rats were weighed, anesthetized by CO_2 exposure and then killed by cervical dislocation.

Uteri were excised, trimmed of fat and connective tissue, weighed and immediately placed in liquid nitrogen for later RNA analysis. Wet uterine weight was determined in all experiments. In experiment 1 dry weight was also determined for each uterus by incubating approximately one-half of the uterus at 100°C for 16 h.

6. Analysis of mammary glands: Rats were weighed, anesthetized by CO_2 exposure, and then killed by cervical dislocation. Mammary glands were prepared according to the procedure of Banerjee et al. (1976). The inguinal mammary gland was excised, fixed in a 3:1 (v/v) solution of ethanol:acetic acid for 2 h, transferred to 70% ethanol for 3 d with ethanol changed each day, rinsed in water and stained in alum carmine for 16 h. The glands were then rinsed in water, placed in 70%, 95% and 100% ethanol for 30 min each, transferred to toluene for 24 h for clarification and then stored in absolute methyl salicylate. Lobulo-alveolar and ductal structure (extent of side branching) were assessed using a dissecting microscope and assigned values from 0 to 4, with the higher number representing maximal development. Analyzes were performed twice on each gland with the identity of the glands unknown to the analyst.

7. Northern blot analysis of c-fos: Total RNA was isolated by the procedure of Chirgwin et al. (1979) as modified by Helferich et al. (1990). Uteri from 4 rats in each group (randomly selected from the following groups: ovariectomized control, 1 µg/g estradiol and 750 µg/g genistein) were removed from liquid nitrogen, placed in 4.0 mL of 4.0 mol/L guaninidium thiocyanate (GITC)and homogenized on ice with a polytron (Brinkman Instruments; Westbury, NY). N-lauryl sarcosyl was added (100 uL 10% solution per mL GITC), the mixture vortexed and cellular debris removed by centrifugation at 12,000 x g (g max) for 10 min. The supernatant was removed and layered over 1.0 mL of 5.7 mol/L CsCl followed by centrifugation at 110,000 x g (g max) for 16 h. The pellet was resuspended in 7 mol/L guanidine-HCl, 20 mmol/L sodium acetate, 1 mmol/L dithiothreitol, 10 mmol/L iodoacetic acid and 1 mmol/L Na_2EDTA and transferred to a 1.5 mL microfuge tube. RNA was precipitated with 2 volumes absolute ethanol and 0.1 volume 300 mmol/L sodium acetate at -20°C. RNA precipitates were washed once with 3 mol/L sodium acetate and 10 mmol/L iodoacetate, pH 5.0 at 4°C, then with 66% ethanol and 33 mmol/L sodium acetate, pH 5.0, and then with absolute ethanol at -20°C. Ethanol was removed and the RNA pellet dissolved in Tris-EDTA (pH 8.0) and spectrophotometrically

quantified at 260/280 nm. Ten µg of RNA was loaded onto a 1.2% agarose formaldehyde denaturing gel and electrophoresed for 8 h at 35 volts. The RNA was transferred to membrane (Hybond-N, Amersham Life Sciences, Cleveland, OH), and the membrane exposed to UV light at 120 kJ/cm^2 for 2 min. Rat c-fos cDNA, 2100bp, was kindly provided by Dr. Tom Curran (Roche Institute of Molecular Biology, Nutley, N.J.) and probes made by random priming (Boeringer Mannheim, Indianapolis, IN) with incorporation of ^{32}P-deoxycytidine 5'-triphosphate. The membrane was blocked by prehybridizing with herring sperm DNA at 42°C for 4 h followed by hybridization for 12 h and subsequent washing at 65°C with 2X saline sodium citrate buffer (SSC) + 0.1% sodium dodecyl sulfate (SDS) 30 min. each, then 0.3X SSC + 0.1% SDS for 25 min. The hybridized membrane was exposed to Kodak X-OMAT film at -70°C for 3 d and then developed.

8. Plasma prolactin analysis: Plasma prolactin concentration was determined in the laboratory of Dr. Keith Lookingland at Michigan State University utilizing a double antibody radioimmunoassay employing reagents and procedures of the National Institute for Diabetes and Digestive and Kidney Disease (NIDDK) assay kit (generously supplied by Drs. A.F. Parlow and Ratti, National Hormone and Pituitary

Program, Rockville, MD.). NIDDK rat PRL RP-3 was used as the standard. Using a 100 µL aliquot of plasma, the lower limit of sensitivity was 10.0 µg/L. Samples from each rat were assayed in duplicate at two different dilutions in a single radioimmunoassay.

9. Competitive binding analysis: Competitive binding analysis with ^{3}H-estradiol was performed using uterine cytosol prepared from untreated rats consuming nonpurified diet (Harlan-Teklad, 22/5 Rodent Diet (w) 8640, Madison, WI). Rat uteri were placed in ice cold TEDG (10.0 mmol/L tris-HCl pH 7.4, 1.5 mmol/L EDTA pH 7.4, 1.0 mmol/L dithiothreitol added fresh, and 10% glycerol) and homogenized with a polytron (Brinkman Instruments, Westbury, NY). The homogenate was centrifuged at 800 x g for 10 min and the resulting supernatant removed and centrifuged at 110,000 x g (g max) for 1.5 h at 4°C. Protein concentration was 5.0 g/L as determined by the Bradford assay (Bradford 1976). Aliquots were quickly frozen in a isopropanol/dry-ice bath and stored at -70°C. Binding assays were composed of 200 µL uterine cytosol (1.0 mg total protein), TEDG, and genistein or estradiol in ethanol vehicle bringing total volume to 500 µL. Assays containing 5.0 nmol/L ^{3}H-estradiol and 0 to 20 nmol/L 17,ß-estradiol or 0 to 50 µmol/L

genistein were incubated at 4°C for 3 h. After incubation the contents were removed and placed in a microfuge tube containing the pellet from 250 µL of dextran coated charcoal (DCC) solution (5% Norit A, 0.5% dextran T-70 in TEDG) to remove the unbound ^{3}H-estradiol. The microfuge tube was vortexed to disperse the DCC pellet and incubated for 3 min at 23°C followed by centrifugation at 13,000 x g to pellet the DCC. Two 200 µL aliquots of the supernatant were collected and counted on a scintillation counter (Beckman Instruments Model LS100, Fullerton, CA). The counts were averaged, divided by the total counts and expressed as a percent of the total radioactivity.

10. Serum genistein analysis:

Rats were anesthetized with diethyl ether and blood (approximately 6 mL/rat) was collected from the tail artery. Blood was placed at 4°C for 16 h to allow clotting. The blood was then centrifuged at 350 x g for 10 min and the serum removed and stored at -70°C. For genistein analysis 50 µL of serum, from each of four rats fed 750 µg/g genistein in experiment 2, was sampled in duplicate with one set receiving 5 µL (515 units) of B-glucuronidase Type H-1 (Sigma, St. Louis, MO). All aliquots were incubated in 0.5 mL microfuge tubes at 37°C for 48 h. Following the

incubation, 50 µL of absolute methanol was added to each tube, the tubes vortexed and then centrifuged at 15,000 x g for 10 min. Approximately 75 µL was removed and placed at -20°C until analysis. For analysis the samples were centrifuged at 15,000 x g for 15 min. and 20 µL injected onto a C_{18} column (Microsorb-MV, 5 µm 100A, Rainin Instrument, Woburn, MA) with a flow rate of 1.0 mL/min of 50:50 methanol:water, with 17 mMol/L acetic acid. Recovery was determined by spiking serum from control rats with genistein and then assessing recovery of genistein. Mean recoveries were determined to be 74% (SEM 1.68%). The data presented are corrected for recovery. No genistein was detected in control rats fed the AIN-76A diet.

11. Statistical analyzes

All statistical tests were performed using a PC-based version of the Statistical Program for the Social Sciences (SPSS) Version SPSS/PC 2.0, Chicago, IL 60611. Uterine weight and plasma prolactin data were analyzed by one-way analysis of variance. Variances in uterine weights presented in Tables 1 and 2 were non-homogeneous with respect to treatment so these data were log transformed prior to ANOVA. Data presented in the tables are means and standard errors before transformation. When a significant

($P < 0.05$) treatment effect was detected, treatment means were compared using the least significant difference method (Steel & Torrie 1980). Mammary gland data were analyzed by the Kruskal-Wallis non-parametric test (Shavelson 1988). When a significant ($P < 0.05$) treatment effect was detected treatment rank means were compared using the least significant difference method (Steel & Torrie 1980). Values in the text are means ±SEM.

D. RESULTS

1. Competitive Binding Analysis

Competitive binding studies utilizing rat uterine cytosol, ^{3}H-estradiol, unlabeled estradiol and genistein were performed to determine the relative binding affinity of genistein to the estrogen receptor. The concentration of unlabeled 17,ß-estradiol required to displace 50% of the bound ^{3}H-17,ß-estradiol in rat uterine cytosol in this study was approximately 5 nmol/L (Figure 1). Competitive binding analysis indicated the relative binding affinity of genistein was approximately 0.01 that of 17ß- estradiol.

2. Uterotrophic Effect of Dietary Genistein

Genistein administered in the diet to ovariectomized adult rats in experiment 1 at 150, 375, and 750 µg/g

produced a dose dependent increase in both uterine wet and dry weights (Table 1). In rats fed genistein at 375 and 750 µg/g, significantly greater uterine wet weights and dry weights than in the control were measured. Rats fed 750 µg/g genistein had similar uterine weights to that of rats fed 1.0 µg/g estradiol.

In experiment 2, the potential of genistein to antagonize the uterotrophic effect of 1.0 µg/g 17,ß-estradiol was evaluated by comparing uterine weights of the 17,ß-estradiol treated group to that of the groups receiving 17,ß-estradiol plus genistein. Uterine weights in the baseline group (rats killed at the start of dietary treatment, 14 d after ovariectomy) indicated that substantial regression had occurred (Table 2) when compared with intact rats at the same age used in experiment one (Table 1). Rats receiving genistein and estradiol at all doses had significantly greater uterine weights than in the control and baseline groups (Table 2). Genistein at 150, 375 or 750 µg/g did not inhibit the uterotrophic effect of concurrently administered 17,ß-estradiol (Table 2).

In experiment 3, the effect of genistein upon the development of the uterus of intact immature rats was evaluated. Ten rats were killed at the beginning of the study to obtain baseline data for uterine weights. Baseline

uterine weights were similar to the uterine weights of the ovariectomized rats observed in experiments 1 and 2, thereby confirming the immaturity of the rats in this study. Dietary genistein administered at either 375 or 750 µg/g for 14 d had no effect on the development of the uterus as indicated by uterine weight, relative to the control, intact rats (Table 3).

D3. Induction of Uterine c-fos

Uterine tissue from ovariectomized rats administered 750 µg/g dietary genistein or 1.0 µg/g 17,β-estradiol and untreated controls were analyzed for the presence of c-fos mRNA (Figure 2). Gels were stained with ethidium bromide to confirm equal loading of RNA and to assess the integrity of the RNA (data not shown). Dietary genistein and estradiol both induced the expression of c-fos mRNA relative to that of the untreated control rats.

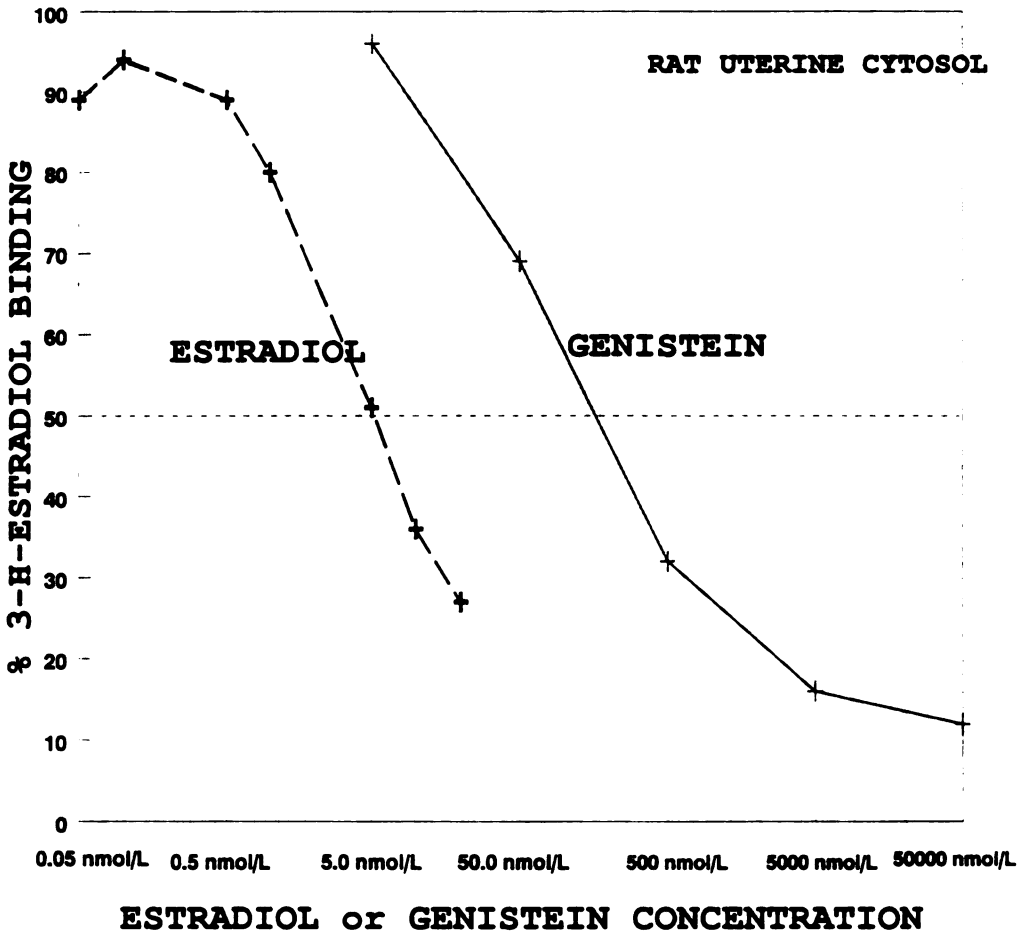

Figure 1: Competitive binding analysis of genistein and estradiol using rat uterine cytosol. Genistein or 17,β-estradiol and ^{3}H-estradiol were incubated with rat uterine cytosol for 2 h. Following incubation, free compounds were removed with dextran coated charcoal and aliquots counted in a Beckman LS-100 scintillation counter. Each point represents the mean of two replicates. Values are expressed as a percent of the total radioactivity.

TABLE 1. Effect of dietary genistein and estradiol on uterine weight in ovariectomized mature rats[1]

TREATMENT	n	UTERINE WET WEIGHT mg	UTERINE DRY WEIGHT mg
CONTROL[2]	8	76.5 ± 3.2[a]	20.7 ± 1.1[a]
INTACT[3]	6	386.6 ± 41.1[e]	73.7 ± 6.5[d]
ESTRADIOL µg/g			
0.5	6	122.1 ± 6.6[b]	17.6 ± 1.7[a]
1.0	6	194.8 ± 8.8[c]	40.4 ± 6.0[c]
1.5	6	255.0 ± 8.8[d]	54.0 ± 2.2[d]
GENISTEIN µg/g			
150	6	92.4 ± 2.6[a]	28.0 ± 2.8[b]
375	6	135.6 ± 9.8[b]	30.0 ± 3.6[bc]
750	6	189.3 ± 26.6[c]	39.0 ± 5.6[bc]

Values are means ± SEM. [1]The experimental groups included a negative control (CONTROL), positive control (INTACT), Genistein 150 µg/g, 375 µg/g, 750 µg/g, Estradiol 0.5 µg/g, 1.0 µg/g and 1.5 µg/g. Values in a column with different superscripts are different ($p<0.05$). ANOVA was performed on log-transformed data followed by multiple means comparison using the least significant difference method. [2]Controls were ovariectomized, non-treated rats. [3]Intact rats were not ovariectomized.

Table 2. Effect of dietary genistein and estradiol on uterine weight and plasma prolactin in adult ovariectomized rats[1].

TREATMENT	n	UTERINE WEIGHT	PLASMA PROLACTIN
µg/g		mg	µg/L
BASELINE[2]	10	130.7 ± 5.5[b]	Not Determined[4]
CONTROL[3]	6	96.5 ± 3.9[a]	5.68 ± 1.05[a]
GEN 750	6	343.6 ± 24.3[d]	20.0 ± 2.73[b]
E_2 1.0	6	220.1 ± 17.3[c]	16.0 ± 2.53[b]
E_2 1.0 + GEN 150	6	241.3 ± 24.9[c]	Not Determined[4]
E_2 1.0 + GEN 375	6	312.4 ± 13.4[d]	16.7 ± 1.52[b]
E_2 1.0 + GEN 750	6	305.6 ± 24.3[d]	18.8 ± 2.33[b]

Values are means ± SEM. [1]The experimental groups included an ovariectomized CONTROL , BASELINE, GENISTEIN (GEN) 750 µg/g, ESTRADIOL (E_2) 1 µg/g), E_2 1 µg/g + 150 µg/g GEN, E_2 1 µg/g + 375 µg/g GEN, and E_2 1 µg/g + 750 µg/g GEN. Values in a column with different superscripts are different ($p<0.05$). ANOVA was performed on log-transformed uterine weight data followed by multiple means comparison using the least significant difference method. [2]Baseline rats were killed at the beginning of dietary treatment. [3]Control rats were ovariectomized, non-treated.
[4]Plasma prolactin was not determined in the baseline and E2 + 150 treated rats. Uterine weights in these groups did not differ.

Table 3. Effect of dietary genistein on uterine weight of intact immature rats[1]

TREATMENT	n	UTERINE WEIGHT
µg/g		mg
BASELINE[2]	8	99.1 ± 14.2[a]
CONTROL[3]	8	230.7 ± 17.9[b]
GENISTEIN 375	8	243.2 ± 10.8[b]
GENISTEIN 750	8	234.4 ± 13.8[b]

Values are means ± SEM. [1]The experimental groups included BASELINE, GENISTEIN 375 µg/g, and GENISTEIN 750 µg/g and CONTROL. Values in a column with different superscripts are different ($p<0.05$). ANOVA was performed on log-transformed data followed by multiple means comparison using the least significant difference method. [2]Baseline rats were killed at the start of dietary treatment. [3]Control rats were not treated.

Table 4. Effect of dietary genistein and estradiol on the mammary gland of ovariectomized adult rats[1]

TREATMENT	n	MEAN RAW SCORE		MEAN RANK SCORE	
		LOB/AV[2]	DUCT	LOB/AV	DUCT
BASELINE[3]	10	3.27	2.89	35.65^{c}	34.35^{c}
CONTROL[4]	6	1.38	2.29	11.75^{ab}	22.83^{abc}
GEN 750 µg/g	6	3.42	2.50	34.42^{c}	27.08^{bc}
E_2 1.0 µg/g	6	0.96	1.63	7.83^{a}	11.83^{a}
E_2 1.0 + GEN 150	6	1.86	1.92	16.58^{ab}	13.42^{a}
E_2 1.0 + GEN 375	6	1.83	1.72	18.75^{b}	19.17^{ab}
E_2 1.0 + GEN 750	6	3.21	2.63	31.25^{c}	28.58^{bc}

[1]The experimental groups included an ovariectomized CONTROL, BASELINE, GENISTEIN (GEN) 750 µg/g, ESTRADIOL (E_2) 1 µg/g , E_2 1 µg/g + 150 µg/g GEN, E_2 1 µg/g + 375 µg/g GEN, and E_2 1 µg/g + 750 µg/g GEN. Values in a column with different superscripts are different ($p<0.05$). Data were ranked and analyzed with Kruskal-Wallis nonparametric ANOVA. Raw mean and rank mean scores are included in the table. When a significant treatment effect was found the rank means were compared with the least significant difference method. [2]Lob/Av = lobular/alveolar.
[3]Baseline rats were killed at the start of dietary treatment. [4]Control rats were ovariectomized non-treated.

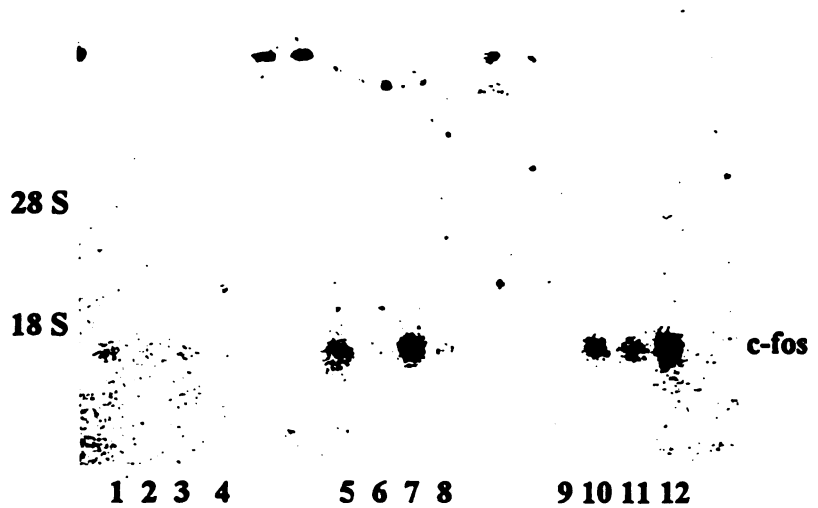

Figure 2: Effect of dietary genistein and estradiol upon the uterine expression of c-fos in ovariectomized mature rats. Total RNA was prepared from four rats randomly selected from each of the following treatment groups: lanes 1-4 control, 5-8 750 µg/g genistein and 9-12 1.0 µg/g 17,β-estradiol. Ten µg of total RNA was electrophoresed on a 1.2% denaturing agarose gel, transferred to nylon and hybridized with random primed rat c-fos DNA probe labeled with ^{32}P. The membrane was exposed for 3 d.

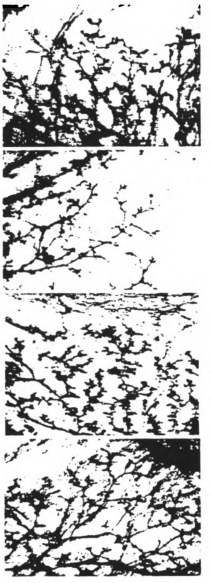

Figure 3: Photographs of the mammary glands from rats treated with dietary genistein and estradiol. Mammary glands were dissected from the rats and stained with alum-carmine. Photographs were taken through a dissecting microscope under 43x magnification. Photograph **a** is from the baseline group (killed at the start of dietary treatments), **b** from the control group (ovariectomized non-treated), **c** from the genistein 750 µg/g treatment group, and **d** from the 1.0 µg/g estradiol treatment group.

4. Mammatrophic Effect of Dietary Genistein

In experiment 2, the mammatrophic effects of dietary genistein was evaluated in ovariectomized rats by analyzing two criteria: 1) lobulo-alveolar structure and 2) ductal structure including side branching and infiltration of ducts into the fat pad of the mammary gland. Dietary treatment of ovariectomized rats for 21 d with 750 µg/g genistein prevented mammary gland regression, seen primarily in lobulo-alveolar structure, relative to that of the ovariectomized, untreated control rats (**Table 4 and Figure 3**). Average lobulo-alveolar development in the 17,β-estradiol treated rats did not differ from controls. Average ductal development did not differ for the genistein or estradiol treated groups compared with the controls. Lobulo-alveolar development was significantly greater for the groups receiving 750 µg/g genistein or genistein 750 µg/g + 17,β-estradiol 1.0 µg/g, compared to the control. The potential of genistein to antagonize the mammatrophic effect of estradiol was also evaluated. Coadministration of dietary genistein at 150, 375 or 750 µg/g, with 1.0 µg/g 17,β-estradiol did not result in lower mammary scores compared with rats receiving 17,β-estradiol alone (Table 4).

In experiment 3, the effect of genistein upon the

mammary gland in immature rats was evaluated. Ten rats were killed at the beginning of the study to obtain baseline data for mammary gland development. Dietary genistein had no effect on the development of the mammary gland, as assessed by lobulo-alveolar and ductal development, relative to the control untreated intact rats (data not shown).

5. Plasma Prolactin Analysis

The effect of dietary genistein on plasma prolactin in ovariectomized rats was determined in experiment 2. Plasma prolactin was significantly higher in the dietary genistein and estradiol treated rats relative to the control group (Table 2). Genistein coadministered with estradiol did not stimulate or inhibit the effects of estradiol.

6. Serum Genistein Analysis

The serum concentration of genistein was assessed in four rats from experiment 2 to determine the concentration present in rats fed 750 µg/g genistein. Total genistein (conjugated plus free) concentration was 2.2 ± 0.01 µmol/L and the free concentration of genistein was 0.4 ± 0.03 µmol/L. Efficiency of recovery was determined by quantifying recovery of a genistein spike from control serum. Average recoveries were 74% ± 1.68%.

E. Discussion

1. Competitive Binding Analysis and Induction of c-fos.

Competitive binding analysis demonstrated a relative binding affinity of genistein for the rat uterine estrogen receptor (ER) to be approximately 0.01 that of estradiol. The binding of a compound to a receptor does not necessarily result in the production of a complex capable of initiating the biological response; therefore, additional studies were undertaken to ascertain whether dietary administration of genistein would induce the expression of an estrogen responsive gene, c-fos (Weisz & Rosales 1990), in an estrogen responsive tissue. Uterine expression of c-fos was induced in ovariectomized rats following the dietary administration of genistein or 17,β-estradiol. The variable expression of c-fos may be due to several factors, including: 1) the timing of food consumption, 2) variability in food consumption, 3) metabolism of genistein, and 4) the short half-life of c-fos mRNA (Greenburg & Ziff 1984). The induction of c-fos by genistein suggests genistein is acting through the ER, similar to estradiol, and is capable of forming an active complex with the ER in uterine tissue.

2. Uterotrophic Effects of Dietary Genistein

Phytoestrogens have long been recognized for their uterotrophic activity in a variety of animal species. These effects range from temporary to permanent infertility (reviewed by Adams 1989). In the present study there was a dose-dependent increase in uterine weight with effects seen at a dietary dose of genistein as low as 375 µg/g diet suggesting that genistein acts in the uterus in a manner similar to that of estradiol. That is genistein binds to the ER and the ligand:receptor complex induces the expression of estrogen responsive genes which ultimately result in increased uterine mass.

Genistein competes with estradiol binding to the ER and has shown estrogenic effects in estrogen responsive tissues. Many antiestrogens, including tamoxifen, possess agonistic properties when administered in low amounts; however, in higher concentrations, they are antagonists (Martinez-Campos et al. 1986). As a weak agonist, genistein also has the potential to antagonize the effects of estradiol. Antagonistic properties have been reported in mice coadministered subcutaneous injections of genistein and estrone. Folman & Pope (1966) inhibited the uterotrophic effect, in mice, induced by subcutaneous injections of 0.4 µg estrone (total dose) by administering concurrent

subcutaneous injections of either 800 or 1600 µg genistein (total dose) twice daily over a 3 day period. In our study the coadministration of 150, 375, or 750 µg/g genistein with 1.0 µg/g 17,β-estradiol did not inhibit the effects of estradiol on uterine weight. In addition dietary genistein did not affect the development of the uterus, when administered to immature rats, as assessed by uterine weight during maturation of the organ. In the studies reported here, genistein was administered in the diet to rats whereas in the study by Folman & Pope (1966), genistein was administered subcutaneously to mice. The dose of genistein administered in our studies, relative to the dose of estradiol, was much lower than that of Folman & Pope. Furthermore, variability in species as well as strain in response to compounds with estrogenic activity is well documented (reviewed by Adams 1989, Farmakalidis & Murphy 1984). The amount of genistein absorbed from the gut is currently unknown. All of these variables could account for the different results obtained by us compared with Folman & Pope.

3. Effects of Dietary Genistein on the Mammary Gland and Plasma Prolactin

Dietary genistein consistently elicits an estrogenic

response in the uterus of ovariectomized and immature rodents; however, the effect of dietary genistein on the mammary gland, another estrogen responsive tissue, has not been assessed. Increased differentiation of the mammary gland has been observed in prepubertal rats by the subcutaneous injection of 5 mg genistein per rat on days 2, 4 and 6 postpartum (Lamartiniere et al. 1995). The model employed in these studies utilized 60 d old rats and assessed the ability of dietary genistein and or estradiol to inhibit mammary gland regression post-ovariectomy.

Development and maintenance of the mammary gland in rats is controlled by many factors including estrogen, progesterone, growth hormone and prolactin (reviewed by Topper & Freeman 1980). Estrogen acts directly at the mammary gland by inducing gene transcription and the subsequent translation of many proteins, including the progesterone receptor required for progesterone action. Estrogen also acts indirectly through the induced synthesis and release of prolactin from the anterior pituitary gland which then exerts its mitogenic effects on the mammary gland. Removal of endogenous estrogen results in regression of the mammary gland, particularly the lobulo-alveolar structures. Mammary gland regression at 35 d post-ovariectomy in untreated control rats was greater than that

in the untreated baseline rats measured at 14 d post-ovariectomy (the start of dietary treatment). Dietary genistein prevented regression of the mammary gland compared to the untreated controls. Our goal in this set of experiments was to evaluate whether the concurrent administration of genistein at different doses would inhibit the effects of estradiol in preventing regression of the mammary gland. Orally administered estradiol (1 μg/g) was effective in increasing uterine weight and plasma prolactin; however, it was ineffective in the mammary gland. The reason dietary estradiol elicits estrogenic effects in the uterus and not the mammary gland is unclear. The ability of genistein to inhibit the effects of estradiol in the mammary gland could not be determined with the dose of estradiol used in this study.

It is unlikely that genistein alone is directly responsible for the effects observed in the mammary gland. Maintenance of the mammary gland in rats is also dependent on prolactin. Prolactin synthesis in the anterior pituitary gland is under tonic inhibition by dopamine produced in the hypothalamus (Jones & Naftolin 1990). Estrogen is thought to decrease the activity of tyrosine hydroxylase, thereby decreasing the concentration of dopamine in the hypothalamus and pituitary (Jones & Naftolin 1990) resulting in increased

plasma prolactin. Genistein or estradiol administered in the diet resulted in increased plasma prolactin compared to the ovariectomized control animals. The increase in plasma prolactin in this study suggests that dietary genistein functions in the hypothalamus and pituitary gland in a manner analogous to that of estradiol, leading to the synthesis and release of prolactin from the anterior pituitary gland.

Estradiol increased uterine weight and plasma prolactin yet did not affect regression of the mammary gland. This suggests that dietary estradiol (1.0 µg/g) is estrogenic in the uterus and on the hypothalamic/pituitary axis. However, this dose was not sufficient to affect the mammary gland.

4. Serum Genistein Analysis

Our results indicate that dietary genistein can elicit uterotrophic effects at a dose as low as 375 µg/g, mammatrophic effects at 750 µg/g and effects upon the hypothalamic/pituitary axis at 750 µg/g. The plasma concentration of genistein in rats fed 750 µg/g genistein was 2.2 µmol/L (conjugated plus free). This concentration was sufficient to elicit estrogenic effects in ovariectomized rodents. The intestinal metabolism, absorption, and half-life of dietary genistein, all of which

could affect its bioactivity, are currently unknown. Genistein is present in soy products at concentrations as high as 1500 µg/g (Eldridge 1982). Women who consumed soymilk powder daily, which contained 227 mg genistein, had plasma genistein concentrations (conjugated plus free) of up to 6.0 µmol/L (Xu et al. 1995).

At present, there are no human studies in which the biological effects of pure genistein have been assessed and only a few which have evaluated the effects of a diet supplemented with soy. Published studies in which post-menopausal women consumed diets supplemented with soy have yielded conflicting results. Wilcox et al. (1990) showed that soy supplementation produced changes in the uterus similar to that of estrogen. However, other studies have failed to show effects different from controls by supplementing soy in the diet of post-menopausal women (Baird et al. 1995).

In the studies described here, genistein did not inhibit the effects of estrogen in either intact or estrogen fed ovariectomized rats. However, in ovariectomized rats, genistein stimulated the growth of estrogen responsive tissues, particularly the mammary gland. Perhaps in premenopausal women genistein would have little, if any, estrogenic activity, whereas in postmenopausal women, the

effects would be more pronounced. This raises some concern with regard to mammary tumorigenesis, which initially requires estrogen, because these studies have demonstrated dietary genistein to have estrogenic effects in the mammary gland of ovariectomized rodents. Further research is needed in this area to clarify the effects of dietary soy, particularly in post-menopausal women in whom circulating estradiol is low.

Genistein is receiving much attention as a potential chemopreventive/therapeutic agent in the treatment and or prevention of various cancers. The results of these studies demonstrate the need for additional experiments on the biological effects of dietary genistein, particularly in tumor models, before any dietary recommendations can be made or supported.

F. Acknowledgment

Sincere appreciation is extended to Keith Lookingland and his laboratory at Michigan State University for their analysis of plasma prolactin. Thanks are also extended to Les Bourquin and Gale Strasburg for their critical review of the manuscript.

Chapter III

Genistein inhibits estrogen receptor negative human breast cancer cell growth in cell culture and in athymic mice.

A. Abstract:

These studies were conducted to assess the effect of the soy derived isoflavone genistein upon the proliferation of estrogen receptor negative human breast cancer cells (MDA-MB-231) utilizing both *in vitro* and *in vivo* models. Genistein (20 μM) inhibited cell proliferation *in vitro* by ca. 50%. Cell cycle progression was blocked in G_2/M with 40 and 80 μM genistein whereas 20 μM had no measurable effect. To determine whether similar plasma genistein concentrations could be achieved (with a reasonable dietary dose) *in vivo*, Balb/C mice were fed genistein (0 to 6000 μg genistein/g AIN-93G) and plasma genistein analyzed by HPLC. Total (conjugated + free) plasma genistein was dependent upon the dose fed and reached a maximum of 7.1 μM with 6000 μg genistein /g diet. Genistein was fed to female athymic mice inoculated with MDA-MB-231, after solid tumor masses had formed, at a dose (750 μg/g AIN-93G) shown to produce a plasma concentration of genistein (ca. 1 μM). Genistein did not significantly affect tumor growth. The plasma concentration of total genistein was 0.933 ± 0.107 μM and

the concentration of free genistein was 0.306 ± 0.141 μM. A higher dose of genistein (3000 μg/g AIN-93G) was then fed to tumor bearing mice. Tumor growth was inhibited ($p<.05$) compared to untreated controls. The plasma concentration of total genistein was 5.88 μM and the concentration of free 0.64 μM. Food intake was 10.7% less ($p<.001$) in the 3000 μg genistein/g AIN-93G fed group; however, weight gain was not significantly different. Genistein has been shown to inhibit angiogenesis: a necessary process for initial solid tumor mass development and subsequent growth. Studies were conducted to assess the effect of dietary genistein on initial tumor development and growth. Genistein (750 μg/g AIN-93G), fed three days before cells were inoculated into mice, did not significantly inhibit tumor formation or growth. The plasma concentration of genistein in mice fed this dose of dietary genistein (750 μg/g AIN-93G) is not sufficient to inhibit tumor formation or growth. These results suggest that feeding 3000 μg genistein/g AIN-93G will inhibit the *in vivo* growth of MDA-MB-231 human breast cancer cells. Food intake was lower in the genistein fed (3000 μg/g AIN-93G) mice however weight gain was not significantly different. Whether the reduction in food intake contributed to the decrease in tumor growth is unknown.

B. Introduction:

Epidemiological studies have shown wide geographical variations in the occurrence of breast cancer in women (Gray et al. 1979, Miller 1977, Wynder & Hirayama 1977). Incidence rates are as high as 1 in 8 in the US and as low as 1 in 30 in Japan. Furthermore, the incidence of breast cancer in migrant Japanese women to the US increases in the first and second generations of offspring to approach that in the US (Buell 1973, Hirayama 1978). These data suggest environmental factors, including diet, might play a primary role in the onset and development of many cancers including mammary cancer. In fact Doll & Peto (1980) have suggested that diet is involved in as many as 70% of cancers. With respect to Japanese migrants to the US, their dietary habits of their home country are abandoned and those of the US adopted as they assimilate into the culture of the US (Nomura et al. 1978). Specifically, these individuals consume less vegetables, in particular soy products, and more fat and meat products.

Soy products contain a number of compounds with the potential to inhibit the carcinogenic process including: protease inhibitors, phytates and isoflavones (Barnes et al. 1990, Kennedy & Manzone 1995). Of the isoflavones, genistein is present in soy products at concentrations as

high as 1.5 mg/g (Eldridge & Kwolek. 1983, Coward et al. 1993, Murphy & Wang 1993).

The initial dose of genistein (750 µg/g AIN-93G) selected for use in these tumor studies was based on data collected from earlier studies in which the biological effects of dietary genistein (750 µg/g AIN-93G) were assessed in estrogen responsive tissues in rats and mice. At this dose (750 µg genistein/g AIN-93G), genistein exerted estrogenic effects in the uterus and mammary gland. The total plasma genistein concentration found in mice consuming 750 µg genistein/g AIN-93G (approximately 1.0 µM) has also been measured in humans consuming soymilk containing 36 mg genistein while plasma genistein concentrations up to 6 µM have been found in humans consuming soymilk with higher amounts of genistein (226 mg) (Xu et al. 1994).

Genistein inhibits the <u>in vitro</u> proliferation of a number of transformed cell lines. The inhibition of cell growth may be due to genistein's ability to inhibit topoisomerase II (Okura et al. 1988, Markovits et al. 1989) and protein tyrosine kinases (Akiyama et al. 1987, Geissler et al. 1990). Genistein concentrations as low as 12 µM can inhibit topoisomerase II activity and concentrations as low as 3 µM inhibit tyrosine kinase activity. Both of these enzymes are involved in cell proliferation. Concentrations

within this range inhibit cell proliferation in a number of different tumor cell lines, for example: genistein inhibited MCF-7 adriamycin resistant, MCF7/WT and MDA-231 human breast cancer cells with IC_{50}'s of between 7 and 37 μM (Monti & Sinha 1994), MCF-7 (estrogen receptor positive) and MDA-468 (estrogen receptor negative) human breast cancer cells with IC_{50}'s of 24 to 44 μM (Peterson & Barnes 1993), stomach and colon cancer cell lines with IC_{50}'s of ca. 25 μM (Yanagihara et al. 1993) and AGS human gastric cells with an IC_{50} of between 7 and 23 μM (Piontek et al. 1993.

Genistein (60 μM) has been shown to reversibly arrest cell cycle progression of human gastric cancer (HGC-27) cells at G_2/M (Matsukawa et al. 1993) and in Jurkat T-leukemia cells at G_2/M (18.5-37.0 μM) while higher doses (74-110 μM) blocked cell cycle progression through S phase (Spinozzi et al. 1994). Blocking progression of the cell cycle is likely due to genistein inhibiting tyrosine kinase activity at key regulatory points in the cell cycle thus preventing progression through mitosis and resulting in inhibition of cell proliferation.

Here we present new information on the effect of genistein on the cell cycle and proliferation of MDA-MB-231 estrogen receptor negative human breast cancer cells in culture. Also presented are studies that evaluated the

effect of dietary genistein on the growth of MDA-MB-231 cells inoculated into athymic mice.

C. Materials and Methods:

1. Chemicals: Genistein was synthesized from organic precursors as described by Chang, et al. (1994). Chemical identity was assessed by NMR and purity assessed at greater than 98%. Analytical grade reagents were used for HPLC analysis. All other chemicals, unless otherwise specified, were purchased from Sigma, St. Louis, Mo.

2. Animals and Diets: Athymic and Balb/C female mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). In all experiments mice were housed 3 or 4 to a cage. Athymic mice were kept under aseptic conditions (enclosed laminar flow hood, sterilized cages, bedding, and water). The food was not sterilized so tetracycline was added to the drinking water at 500 µg/ml. All mice were kept in a temperature controlled (22°C ± 2°C), relative humidity controlled (40%-70%) and light controlled (12hr/12hr light/dark cycle) animal facility. All mice were fed the American Institute of Nutrition 93G (AIN-93G) semi-purified diet (corn oil replaced soybean oil) containing genistein at

the levels specified below (Reeves et al. 1993).

3. Experimental Design

a. Cell proliferation: MDA-MB-231 human breast cancer cells were a kind gift from Clifford Welsch at Michigan State University. Cells were maintained in minimal essential medium (MEM) with the following: Na-bicarbonate 2.2 g/L, L-glutamine 0.292 g/L, fetal bovine serum (FBS) 10%, Na-Pyruvate 8.8 mg/L, bovine insulin 10 mg/L, penicillin 10000 units/L, and streptomycin 10 mg/L. Cells were collected from 100 mm x 20 mm tissue culture plates at 80% confluence by washing two times with 1X PBS followed by trypsinization. Cell number was determined using a hemocytometer and the cell suspension diluted to approximately 500k cells/ml.

The cells were plated in 24 well plates at 15k cells per well and incubated for 16 hours in maintenance medium (described above). After 16 hours the medium was removed and replaced with similar maintenance medium but with only 0.1% FBS. Cells were incubated for 48 hours after which the medium was replaced with fresh maintenance medium (MEM +10% FBS) containing genistein (in ethanol) at 0, 10, 20, 40, and 80 μM. Ethanol vehicle for the treatments did not amount to more than 0.1% total volume of the medium in the well.

Cells were collected at 1, 3, 5 and 7 days for DNA analysis as described by West & Kumar (1985) with slight modification. For the DNA assay the medium was removed, cells washed two times with 1X PBS and then lyzed with 2.7 ml 10 mM EDTA pH 12.3. Following a 30 minute incubation at 37°C the pH was adjusted to 7.0 with KH_2PO_4 (~150 µl) and 10 µl (1000 ng) of a stock solution of Hoechst-33258 dye (200 µg/ml) was added to each well. Aliquots of 200 µl were transferred to Labsystems black 96-well Cliniplate for reading on a fluorescent plate reader (Cytofluor II, Perspective Biosystems, Framingham, MA.). The fluorophore was excited with 350 nm and emission measured at 455 nm. Fluorescence values were converted to DNA values from a standard curve made from salmon testes DNA.

b. Flow Cytometry: MDA-MB-231 cells were collected from 100 mm x 20 mm tissue culture plates at 80% confluence by washing two times with 1X PBS followed by trypsinization. Cell number was determined using a hemocytometer and the cell suspension diluted to approximately 500k cells/ml. Three ml maintenance media (described above) was added to each 60 mm x 15 mm issue culture plate and 1 ml of the cell suspension added (500k cells/plate). The cells were incubated for 16 hours, the medium removed and replaced with

similar maintenance medium but with only 0.1% FBS. Cells were incubated for 48 hours after which time the medium was replaced with fresh maintenance medium (MEM +10% FBS) containing genistein at 0, 10, 20, 40, and 80 µM. The ethanol vehicle for the treatments did not amount to more than 0.1% total volume of the medium in the well.

Cells were collected at 12, 24, 48 and 72 hours for cell cycle analysis. Cells were collected by removing the medium from the tissue culture plates into flow cytometry tubes (13 mm x 75 mm), trypsinizing the cells, collecting them into the same tubes and centrifuging at 350 x g for 5 min. The supernatant was aspirated and the pellets washed with MEM containing 10% FBS. For analysis of cell viability two 90 µl aliquots were collected into 0.5 ml microfuge tubes, 10 µl of 40% trypan blue added, the samples incubated at room temperature for 5 min., and cell viability determined by the exclusion of trypan blue.

For flow-cytometry the remaining samples were centrifuged at 350 x g for 5 min. and the supernatant removed. The cellular pellet was fixed in 1.5 ml of 70% ethanol and then placed at 4°C for 96 hours. The cells were removed from 4°C, centrifuged at 350 x g, the ethanol removed, the cellular pellet washed with 1X PBS, and then flow cytometry staining reagents added: 945 µl reagent A

(stock solution of reagent A contained the following: 100 µl of 100 mM EDTA pH 7.4, 100 µl of Triton X-100 in 99.8 ml 1X phosphate bufferred saline (PBS)), 50 µl reagent B (stock solution of reagent B contained the following: propidium iodide 1 mg/ml in H_2O) and 10 µl of RNase that had been boiled for 6 minutes. The tubes were gently vortexed and placed in the dark at 4°C until reading on the flow activated cell sorter (FACS). Fluorescence was assessed on a FACS Vantage (Beckton Dickinson) by excitation with an Argon laser at 488 nm and the emission measured at 630 ± 10 nm. Data was collected with Lysis II software and percent of cells in each phase of the cell cycle calculated with MPLUS software (Phoenix Flow).

c. Mice fed 0-6000 µg genistein/g diet: Female Balb/C mice (n=36) were received at four weeks of age and allowed unrestricted access to water and American Institute of Nutrition (AIN)-93G diet. Two-weeks later mice were weighed, sorted 3 mice to a cage, 2 cages per group, and provided 6 grams AIN-93G per mouse/day. Cages and food cups were collected at 17:00 each day for four days, the bedding sifted for food, and baseline control food intake determined. Genistein was then mixed into the AIN-93G in the following amounts: 0, 375, 750, 1500, 3000, and 6000

µg/g and fed to each group for four days. Food intake was determined each day as described above. At 10:00, on the fifth day of treatments, mice were anesthetized, weighed, bled by cardiac puncture (approximately 800 µl/mouse), and then killed by overexposure to anesthesia.

d. Genistein Fed to Mice Inoculated with MDA-MB-231 Cells: To evaluate the effect of dietary genistein on the growth of solid tumor masses the following study was conducted. Female athymic mice (n=30) were received at 3-4 weeks of age and allowed unrestricted access to water and the AIN-93G diet for 7 days prior to inoculation of MDA-MB-231 cells. MDA-MB-231 cells were collected from 80% confluent 100 mm x 20 mm plates by washing two times with 1X PBS, trypsinizing, and then collecting and pooling the cells in MEM supplemented with 10% FBS. Cell counts were determined using a hemocytometer and the cells diluted to 5 x 10^6 cells per ml. The cells were kept at 37°C for the duration of the injections. The mice were anesthetized and injected subcutaneously with 200 µl of the cell suspension (10^6 cells/site), using a 1 ml syringe with 25 gauge needle (5/8 inch), in each of their four flanks. After five weeks the mice were grouped, to equalize tumor number and cross-sectional area, into a 750 µg genistein/g AIN-93G group and an AIN-93G control group and treatment began. Only mice

with tumors >3 mm (measured by caliper) at the time of grouping were used in the study (n=27). Tumor length (l) and width (w) were measured weekly for 5 weeks and cross-sectional area determined by the following formula $(l*w/2)\ \pi$. Food intake was measured during the last 3 days of the study. At the end of the study mice were anesthetized, weighed, bled via cardiac puncture, and killed by overexposure to anesthesia.

Genistein did not affect the growth of tumors in the previous study so the amount of genistein fed to mice was increased to 3000 µg/g AIN-93G. Female athymic mice (n=27) were received at four to five weeks of age, sorted 3 or 4 mice to a cage and allowed unrestricted access to water and AIN-93G diet. One week later the mice were injected subcutaneously with MDA-MB-231 human breast cancer cells (as described above). After five weeks the mice were grouped, to equalize tumor number and cross-sectional area, into a 3000 µg/g genistein/AIN-93G group (n=14) and an AIN-93G control group (n=13), and treatments began. Tumor measurements and analysis were performed as described above.

To evaluate the effect of dietary genistein on the initial formation and growth of tumors the following study was conducted. Female athymic mice (n=30) were received at 3-4 weeks of age and allowed unrestricted access to water

and the AIN-93G diet for 7 days. One group of mice (n=15) were fed AIN-93G diet supplemented with 750 µg genistein/g AIN-93G, 3 days prior to injecting the MDA-MB-231 cells. Inoculation of tumor cells and tumor measurements were performed as described above.

e. High Pressure Liquid Chromatography Analysis (HPLC) of Plasma Genistein: Mice were anesthetized and bled by cardiac puncture. Blood (approximately 800 µl/mouse) was placed in microfuge tubes containing 10 µl 15% EDTA, centrifuged at 12,000 x g and the plasma removed and stored at -20°C. To determine conjugated and unconjugated genistein 50 µl of plasma was aliquotted in duplicate and one set received 5 µl (515 units) of B-glucoronidase Type H-1 (Sigma, St. Louis, MO.). All aliquots were incubated in 0.5 ml microfuge tubes at 37°C for 24 h. Following the incubation, 50 µl of absolute methanol was added to each tube, the tubes vortexed and then centrifuged at 15,000 x g for 10 min. Approximately 75 µl was removed and placed at -20°C until analysis. For analysis of genistein the microfuge tubes were centrifuged at 15,000 x g for 10 min. and 20 µl injected onto a C_{18} column (Microsorb-MV, 5 µM 100A, Rainin Instrument in Woburn, MA) with a flow rate of 1.0 ml/min of 50:50 methanol:water with 0.1% acetic acid.

Recovery was determined by adding a known amount of genistein (28 µl of 21.1 µg/ml in ethanol) into 600 ul plasma from control mice and diluting this with plasma to a final concentration of 1.82 µM genistein. Mean recoveries were determined to be 96 ±5.87%. The data presented are not corrected for recovery. Genistein was not detected in the plasma of control mice fed the AIN-93G diet. To avoid artificially manipulating the data obtained from HPLC analysis of plasma genistein, mean plasma genistein concentrations are reported only for samples in which genistein was detected. The level of detection for plasma genistein was ca. 130 nM.

f. Statistical Analyzes: All statistical tests were performed using a PC-based version of the Statistical Program for the Social Sciences (SPSS) Version SPSS/PC 7.5, Chicago, IL 60611. At the end of each experiment tumor area per mouse, food intake per cage and mouse weight were analyzed by student's T-test. Values in the text and figures are means ± SEM.

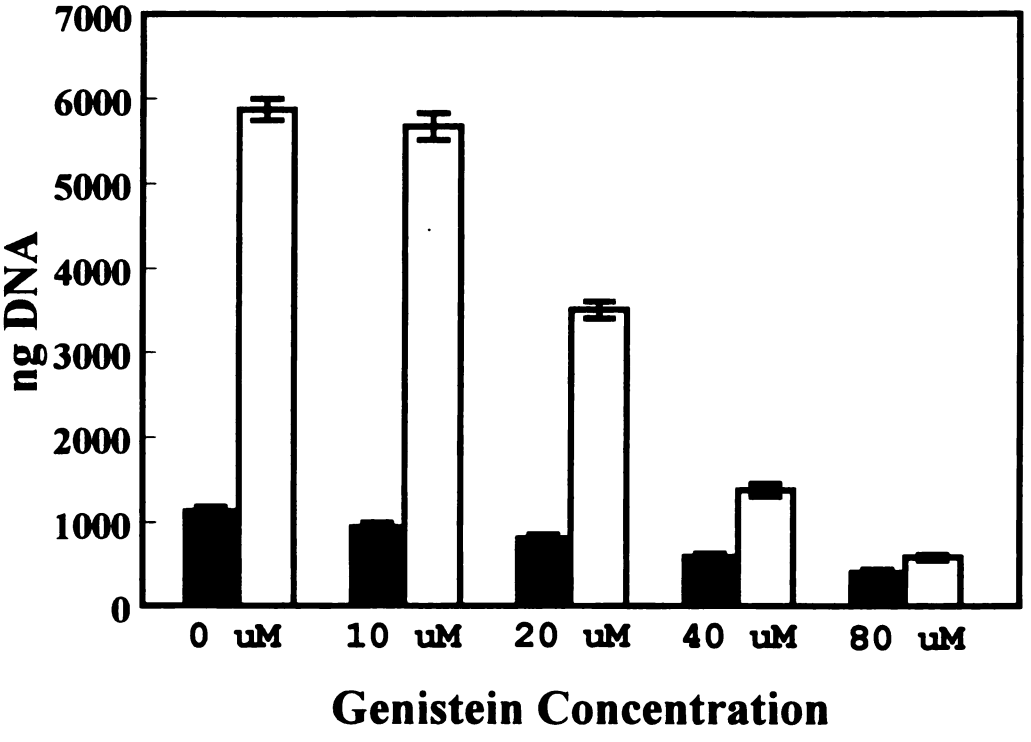

Figure 4. Effect of Genistein on MDA-MB-231 Cell Proliferation. MDA-MB-231 human breast cancer cells were treated with 0, 10, 20, 40 and 80 μM genistein in MEM containing 10% FBS for a period of 7 days. Fluorometric analysis of cellular DNA was performed on days three and seven. Solid bars represent cells cultured for three days and open bars are data collected after seven days. Each point represents the mean ± SEM of eight measurements.

D. Results

1. In Vitro MDA-MB-231 Cell Proliferation and Cell Cycle Analysis: In order to assess the effect of genistein on cell proliferation the following studies were conducted. Genistein inhibited proliferation of MDA-MB-231 cells with an IC_{50} of approximately 20 μM (Figure 4). Genistein at 10 μM had no effect on cell proliferation whereas 20, 40 and 80 μM resulted in a progressive decline in proliferation.

To evaluate a potential mechanism for growth inhibition studies were conducted to determine whether the inhibition of cell proliferation by genistein resulted from perturbations in the cell cycle or perhaps cytotoxicity. Genistein at 40 and 80 μM produced a marked block in the G_2/M phase of the cell cycle (Figure 5 and Table 5). Cell viability, as assessed by the cellular exclusion of trypan blue dye, was greater than 86% at all concentrations of genistein. Viability was actually greater than 93% at all time points with the exception of 48 hours where samples treated with 40 and 80 μM genistein had mean viabilities of 86%.

2. Dose Response Study of Dietary Genistein on Plasma Genistein and Food Intake: The concentration of genistein required to inhibit MDA-MB-231 cell proliferation in vitro

Table 5. Effect of Genistein Upon the Cell Cycle of MDA-MB-231 Cells.

Cell Cycle Analysis															
[genistein] μM	0			10			20			40			80		
Time hrs.	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
0	60	28	12												
12	71	19	11	68	19	13	67	18	15	65	16	19	65	14	22
24	41	31	28	38	37	25	36	44	20	40	51	10	52	37	11
48	63	33	5	59	29	13	47	32	21	33	27	40	23	37	44
72	56	31	14	49	42	13	45	45	11	31	34	35	17	31	53

* Data is expressed as a percent of the cells in each phase of the cell cycle. Viability assessed by trypan blue exclusion and found to be greater than 93% at all concentrations and time points.

MDA-MB-231 cells were treated with 10, 20, 40 and 80 μM genistein in MEM containing 10% FBS for a period of 12, 24, 48 and 72 hours. Cells were stained with propidium iodide and analyzed by flow cytometry. Percent of cells in each phase of the cycle was determined by M-Plus software as described in materials and methods. All values are the mean of three replications.

Figure 5. Effect of Genistein Upon the Cell Cycle of MDA-MB-231 Cells. MDA-MB-231 cells were treated with 10 (data not shown), 20, 40, and 80 μM genistein in MEM containing 10% FBS and samples collected at 12, 24 (data not shown), 48 and 72 hours. Cells were stained with propidium iodide and analyzed by flow cytometry. Histograms of cell cycle phase were prepared with M-Plus software as described in materials and methods. The actual percentages of cells in the various phases of the cell cycle at each time point are presented in Table 5.

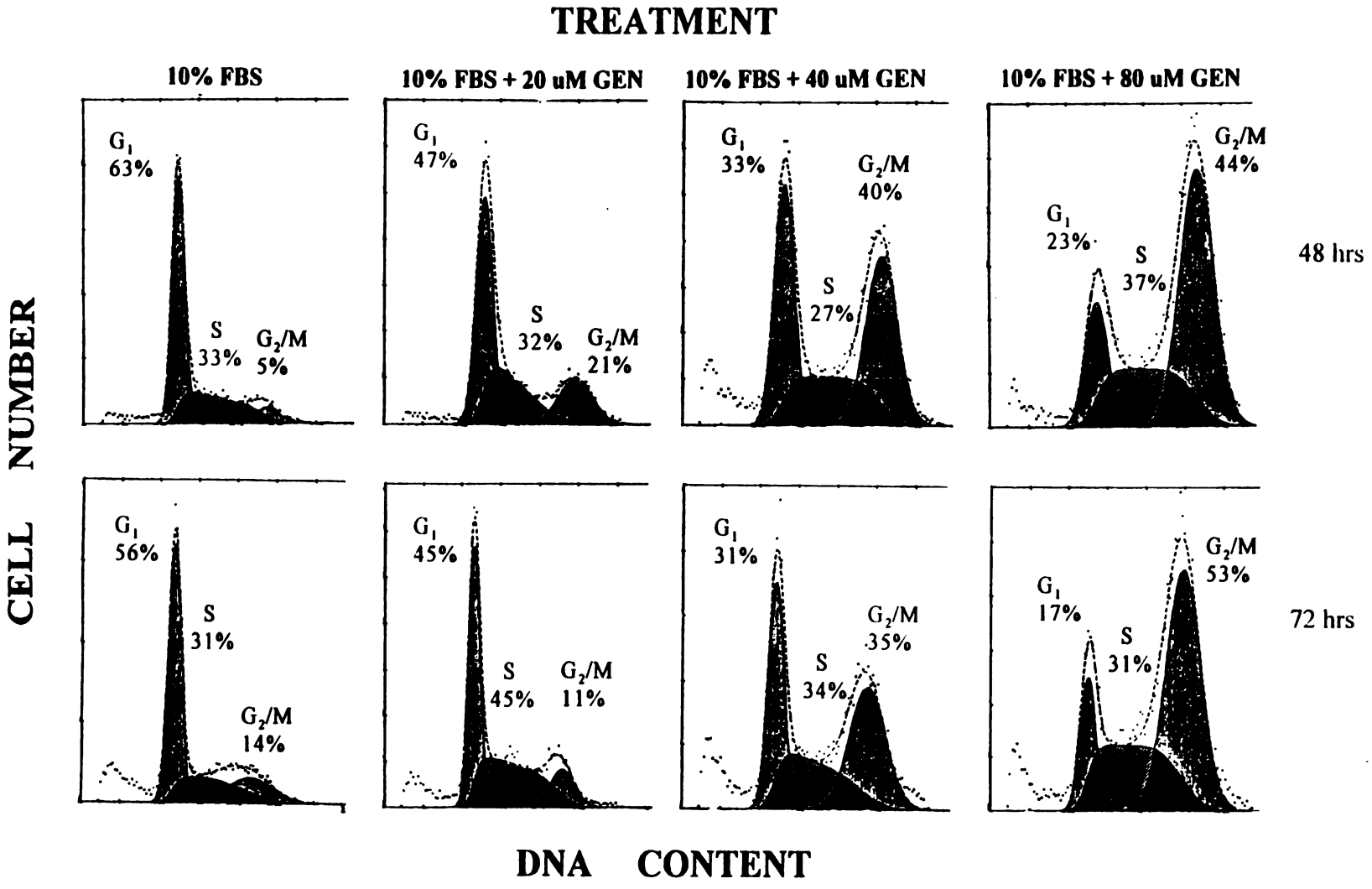
TREATMENT
10% FBS
10% FBS + 20 uM GEN
10% FBS + 40 uM GEN
10% FBS + 80 uM GEN
CELL NUMBER
G1 63%
S 33%
G2/M 5%
G1 47%
S 32%
G2/M 21%
G1 33%
S 27%
G2/M 40%
G1 23%
S 37%
G2/M 44%
48 hrs
G1 56%
S 31%
G2/M 14%
G1 45%
S 45%
G2/M 11%
G1 31%
S 34%
G2/M 35%
G1 17%
S 31%
G2/M 53%
72 hrs
DNA CONTENT

Figure 5

was approximately 20 μM. Whether a similar plasma concentration can be produced in mice fed genistein is unknown. To determine the plasma concentration of genistein produced by feeding different amounts of genistein, Balb/C mice were fed genistein at doses ranging from 0-6000 μg/g AIN-93G. The plasma concentration of genistein (free + conjugated) in mice fed genistein at doses from 0 to 6000 μg/g AIN-93G ranged from 0 to 7.1 ± 1.25 μM (Figure 6). The plasma concentration of unconjugated genistein ranged from 0 to 1.76 ± 0.339 μM.

To assess whether genistein would alter food consumption, groups of mice were fed the control diet (AIN-93G) for 4 days and then switched to diets containing from 0-6000 μg genistein/g AIN-93G for 4 days. Food intake was assessed each day. Food intake did not differ with dietary genistein at any of the doses tested (data not shown).

3. Effect of Genistein Fed to Mice Inoculated with MDA-MB-231 Cells: To determine whether the observed antiproliferative effects of genistein in cell culture could also occur <u>in vivo</u> the following studies were conducted.

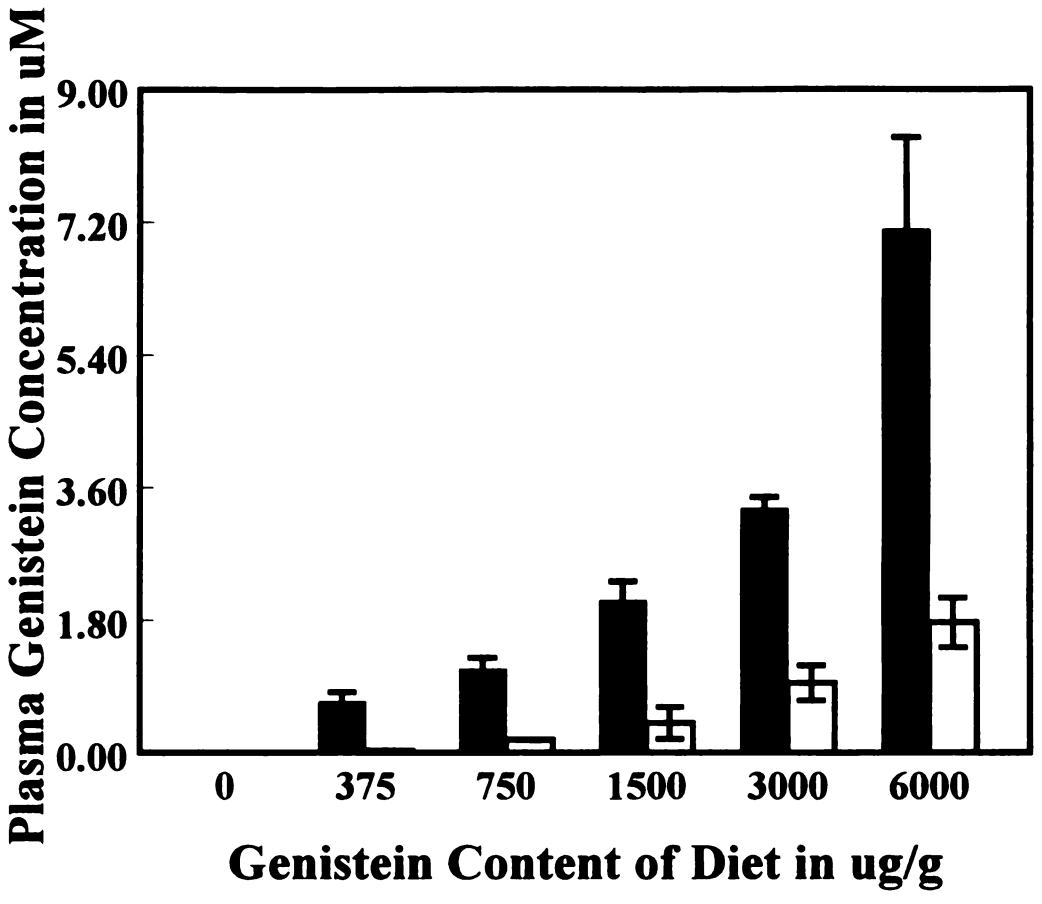

Figure 6. Plasma Genistein Concentration in Mice Fed Genistein. Balb/C mice were received at 4 weeks of age, sorted to equalize weights (3 mice/cage, 2 cages/group, n=6), and fed AIN-93G (6 g/d) for two weeks. Mice were then given 6 g of AIN-93G per day without genistein for four days and then switched to AIN-93G diet containing genistein at the doses noted above for four days. Blood was collected, plasma prepared for analysis of genistein content by reverse phase HPLC. Dark bars represent total genistein (free + conjugate) and open bars represent free genistein. Values are expressed as mean ± SEM.

Dietary genistein was fed at a dose (750 µg genistein/g AIN-93G) which produced a plasma genistein concentration of approximately 1 µM. Xu et al. (1994) have found total plasma genistein concentrations of approximately 1 µM in women consuming soymilk which contained 36 mg of genistein. This dose also exerted biological activity in estrogen responsive tissues in mice. This dose of dietary genistein did not significantly affect the growth of solid tumors (Figure 7). Plasma genistein (free + conjugated) was 0.933 ± 0.107 µM and the free form was 0.306 ± 0.141 µM. A plasma concentration of approximately 1 µM genistein appears to be insufficient to inhibit the growth of solid tumor masses. To determine the effect of a higher dose of genistein on tumor growth, genistein was fed at 3000 µg/g AIN-93G. This lead to an increase plasma genistein concentrations (5.88 µM ± 0.56, conjugated + free form, and 0.64 µM ± 0.18 free form) and allowed us to assess the effect a larger dose of genistein would have on tumor growth. Tumors in the group of mice fed 3000 µg genistein/g AIN-93G grew significantly slower and at the end of five weeks were 23% smaller than tumors in the control group (Figure 8).

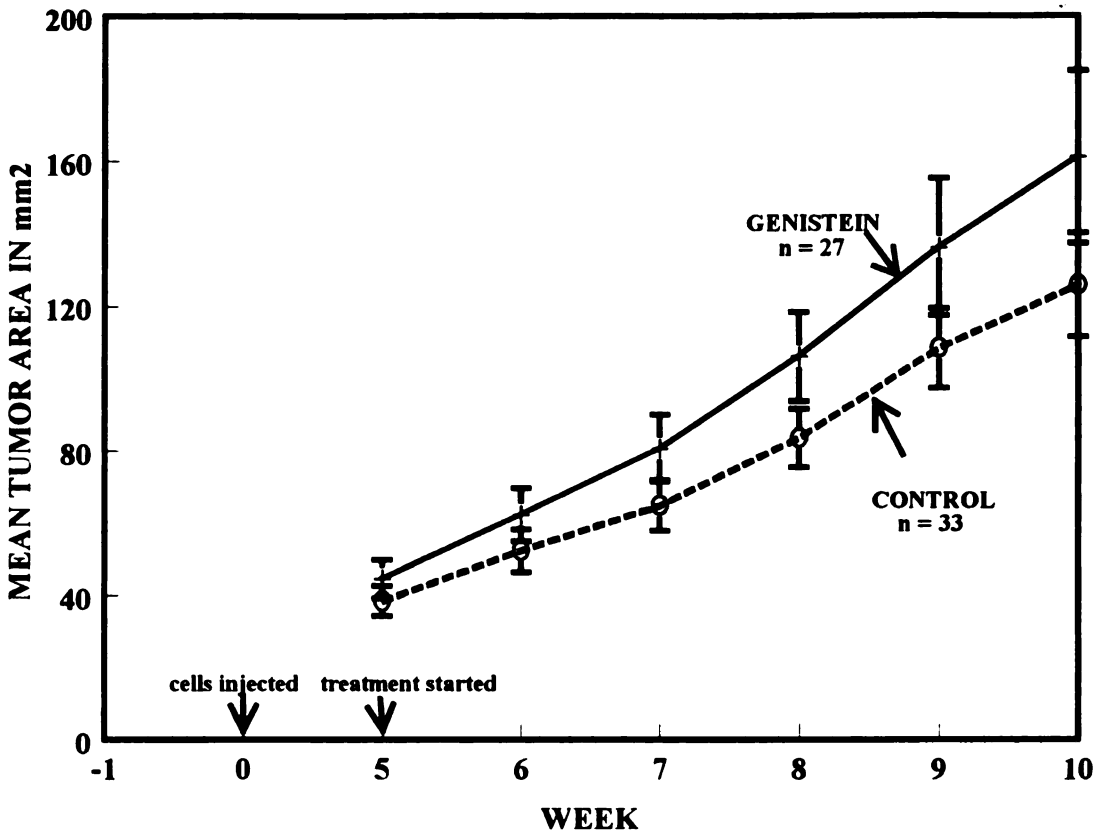

Figure 7. Tumor Growth in Mice Fed 750 µg/g Genistein After Tumor Formation. MDA-MB-231 cells were inoculated into the mice as described in the materials and methods. Mice were grouped to equalize tumor number and size into AIN-93G and AIN-93G plus genistein. The solid line represents mice (n=13) fed dietary genistein (750 µg/g) and the dashed line represent control-fed mice (n=14). Tumor growth was measured weekly. Values represent mean tumor area/mouse ± SEM.

Food intake per cage was 10.7% ($p \leq 0.001$) lower in the genistein fed mice compared to control mice (13.24 ± 0.19 vs. 14.83 ± 0.22). Weight gain, although lower in the genistein fed mice (2.06 ± 0.33 g vs. 2.69 ± 0.59 g), did not differ significantly ($p=0.36$) between the groups.

Dietary genistein (750 µg genistein/g AIN-93G) did not significantly affect tumor growth (Figure 7) when given after the formation of solid tumor masses. Development of a vasculature is required for solid tumor growth in excess of 2-3 mm (Folkman 1989). Genistein has been shown to inhibit the process of angiogenesis thus genistein may inhibit the initial development of a tumor (Fotsis et al. 1993). To assess the effect of genistein upon initial tumor development 750 µg genistein/g AIN-93G was fed three days before the MDA-MB-231 cells were inoculated into the athymic mice and continued throughout the experiment. Dietary genistein (750 µg genistein/g AIN-93G) did not significantly affect the development (number of tumors per group) or growth of tumors (Figure 9). In both of the studies where mice were fed 750 µg genistein/g AIN-93G, food intake or weight gain did not significantly differ between the two groups (data not shown).

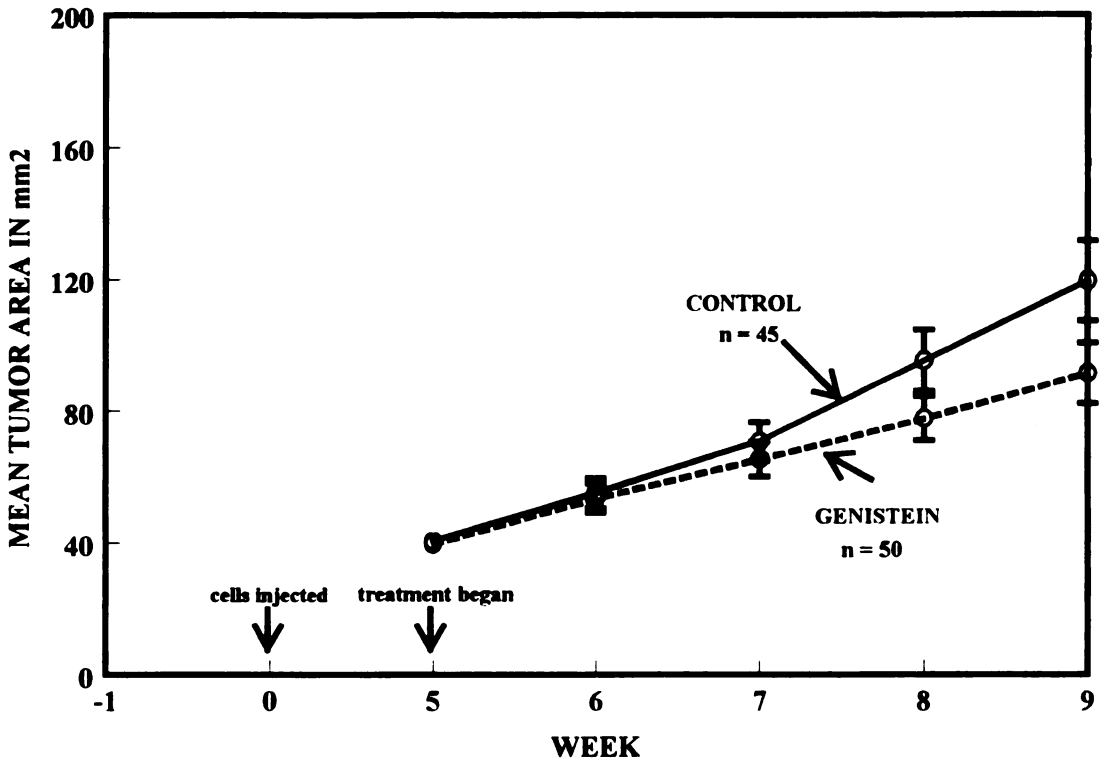

Figure 8. Tumor Growth in Mice Fed 3000 µg/g Genistein After Tumor Formation. MDA-MB-231 cells were inoculated into the mice. Mice were sorted to equalize tumor number and size into AIN-93G and AIN-93G plus genistein groups. Tumor growth was measured weekly. The dashed line represents mice (n=14) fed dietary genistein (3,000 µg/g) and the solid line represents control-fed mice (n=13). Values represent mean tumor area/mouse ± SEM.

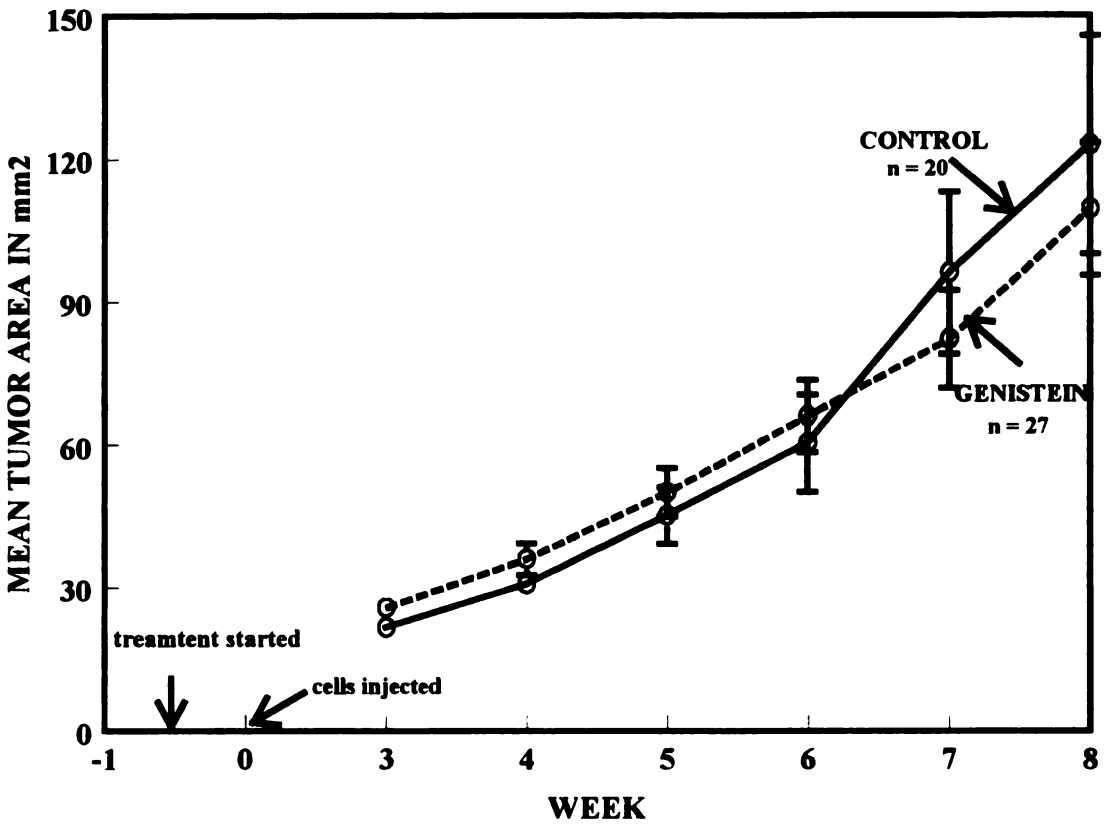

Figure 9. Tumor Growth in Mice Fed 750 µg/g Genistein Before Tumor Formation. Mice received dietary genistein three days before cell inoculation. MDA-MB-231 cells were inoculated into the mice and tumor growth measured weekly. The dashed line represents mice (n=10) fed dietary genistein (750 µg/g) and the solid line represents control-fed mice (n=9). Values in the graph represent mean tumor area/mouse ± SEM.

E. Discussion

We have demonstrated that genistein will inhibit the proliferation of MDA-MB-231 human breast cancer cells in vitro (Figure 4) and that the probable mechanism is inhibition of the cell cycle at G_2/M (Figure 5 and Table 5). These data are in agreement with the work of Peterson & Barnes (1991) using MDA-468 and MCF-7 cells and that of Monti & Sinha (1994) using MCF-7/WT, MCF-7/ADR and MDA-231 human breast cancer cells. In these studies the concentration of genistein required to inhibit the proliferation of human breast cancer cells by 50% ranged from 7 µM to 40 µM. In addition, others have shown that similar concentrations of genistein (7 µM to 68 µM) will inhibit the proliferation of various cell lines (Yanagihara et al. 1993, Piontek et al. 1993). In the present studies 40 µM and 80 µM genistein blocked cells in the G_2/M phase of the cell cycle. Hunakova et al. (1994) conducted studies with leukemic K-562 cells and demonstrated that 10 µM genistein produced a block at G_2/M. Matsukawa et al. (1993) treated HGC-27 gastric cells with 25 µM to 60 µM genistein and found a progressive block in G_2/M. Traganos (1992) however, has shown that 74 µM genistein will block lymphocytic leukemia MOLT-4 in S phase in addition to a G_2/M block with 18.5 to 74 µM genistein. Spinozzi (1994) has

also demonstrated that 18.5 to 37 μM genistein will block Jurkat T-leukemia cells in G_2/M while 74 to 111 μM genistein will block the cell cycle in S phase. It is likely that genistein is inhibiting one or more tyrosine kinases required in the transition through different phases of the cell cycle and that the perturbation in the cell cycle produced by genistein depends on the specific cell line utilized.

To date no studies have been reported in which dietary genistein was fed to tumor bearing mice. To assess the effect of genistein upon the proliferation of MDA-MB-231 tumors female athymic mice were fed 750 μg genistein/g AIN-93G after solid tumor masses had formed. This dose was selected based on data from previous studies in which 750 μg genistein/g AIN-93G exerted biological effects in estrogen responsive tissues (Santell et al. 1997). In addition 750 μg genistein/g AIN-93G produces a plasma genistein concentration of approximately 1 μM in mice which is similar to that seen in humans consuming soymilk containing 36 mg of genistein (Xu et al. 1994). However, this plasma concentration of genistein is much less than that required to inhibit MDA-MB-231 cell proliferation *in vitro* (Figure 4). Direct comparisons from *in vitro* data to conditions *in vivo* cannot be made with certainty due to a number of

factors, one of which is serum concentration. Fetal bovine serum is typically supplemented at 10% v/v to maintain MDA-MB-231 cells. Whether mitogens, such as growth factors and steroids, contained in this formula are representative of those occurring *in vivo* is unlikely.

Treatment of the mice with this dose of genistein (750 µg genistein/g AIN-93G) did not significantly affect the growth of solid tumor masses. In order to assess the effect a higher plasma concentration of genistein would have on tumor growth the amount of genistein fed to mice was increased to 3000 µg/g AIN-93G. Results from feeding Balb/C mice different amounts of genistein, indicate that a total plasma genistein concentration of 3.3 µM could be achieved in mice fed 3000 µg genistein/g AIN-93G (Figure 6). There are data that indicate that human consumption of soymilk containing 226 mg genistein (total)/day will result in total plasma genistein concentrations of up to 6 µM (Xu et al. 1995). Thus, it is possible to achieve relatively high plasma concentrations of genistein in humans. Treatment of athymic mice with 3000 µg genistein/g AIN-93G significantly inhibited the growth of solid tumor masses when compared to control mice (Figure 8).

Total plasma genistein concentration in the athymic mice fed 3000 µg genistein/g AIN-93G was 5.88 µM, 78% higher

than that in the Balb/C mice (3.31 μM) fed the same amount of genistein (3000 μg/g). The plasma concentration of free genistein was 0.64 μM in the athymic mice compared to 0.70 μM in the Balb/C mice. Whether the absence of microflora in the sterile gut of the athymic mouse is responsible for difference in total plasma genistein concentration, compared to Balb/C mice, is unknown.

Food intake did not differ in the study in which genistein was fed to Balb/C mice from 0-6000 μg/g AIN-93G, however the mice were only fed for a period of 4 days. Caloric restriction has been shown to inhibit tumor formation and growth (Tannenbaum 1945) so food intake was monitored for 3 days in every week over the duration of the 5 week study. Food intake in the genistein treated mice was 10.7 % lower and this difference was significant; however, weight gain per mouse was not significantly different. Weight gain has a slightly higher correlation to tumor incidence than caloric intake although both weight gain and caloric intake are obviously related (Albanes 1987).

In the previous study 750 μg genistein/g AIN-93G was administered to the mice after the formation of solid tumor masses. Solid tumor masses greater than 2-3 mm require a vasculature for nourishment. This vasculature is formed (angiogenesis) through an intricate balance of protease

activity resulting in degradation of the basement membrane in a controlled fashion thus permitting the generation and infiltration of blood vessels. Genistein has been shown to inhibit this process although the concentration required *in vitro* was approximately 150 µM (Fotsis et al. 1993). This concentration is much greater than that observed in the feeding studies with genistein; however, the effect of lower concentrations of genistein upon the initial stages of solid tumor development *in vivo* has not been investigated. In addition *in vitro* endothelial cell proliferation was inhibited with IC_{50}'s of 5 µM (Fotsis et al. 1993) and 12 µM genistein (Koroma et al. 1994). To assess the effect of genistein on initial tumor growth a study was conducted in which 750 µg genistein/g AIN-93G was given 3 days prior to inoculating the MDA-MB-231 cells. Genistein fed before inoculating the MDA-MB-231 cells and then continued throughout the experiment did not affect the appearance or growth of the resultant tumors (Figure 9).

This is the first study that has demonstrated dietary genistein to inhibit the growth of MDA-MB-231 human breast cancer cells implanted into athymic mice. The amount of dietary genistein required to inhibit tumor growth was high (3000 µg/g) and would not be attained through a normal diet; however, as mentioned above it is possible to achieve plasma

genistein concentrations in humans (6 μM). Food intake was lower in the genistein fed mice and this could have contributed to the decrease in tumor growth in these mice; however weight gain was not different. This could be due to little variation in food intake within treatment groups and a wide variation in weight gain. The long-term physiological effects of a large dose of genistein (3000 μg genistein/g AIN-93G) are unknown. Genistein is a phytoestrogen and at the doses used in these studies does produce estrogenic responses in various organs, including the mammary gland, of ovariectomized rodents (Santell et al. 1997). Furthermore genistein has been shown to induce estrogen receptor positive human breast cancer (MCF-7) cell proliferation *in vitro* and (MCF-7) tumor growth *in vivo* (Hsieh et al. 1996). The effect of administering genistein to post-menopausal women, particularly those susceptible to estrogen dependent cancers, is unknown. Before any recommendations can be made on the use of genistein as a prophylactic or treatment for estrogen receptor negative breast cancer, much more research is required.

SUMMARY AND FUTURE RESEARCH

Summary

The incidence of breast cancer is approximately 1 in 30 in Japanese women while it is approximately 1 in 8 in the US. As Japanese women migrate to the US there is an increase in breast cancer incidence in each of two subsequent generations approaching the incidence rate of breast cancer in the US. This suggests environmental changes, including dietary changes, are responsible for the increase in breast cancer. The diet of Japanese immigrants changes from one in which vegetables, including soy products, are largely consumed in Japan to one in which more meat and fat and less vegetables are consumed in the US. At the present time the role of fat in human mammary tumorigenesis is not clear. A number of prospective studies suggest that fat intake is not correlated with the incidence of breast cancer, however there are also a couple of studies that show a slight positive relationship. The consumption of soy products in Japan is many fold greater than that in the US. In addition Japanese immigrants to the US consume

less soy products than they do in Japan. Therefore the change in consumption of soy products could be one factor responsible for the changing breast cancer incidence pattern seen in Japanese immigrants to the US. Of the possible anticarcinogenic compounds in soy the one component that has demonstrated the greatest ability to inhibit cell growth *in vitro* is the isoflavone genistein. I therefore conducted studies to assess the ability of this compound to inhibit breast cancer cell growth *in vitro* and *in vivo*.

Genistein is a phytoestrogen and is capable of exerting estrogenic effects in the rodent uterus, however the biological effects of dietary genistein are not well characterized in other estrogen responsive organs. Therefore studies were conducted to assess the effect different doses of dietary genistein would have in the rodent by using estrogen responsive tissues such as the uterus, mammary gland and the hypothalamic/pituitary axis as indicators. In addition the plasma concentration of genistein responsible for these effects was determined. Dietary genistein (750 µg/g diet)increased uterine weight and uterine c-fos mRNA expression, stimulated lobular/alveolar development of the mammary gland and increased plasma prolactin. The plasma concentration of total genistein (free + conjugated) in rats fed 750 µg

genistein/g AIN-76A was 2.54 µM and the concentration of the free form was 0.4 µM. Competitive binding studies indicated that genistein competes with estradiol for the estrogen receptor with an affinity approximately 1/100th that of estradiol. Genistein (750 µg/g AIN-76A) did not antagonize the action of concurrently fed estradiol (1.0 µg/g AIN-76A) in the organs studied in ovariectomized rats nor did dietary genistein (750 µg/g AIN-76A) inhibit the development of the mammary gland or uterus in immature intact rats fed genistein through sexual maturation (30 to 44 days of age). These studies have shown that 750 µg genistein/g diet is capable of exerting biological effects *in vivo*. They have also provided useful data that will help in deciding the initial dosage of genistein that will be used in conducting *in vivo* tumor studies.

Antiproliferative effects of genistein have been observed in both estrogen dependent and independent cells *in vitro*. I decided to conduct my *in vitro* and *in vivo* studies using estrogen independent MDA-MB-231 human breast cancer cells. By using estrogen receptor negative cells the potential estrogenic activity of genistein in affecting estrogen dependent tumor formation and growth is eliminated. Genistein (20 µM) inhibited MDA-MB-231 cell proliferation *in vitro* by 50%. The cell cycle was blocked at G_2/M when 40 µM

or 80 μM genistein was added to the medium. To evaluate the effect of genistein on tumor growth *in vivo*, athymic mice were inoculated with MDA-MB-231 cells and fed (750 μg/g AIN-93G) genistein. Genistein at this dose did not affect tumor growth. Next I conducted a dose response study to determine the plasma genistein concentration in Balb/C mice fed 0 to 6000 μg genistein/g AIN-93G. Genistein at 3000 μg genistein/g AIN-93G produced a plasma genistein concentration (free + conjugated) of 3.3 μM. When this dose was fed to mice an inhibition of tumor growth was observed when compared to untreated control mice; however, there was a 10.7% reduction in food intake in the genistein group. Although food intake was lower in the genistein treated mice compared to control mice there was no difference in weight gain. To assess the effect of dietary genistein on initial tumor development 750 μg genistein/g AIN-93G was fed before tumor cells were inoculated into the mice. This dose of genistein did not inhibit the development or growth of tumors.

This research demonstrates the disparate characteristics of genistein. Genistein is mitogenic in estrogen responsive tissues in ovariectomized rodents yet is also antiproliferative in both estrogen dependent and independent cell lines *in vitro*. Other researchers have

demonstrated that low doses of genistein will stimulate the growth of estrogen dependent breast tumor cells *in vitro* and *in vivo*; however, when higher doses of genistein are given estrogen dependent cell growth is inhibited *in vitro*. The stimulatory or inhibitory effect of genistein on cell growth are dependent on the dose. In the present studies 750 µg genistein/g diet did not inhibit tumor growth however tumor growth was inhibited with 3000 µg genistein/g diet. Food intake was lower in the genistein fed mice compared to the control mice and this could have contributed to the inhibition of tumor growth; however, weight change was not different between the two groups.

The effects of dietary genistein upon development of mammary cancer in humans is unknown. These studies suggest genistein could either enhance (due to its estrogenic activity) or inhibit (due to its antiproliferative activity) mammary tumorigenesis. Additional research is needed on the effects of dietary genistein in tumor models that simulate tumor development in pre- and postmenopausal conditions.

Future Research

These studies have answered some of the questions regarding genistein and its effect on breast cancer yet have also created additional ones. For example:

1) Relatively large doses of dietary genistein (750 µg/g) did not affect estrogen independent tumor growth yet did exert estrogenic effects in estrogen responsive organs in ovariectomized rodents. This suggests that this does of dietary genistein could potentially stimulate estrogen dependent breast cancer in ovariectomized rodents. Whether dietary genistein could have similar effects on the development of breast cancer in postmenopausal women is unknown yet clearly worthy of study in an appropriate animal model. The ovariectomized athymic mouse implanted with estrogen dependent breast cancer cells would be a useful model in which to conduct these studies.

2) Genistein did not antagonize estradiol in intact or estradiol supplemented ovariectomized rats. Nor was the estrogenic effect of genistein additive to that of estradiol. This suggests dietary genistein was not estrogenic, or the estrogenic activity was not measurable, in the presence of high levels of estradiol found in premenopausal women. Whether genistein would have a role in the promotion of breast cancer in premenopausal women is

unknown yet once again worth studying in the appropriate model. The intact female athymic mouse implanted with estrogen dependent human breast cancer cells would be a useful model in which to conduct this study.

3) A large dose of dietary genistein (3000 µg/g diet) did inhibit the growth of estrogen independent human breast cancer cells in the athymic mouse. However, food intake was lower in this group of mice compared to the control mice. Mouse weight did not differ between the two groups and weight is thought to be more highly correlated with breast cancer incidence. Nevertheless, it is unclear whether the decrease in food consumption could be partly responsible for the observed inhibition of tumor growth. This study should be repeated with the following conditions. Mice should be housed individually to enable a more accurate analysis of food intake per mouse. The amount of food provided to mice in the control and treatment groups should be slightly less than that consumed by the mice fed 3000 µg/g in this study. Furthermore a positive control group of mice provided with food ad lib should be included. This would eliminate the potential confounding variable of food intake upon tumor growth.

4) Genistein has been shown to inhibit both estrogen dependent and independent human breast cancer cells <u>in</u>

vitro. Therefore the research described in number 3 should also be conducted utilizing estrogen dependent human breast cancer cells.

Appendices

Introduction to the Appendices

The data presented in the attached appendices are from experiments that were not included in manuscripts or were from preliminary studies. These include a number of studies that evaluated the effect of administering genistein via different routes upon tumor growth, for example: gavaging genistein or implanting genistein pellets. Studies are presented that assessed the effect the gavage vehicle has on food intake. A study assessed the plasma genistein concentration over a 24 hour period after gavaging a genistein bolus to determine the timing of maximum plasma concentration. The eating behavior of mice was assessed to find out when they consume the greatest amount of food. These studies allowed us to determine what time to kill the mice in order to measure the highest plasma concentration that could be achieved from the diet. In addition the effect feeding different amounts of dietary genistein would have on food intake and weight gain was assessed.

Appendix A

Effect of Genistein, with 1% or 10% Fetal Bovine Serum, on MDA-MB-231 Cell Proliferation:

Previous work presented has demonstrated that genistein will inhibit MDA-231 cell growth with an IC_{50} of ~25 μM. Those experiments utilized standard passing media which contained 10% FBS plus genistein at 0, 10, 20, 40, or 80 μM. It was of interest to determine the effect of genistein upon cell growth in media containing a lower percent of FBS. FBS contains various growth factors that are required for the maintenance and growth of cells in culture and it also contains serum albumin. In the past I have demonstrated a FBS dependent increase in cell proliferation with increasing percent of FBS in the media: this suggests increased mitogenic stimulation of the tumor cells in the presence of increased growth factors. It was of interest to determine the effect of genistein upon cell proliferation in the presence of media containing different amounts of FBS. Will genistein inhibit cell proliferation at the same IC_{50} when the FBS concentration is lowered to 1%?

Experimental Protocol:

3-5-95 collected MDA-231 cells from 4 p100 plates, determined cell concentration and plated cells in 24-well plates at 15k cells per well in MEM containing 10% FBS

3-6-95 changed media to MEM containing 0.1% FBS

3-8-95 collected a plate to determine day 0 values by analyzing DNA content, began treatment of cells with MEM containing either 10% FBS or 1% FBS with 0, 10, 20, 40, or 80 μM genistein

3-9-95 collected plates from the 10% and 1% FBS groups and ran DNA assays to assess cell proliferation, day 1

3-10-95 changed media in all plates

3-11-95 collected plates from the 10% and 1% FBS groups and ran DNA assays to assess cell proliferation, day 3

3-12-95 changed media in all plates

3-13-95 collected plates from the 10% and 1% FBS groups and ran DNA assays to assess cell proliferation, day 5

3-14-95 changed media in all plates

3-15-95 collected plates from the 10% and 1% FBS groups and ran DNA assays to assess cell proliferation, day 7

Results:

Genistein administered in MEM containing 10% FBS inhibited cell proliferation with an IC_{50} of ~25 μM (Figure A1) which agrees with previous studies. In the MEM containing 1% FBS, genistein inhibited cell proliferation with an IC_{50} of ~10 μM (Figure A2).

Conclusion:

The results of this study suggest that the ability of genistein to inhibit cell proliferation is dependent upon the concentration of mitogens in the media. This is important when attempting to compare *in vitro* results to the *in vivo* condition. The relationship between the concentration of growth factors in the media to the concentration found *in vivo* is unknown. Furthermore the concentration *in vivo* is continually changing. The inhibitory affect of genistein seen in this study is dependent upon the concentration of mitogens the cells are exposed to, so although we see IC_{50}'s ranging from ca. 10 to 25 μM depending on culture conditions, the inhibitory concentration required or expected *in vivo* is not known.

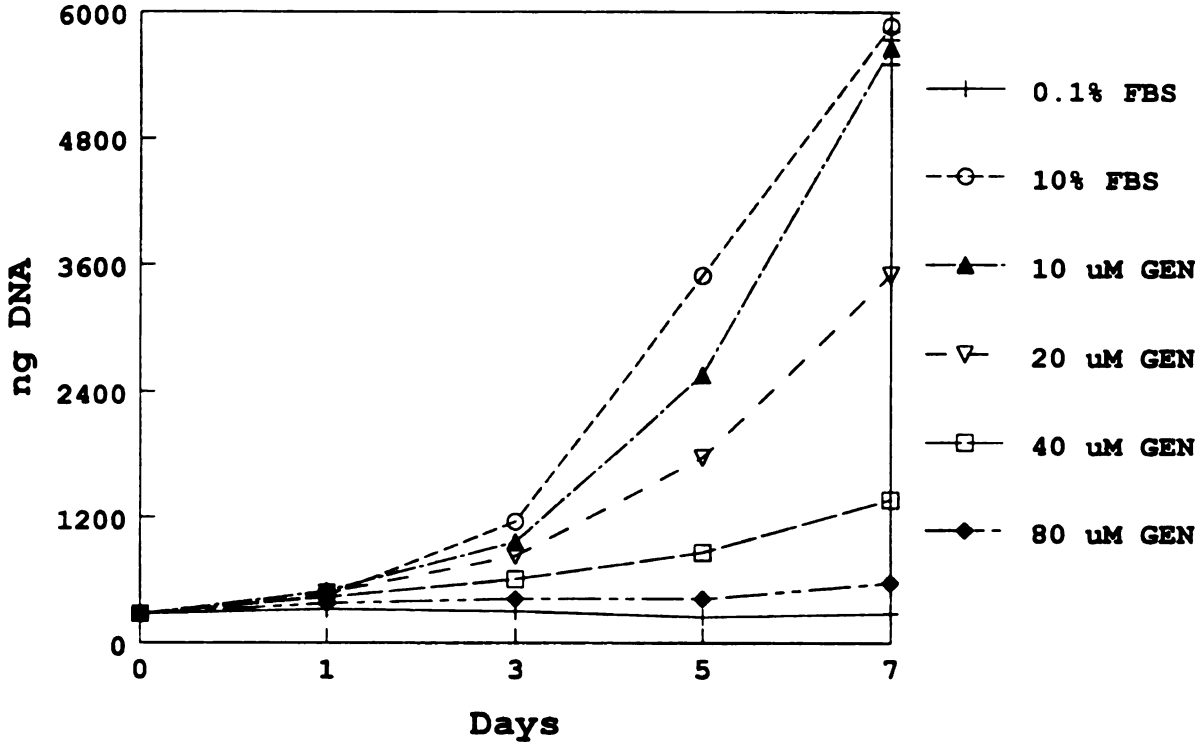

Figure A1. Effect of genistein on MDA-MB-231 Cell Proliferation. MDA-MB-231 human breast cancer cells were treated with 0, 10, 20, 40 and 80 μM genistein in MEM containing **10% FBS** for a period of 7 days. Fluorometric analysis of cellular DNA was performed on days three and seven. Solid bars represent cells cultured for three days and open bars are data collected after seven days. Each point represents the mean ± SEM of eight measurements.

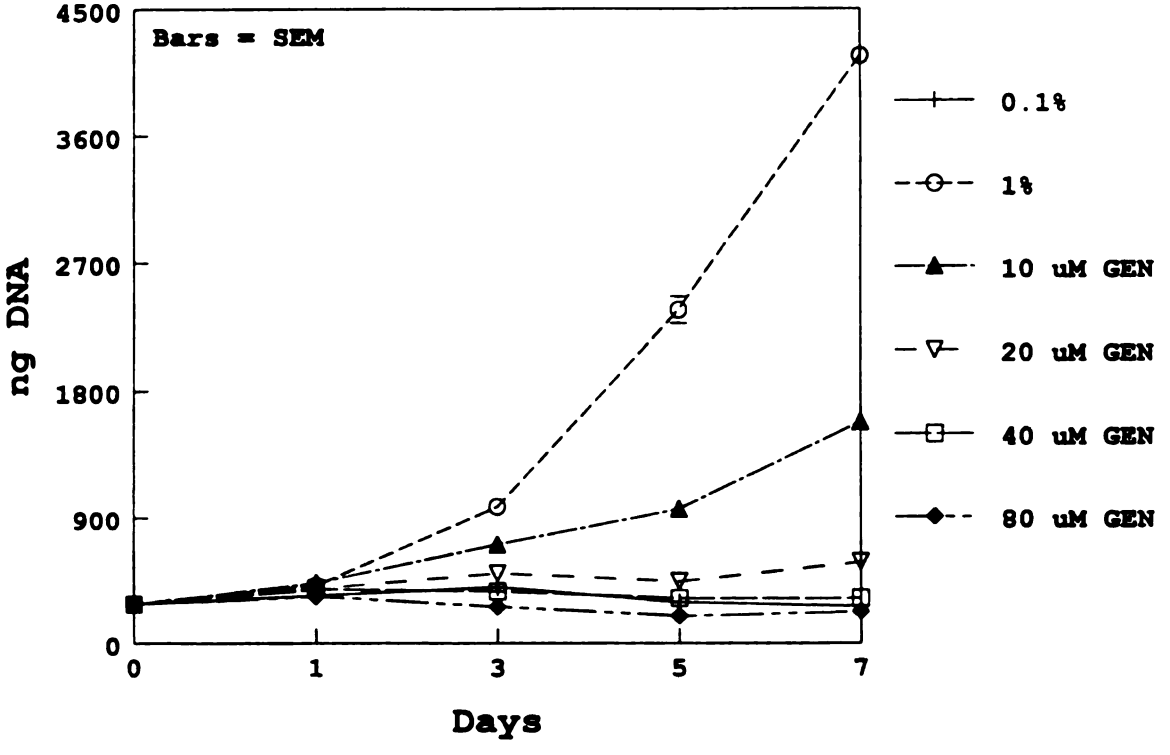

Figure A2. Effect of genistein on MDA-MB-231 Cell Proliferation. MDA-MB-231 human breast cancer cells were treated with 0, 10, 20, 40 and 80 µM genistein in MEM containing **1% FBS** for a period of 7 days. Fluorometric analysis of cellular DNA was performed on days three and seven. Solid bars represent cells cultured for three days and open bars are data collected after seven days. Each point represents the mean ± SEM of eight measurements.

Appendix B

Kinetic Analysis of B-Glucoronidase:

Optimal ph for B-glucoronidase function is approximately pH 5. At this ph the isolation and preparation of serum samples for HPLC analysis of genistein produce chromatograms which are very 'dirty', however at approximately ph 7 the chromatograms look good. However, the activity of B-glucoronidase at this pH in serum samples was not known. Therefore the following study was conducted to assess B-glucoronidase activity at the higher ph.

Experimental Protocol:

Rat serum, from a study in which rats were gavaged with 40 mg genistein, was aliquotted into three 550 ul samples in 1.5 ml microfuge tubes. One tube was served as the control, no enzyme, one tube received 0.5 ul enzyme/10 ul serum (1x) and another tube received 1.0 ul enzyme/10 ul serum (2x). 50 ul samples were taken from all tubes at time 0, 15 and 30 min., and 1, 2, 4, 8, 12, 24 and 48 hours. Following the incubation, 50 µL of absolute methanol was added to each tube, the tubes vortexed and then centrifuged at 15,000 x g for 10 min. Approximately 75 µL was removed and placed at -20°C until analysis. For analysis of genistein the

microfuge tubes were centrifuged at 15,000 x g for 10 min. and 20 µL injected onto a C_{18} column (Microsorb-MV, 5 µM 100A, Rainin Instrument in Woburn, MA) with a flow rate of 1.0 ml/min of 50:50 methanol:water with 0.1% acetic acid.

Results:

see Figure B.

Conclusion:

The data suggests the reaction had gone to completion in approximately 24 hours in the 2x reaction while it took around 48 hours in the 1x reaction. This study shows that the enzyme is functional at pH 7, the concentration required for greater effect, and the time required for completion of the reaction. These data will be used in future procedures for analyzing plasma genistein.

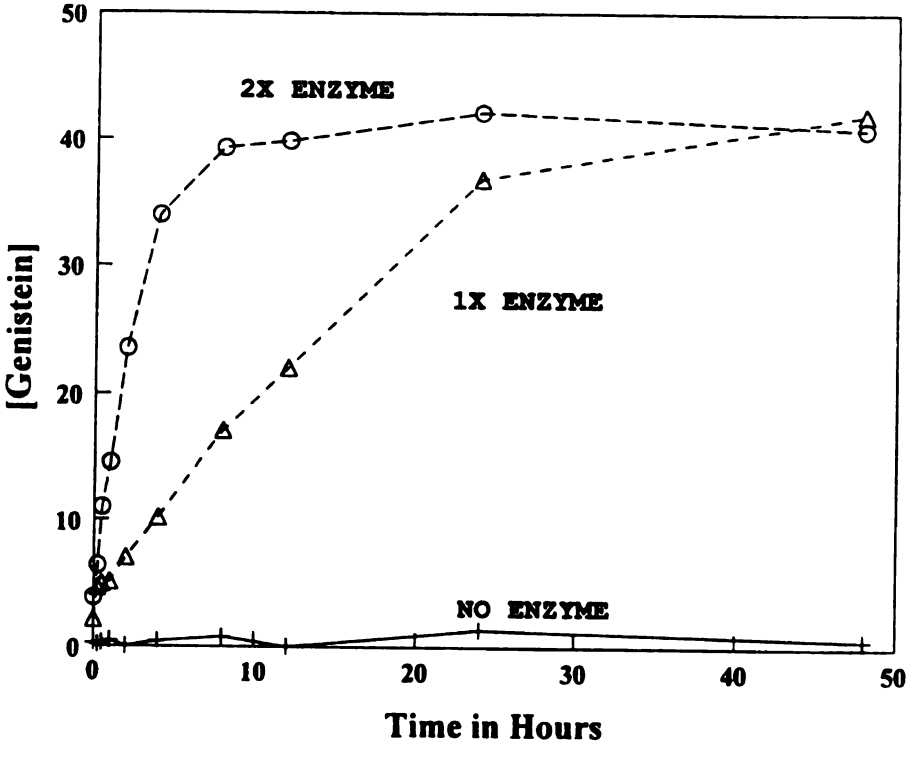

Figure B. Kinetic analysis of B-glucuronidase in serum from rats gavaged with genistein. Serum samples (50 ul) were treated with B-glucuronidase (208 (1X) or 515 (2X) Fishman Units) or without, incubated for different times and samples analyzed at for genistein with HPLC.

Appendix C

Food Consumption Pattern in the Mouse:

Although feeding 750 µg/g genistein does not affect the growth of existing tumors feeding higher amounts of genistein may be effective. It is necessary to determine the plasma concentration of genistein in mice fed various amounts of genistein in order to evaluate the plausibility of feeding higher amounts...is there a plasma genistein concentration that could be effective. In order to determine the time where plasma genistein concentration reaches a maximum it is necessary to establish the food intake pattern of the mouse.

Experimental Protocol:

18:00 provide 2 cups of food to mice, 5 per cage, 4 cages

20:00 weigh food cups

24:00 weigh food cups

06:00 weigh food cups

12:00 weigh food cups

18:00 weigh food cups

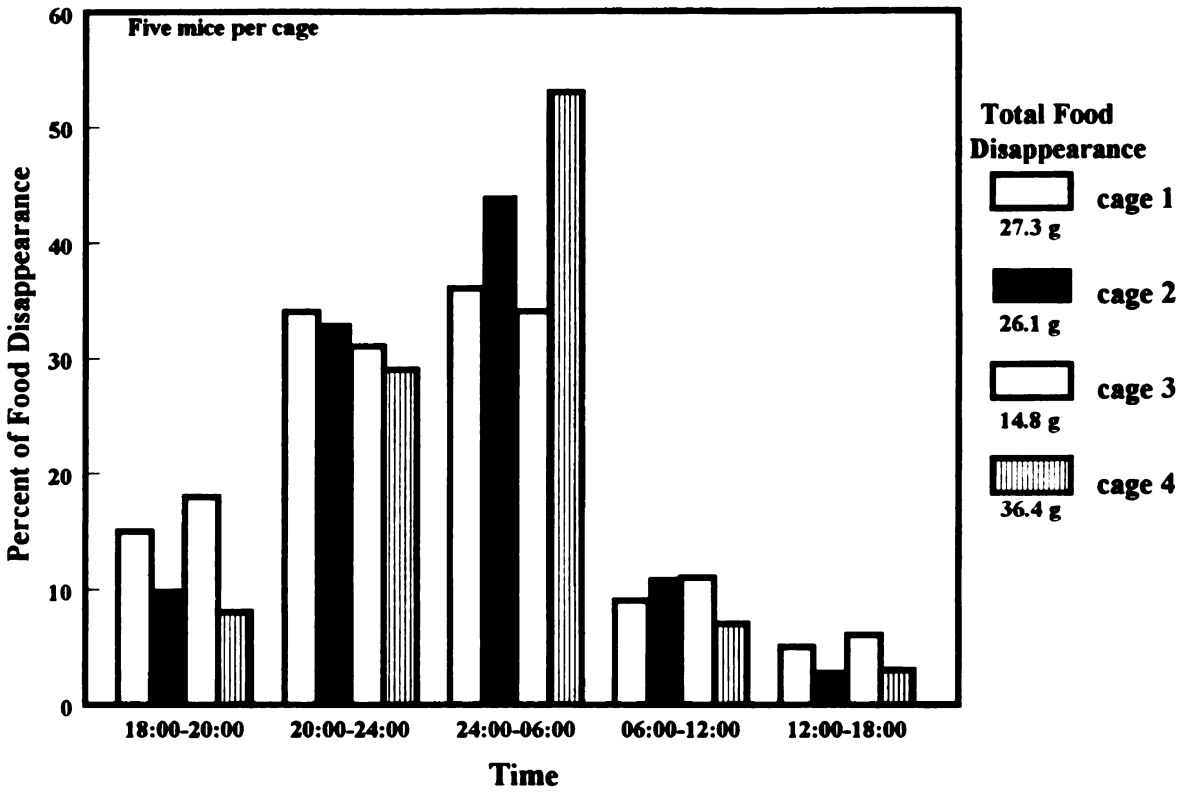

Figure C. Food intake of mice over a 24 hour period. Two cups of food were weighed and provided to each cage of five mice at 6:00 pm. Food disappearance was monitored by weighing the food cups at the indicated times. At 6:00 am new cups were provided.

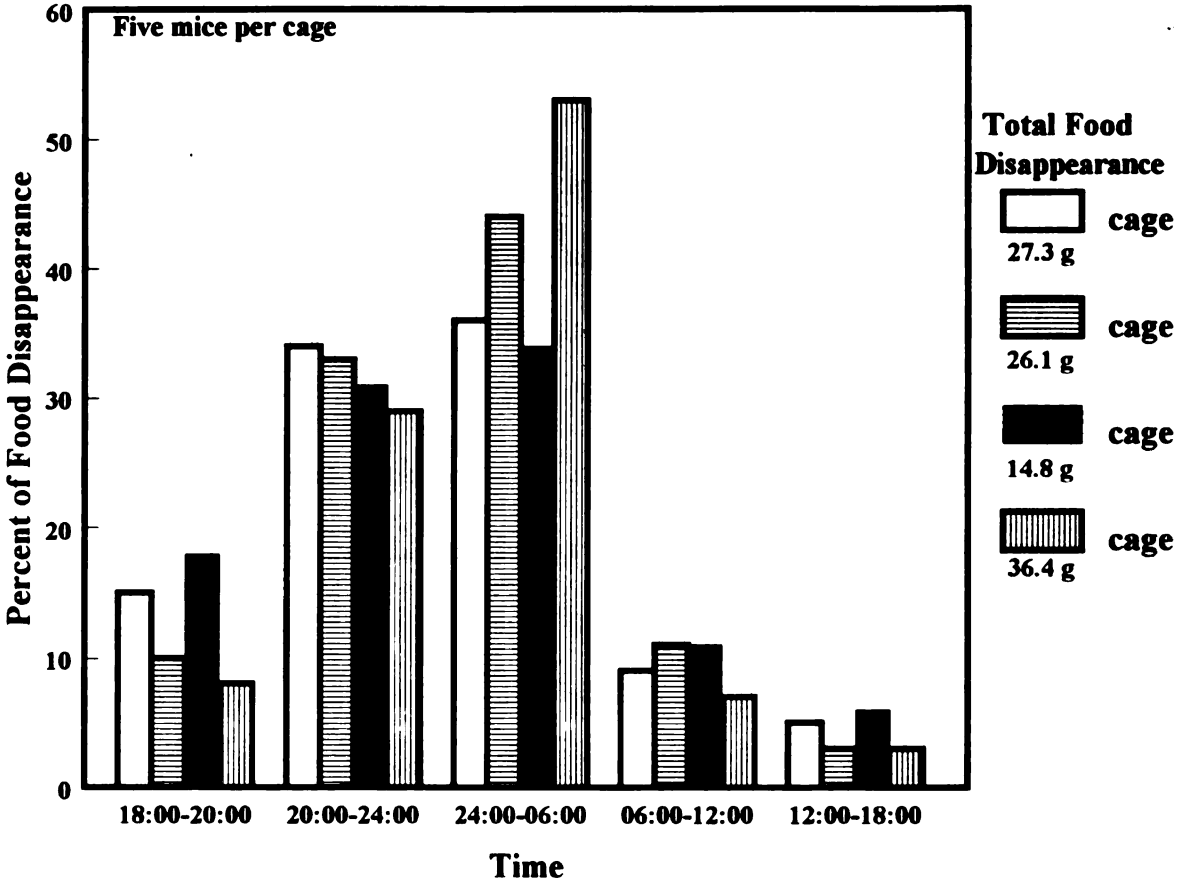

Figure C. Food intake of mice over a 24 hour period. Two cups of food were weighed and provided to each cage of five mice at 6:00 pm. Food disappearance was monitored by weighing the food cups at the indicated times. At 6:00 am new cups were provided.

Appendix D

Athymic Mice Fed Genistein for 10 Weeks:

Genistein at 750 µg/g did not affect the growth of tumors in the above study. Feed intake was measured over three days and was similar among the groups. Weight change was also similar among the groups. To assess the effect of higher concentrations of genistein upon feed intake in the athymic nude mouse a feeding study was conducted.

Experimental Protocol:

1-16-95 Receive 12 mice at 21 days of age and grouped 2 mice/cage, 2 cages/group.

1-31-95 Began feeding AIN-93G, AIN-93G + 1500 µg/g genistein and AIN-93G + 3000 µg/g genistein

2-1-95- Began assessing feed intake three days of every week for ten weeks

4-13-95 Killed and weighed all mice, weighed uterus and collected mammary gland for analysis of development

Results:

Food Intake: Food intake was 10.17 ± .17 g/cage/day in the control group, 8.82 ± .14 g/cage/day in the

1500 µg genistein/g group and 7.43 ± .16 g/cage/day in the 3000 µg/g genistein group. (See Figure D)

Weight Change: There were no significant differences in weight change among the groups, control ± 4.11 g ± .57, 1500 µg/g 5.26 g ± .35 and 3000 µg/g ± 4.24 ± .76 (See Figure D).

Uterus: Uterine weight in the treatment groups was not significantly different from the control: control 0.09 ± .04, 1500 µg/g 0.10 ± .02 and 3000 µg/g 0.86 ± .01.

Mammary Gland: The inguinal mammary gland was removed from each mouse and stained. Glands were analyzed and scores from 1 to 4 assigned for endbud analysis and ductal growth.

Endbud Analysis: There were no significant differences among the treatment groups: control 1.75 ± 1.5 (SD), 1500 µg/g 1.5 ± .58 and 3000 µg/g 2.88 ± .63.

Duct Analysis: There was no significant difference in duct infiltration into the mammary fat pad among the control and the 1500 µg/g groups, control 3.95 ± .06 and 1500 µg/g 3.63 ± .48. The 3000 µg/g group did have a significant decrease in ductal

development compared to the control, control 3.95 ± .06 and 3000 µg/g 2.88 ± .63.

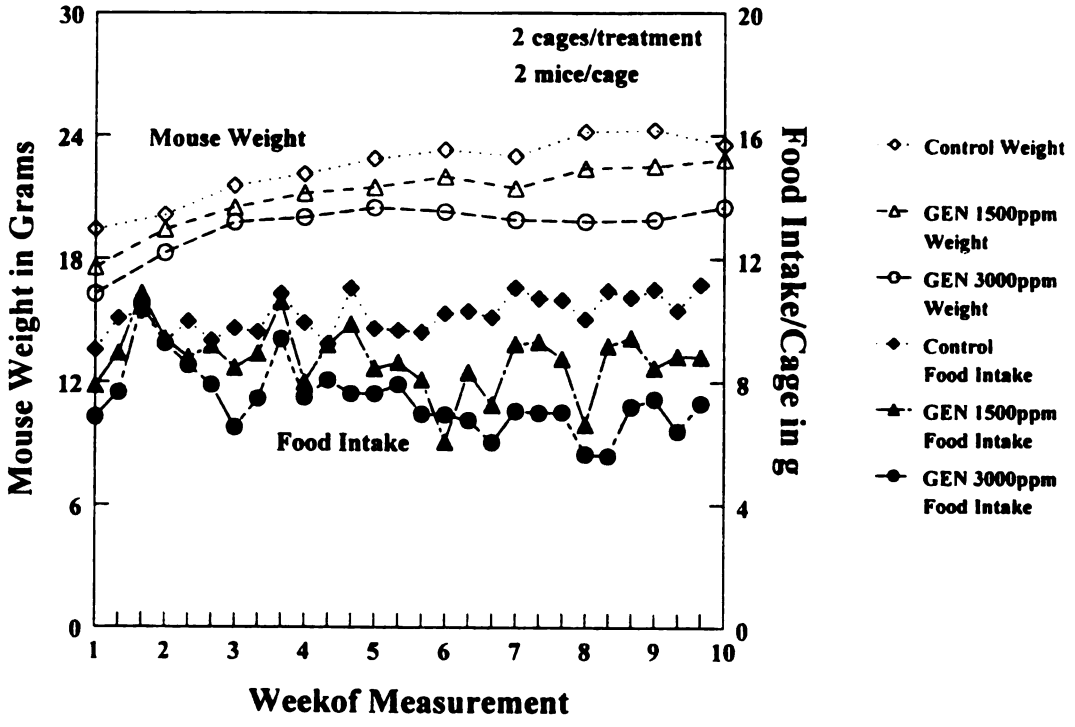

Figure D. Food intake and weight of mice fed 0, 1500 and 3000 µg genistein/g food. Athymic mice were fed genistein at the doses noted for a period of ten weeks. Food disappearance was monitored for 3 days of each week and weight assessed weekly. Open figures are mouse weight and closed figures are food intake per cage.

Conclusion:

This study was conducted to determine food intake of mice fed 1500 and 3000 µg/g genistein over a long period (10 weeks). Although food intake was 2.7 g/cage/day (averaged throughout the experiment) ($p \leq 0.001$) less in the mice receiving 3000 µg genistein/g AIN-93G, compared to control, and 1.35 g/cage/day in the mice receiving 1500 µg/g diet, weight change between the groups was not significantly different. However, the mice in the genistein treated groups weighed less at the beginning of the study than the mice in the control group: control 19.4 g, 1500 µg/g 17.6 g and 3000 µg/g 16.3 g. This could have affected weight change in the experiment. These data suggest food intake decreases with increasing concentration of genistein in the diet; however, the change in food intake does not produce an immediate change in weight gain.

Appendix E

Plasma Analysis of Genistein from Mice Given a Bolus of 6 mg Genistein:

The uptake, availability and metabolism of dietary genistein are unknown. This study sought to determine the point at which the maximum concentration of genistein would be present in the plasma from a bolus of 6 mg genistein in DMSO. This information will be used to determine the feasibility of this approach in giving genistein to mice in future tumor studies.

Experimental protocol:

09:00	6 mg genistein administered as a bolus via gavage
10:00	kill three mice and collect plasma
11:00	kill three mice and collect plasma
13:00	kill three mice and collect plasma
17:00	kill three mice and collect plasma
21:00	kill three mice and collect plasma
09:00	kill three mice and collect plasma

Results:

Genistein is absorbed into the blood within 1 hour after administration and reaches a peak at 4 hours (Figure E). Concentrations in excess of 20 μM are present for at least 12 hours and decrease to about 3 μM after 24 hours.

Conclusion:

This approach will produce plasma genistein concentrations in excess of 20 μM for at least 12 hours; however, it is not practical to gavage mice daily over the course of a long term study due to a potential mortality occurring during the gavage procedure.

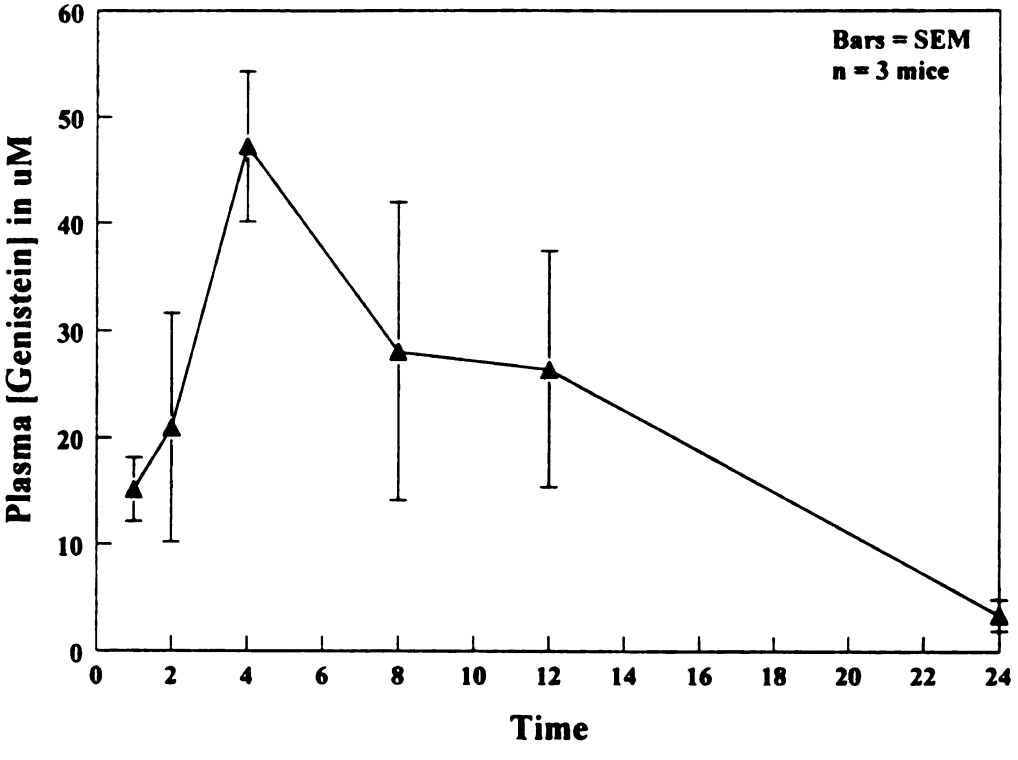

Figure E. Plasma genistein in mice. Balb/C mice were gavaged with 6 mg genistein in 100 ul DMSO and killed at the indicated time points. Blood was collected and analyzed for genistein with HPLC. Each point represents the mean plasma genistein concentration of 3 mice.

Appendix F

Gavage Vehicle and Its Affect on Food Intake in the Mouse:

Earlier experiments in which genistein was gavaged to rats and mice utilized DMSO as a vehicle for the genistein. Given the effect of caloric restriction on tumor growth it was necessary to assess the effect of DMSO on food intake. The following experiment was conducted to evaluate the effect of DMSO on food intake.

Experimental Protocol:

Two cages each containing five mice were included in the study. Mice in one cage were gavaged daily at 14:30 with 100 µl DMSO. Mice were then placed in new cages with new food cups, two per cage, and the food cups weighed. Each day for 3 days mice in the one cage were gavaged. Each 24 hour period the cups were removed from both cages and weighed. After 3 days the gavaging was discontinued but food intake monitored for an additional 5 days. Three days later the mice initially receiving no gavage were gavaged with 100 µl corn oil for two days.

Results:

CAGE	TREATMENT	MEAN FOOD INTAKE/CAGE (SEM)	
1	DMSO Gavage	(3 days)	12.6 (2.22)
1	no gavage	(3 days)	23.9 (1.4)
2	no gavage	(5 days)	24.5 (1.38)
2	corn oil gavage	(2 days)	19.7 (3.65)

Food disappearance see Figure F

Conclusion:

The data indicate that gavaging reduces food intake especially with the DMSO gavage. If gavaging is to employed in future experiments to administer genistein the vehicle used is important. DMSO resulted in a large decrease in food intake whereas the corn oil slightly decreased food intake. Genistein is not soluble in corn oil and the suspension is too viscous for use in the gavage tube so an alternate vehicle had to be found. Medium chain triglyceride oil was found to be marginally useful. Genistein could be added to MCT-oil at 6 mg/100 µl with the resultant suspension capable of passing through the gavage tube however with difficulty at times.

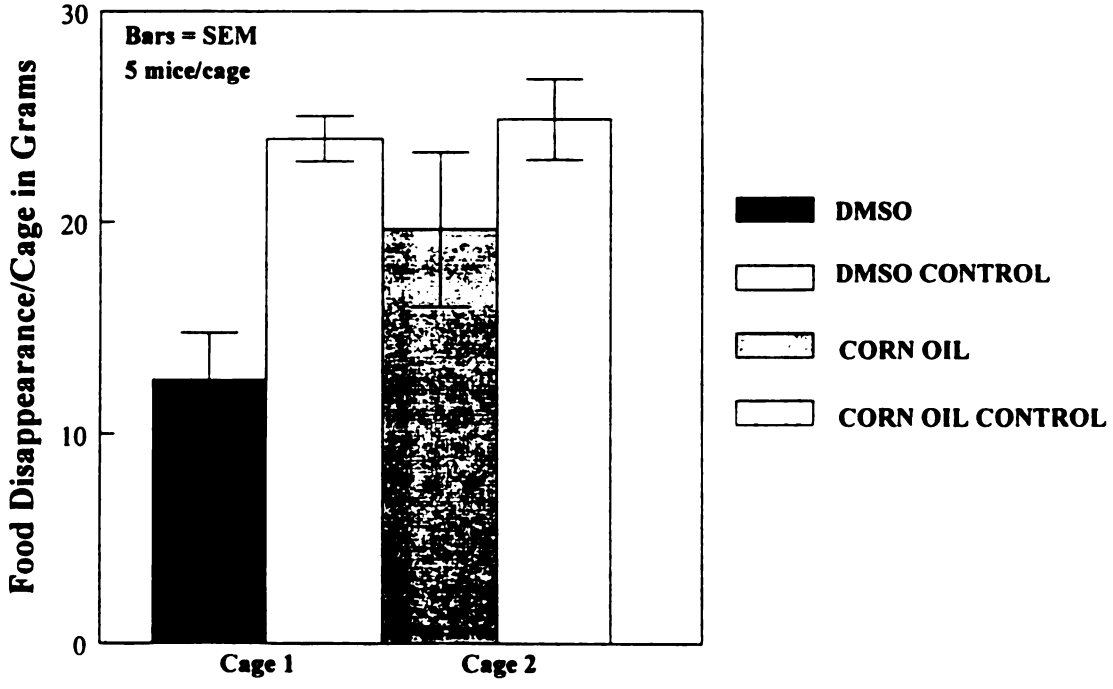

Figure F. Food intake in mice gavaged with corn oil or DMSO. Food intake was monitored in five mice (cage 1) that were gavaged with 100 µl DMSO for 3 days and then monitored for an additional 3 days while not gavaged. Food intake was monitored in another group of 5 mice (cage 2) for 5 days and then the mice were gavaged with 100 µl corn oil for a 2 day period and food intake monitored. Data are expressed as mean food intake per group.

Appendix G

Genistein Gavaged to Tumor Bearing Mice:

A previous study had found that plasma genistein concentrations in excess of 20 μM could be achieved in mice by gavaging 6 mg genistein. This study was conducted to assess the feasibility of this approach over a long period and also to assess the effect upon tumor growth.

Experimental Protocol:

3-6-96	Mice were received at 23 days of age.
3-9-96	Tumor cells injected at 26 days of age.
4-22-96	100% take rate.
4-24-96	Began gavaging mice daily with 6 mg genistein in 100 μl MCT-oil or 100 μl MCT-oil, 2 cages 5 mice/cage, measured food disappearance daily
4-29-96	Killed mice, measured tumors, collected tumors and blood.

Results:

Change in tumor area see Figure G1

Plasma genistein concentration see Figure G2

Food intake see Figure G3

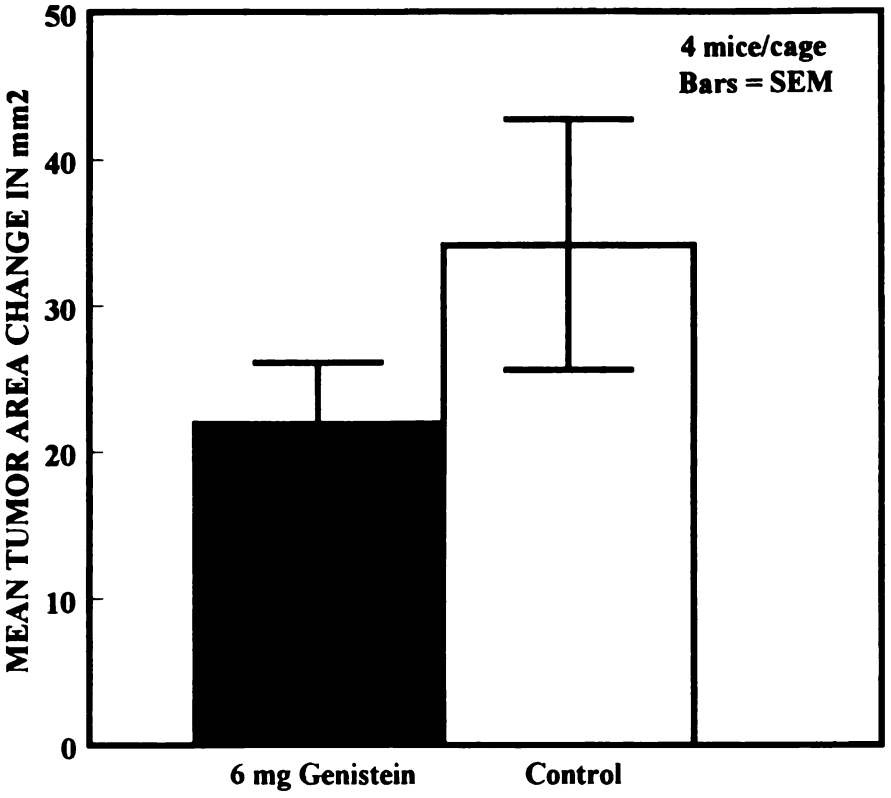

Figure G1. Tumor area change. Tumor bearing athymic mice (4 mice/group) were gavaged with 6 mg genistein in 100 µl MCT-oil or 100 ul MCT-oil for a period of 6 days. Data is expressed as mean tumor area change per group.

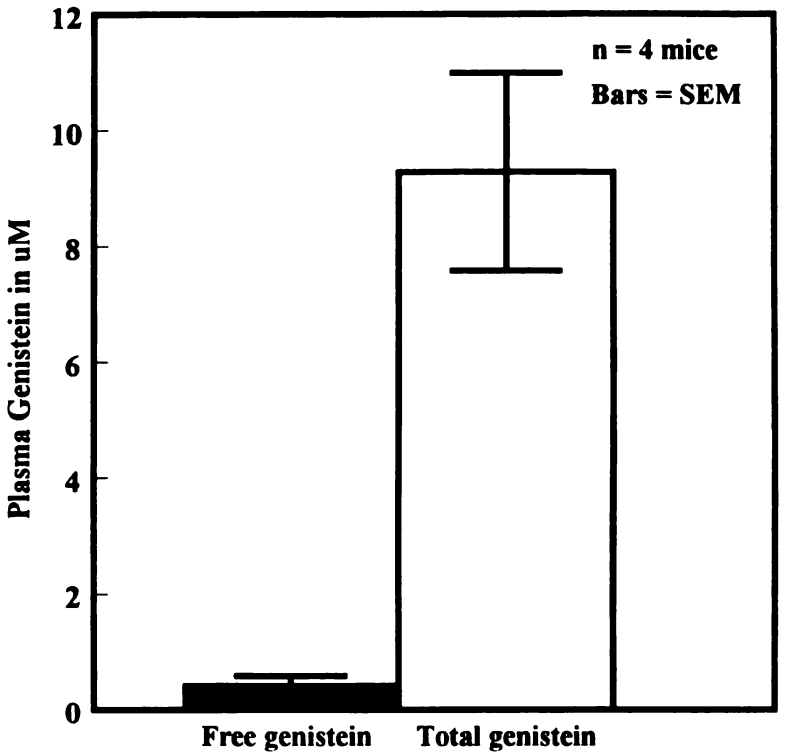

Figure G2. Plasma genistein in mice. Tumor bearing athymic mice (4 mice/group) were gavaged with 6 mg genistein in 100 µl MCT-oil or 100 µl MCT-oil for a period of 6 days. Blood was collected and analyzed for genistein with HPLC. Each point represents the mean plasma genistein concentration of 3 mice.

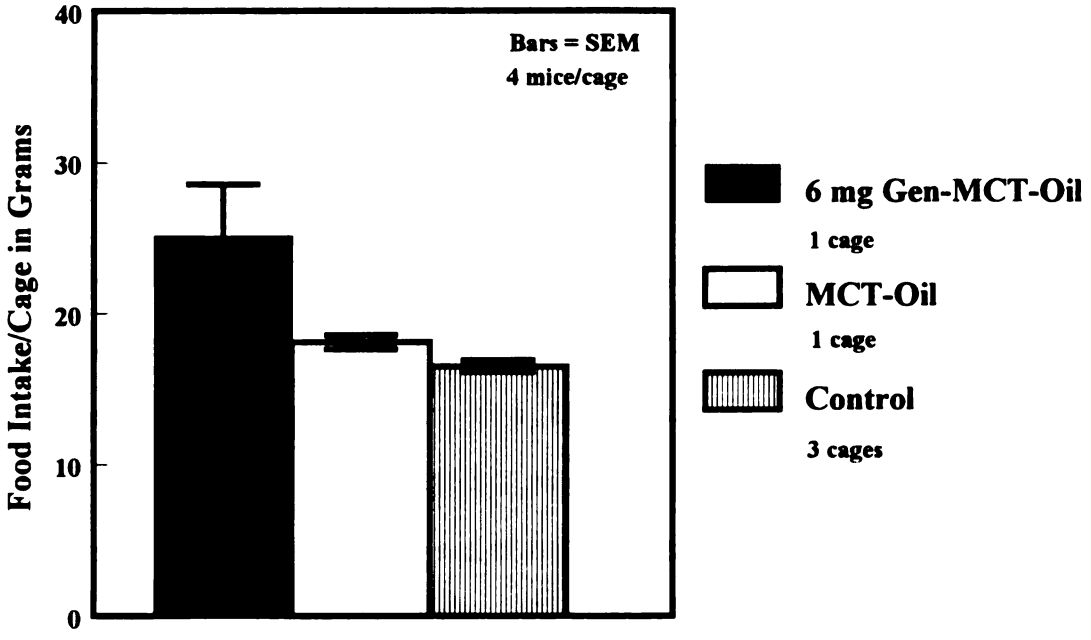

Figure G3. Food intake. Tumor bearing athymic mice (4 mice/group) were gavaged with 6 mg genistein in 100 µl MCT-oil or 100 µl MCT-oil alone for a period of 6 days. Food intake was determined for 4 days in a control group in addition to the two gavage groups. Data are expressed as mean food intake per cage over 4 days. Asterisk denotes significance at $p \leq .05$.

Conclusion:

These results suggest that this dose of genistein could have an effect upon tumor growth although the difference in this study was not significant. Even though the control mice consumed significantly ($p \leq .005$) less food tumor growth appears to be more rapid in this group. Gavaging 6 mg genistein in MCT-oil produced plasma genistein concentrations of ca. 10 μM, 4 hours after gavaging, a value much less than that seen in the DMSO gavage study (ca. 47 μM). It is possible that DMSO might facilitate the absorption of genistein resulting in higher plasma concentrations. Gavaging is not an appropriate means of administering chemicals over a long period of time for the following reasons: 1) damage to the mice, 2) potential fatality to mice due to the procedure, and 3) stress on the mice. In addition the resulting genistein/MCT-oil suspension was at times difficult to pass through the gavage tube. Higher concentrations of genistein in MCT-oil are unlikely to be successful.

Appendix H

Genistein Release from Elvax Pellets:

To assess the release of genistein from elvax pellets the following study was conducted.

Experimental Protocol:

Genistein containing elvax pellets were incubated in phosphate buffered saline and samples taken periodically. Samples were then dried, brought up in methanol, and analyzed by high pressure liquid chromatography (HPLC). 40 ul were injected onto a C_{18} column (Microsorb-MV, 5 µM 100A, Rainin Instrument in Woburn, MA) with a flow rate of 1.0 ml/min of 50:50 methanol:water with 0.1% acetic acid.

Results:

Genistein is released from the pellet at the following rate:

ELAPSED TIME	PERCENT OF REMAINING GEN RELEASED
4 hrs	26
24 hrs	38
48 hrs	33
168 hrs	22

Total release after 168 hrs is equivalent to 75% of the genistein content of the pellet. See Figure H.

Conclusion:

Genistein is released from the plastic elvax matrix into an aqueous environment.

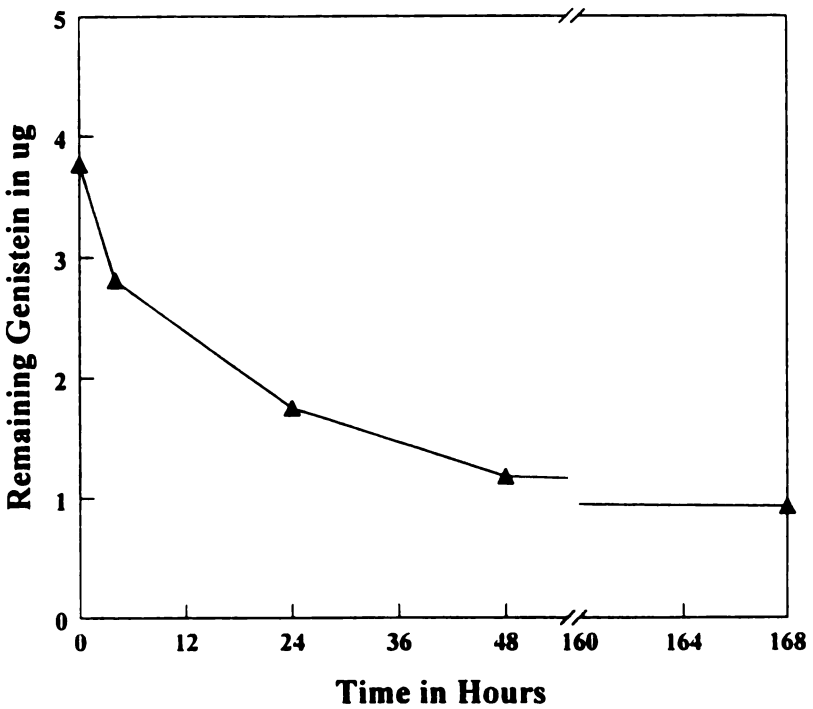

Figure 8. Genistein release from an Elvax pellet. A 1.9 mg Elvax pellet containing 3.77 µg genistein was incubated in phosphate buffered saline at 37°C and samples withdrawn at the indicated times and analyzed for genistein with HPLC.

Appendix I

Effect of Genistein Pellet Implants on Mammary Gland Development in the Mouse:

The following study was conducted to assess the effect of genistein (GEN) on the mammary gland of female intact and ovariectomized mice. Dr. Haslam's laboratory personnel collaborated in the experiment. Intact and ovariectomized (OVEX) immature female mice were implanted with elvax pellets containing either genistein (5 µg) or estradiol (E_2)(5 ng) into the number four mammary gland fat pad. The contralateral mammary gland was implanted with an elvax pellet and served as the control.

Experimental Protocol:

2-15 ovariectomized group DOB

3-2 intact group DOB

3-23 ovariectomies performed

4-3 pellets prepared

4-7 mice were divided into the following groups and the following treatments initiated

group 1 (OVEX) implanted with elvax pellets containing 5 µg GEN

group 2 (OVEX) implanted with elvax pellets containing 5 ng E_2

group 3 intact implanted with elvax pellets containing 5 µg GEN

group 4 intact implanted with elvax pellets containing 5 ng E_2

4-11 mice were killed and mammary tissue mounts prepared

Results:

There were 5 mice per group. The mammary gland mounts were examined for end bud development and number, and ductal growth. Each mouse was implanted with the plastic matrix alone into the other number four mammary gland fat pad and thus served as its own control.

group 1: (OVEX) Genistein had no effect compared to the control gland (Figure I1)

group 2: (OVEX) E2 stimulated development compared to the control gland (Figure I1)

group 3: (Intact) Genistein did not stimulate development compared to the control gland and appears to have slightly inhibited development (Figure I2)

group 4: (Intact) E2 did not stimulate development compared to the control gland (Figure I2)

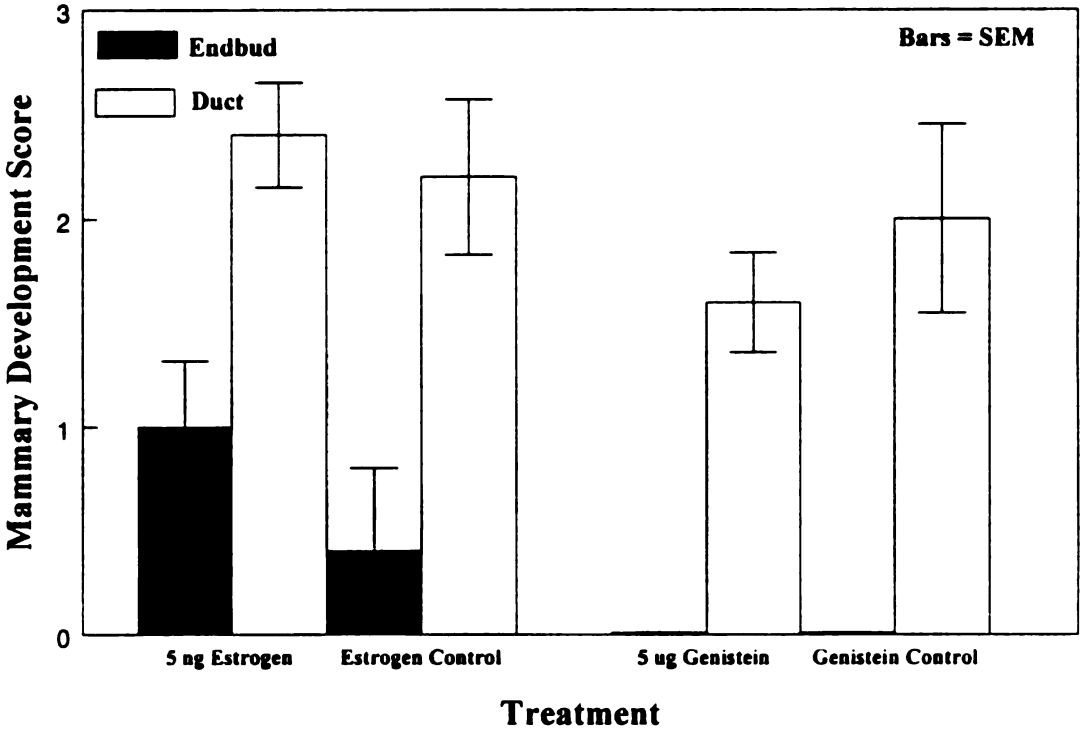

Figure I1. Effect of genistein pellet implant on the mammary gland in ovariectomized mice. Female Balb/C mice (n=5) were ovariectomized at 36 days of age. Plastic pellets containing either 5 ng estradiol or 5 µg of genistein were implanted into the mammary gland, at 51 days of age, for a period of 5 days. The contralateral mammary gland was implanted with plastic pellets to serve as a control. The mammary glands were removed, stained and analyzed for development by assessing endbud size and number and extent of ductal growth.

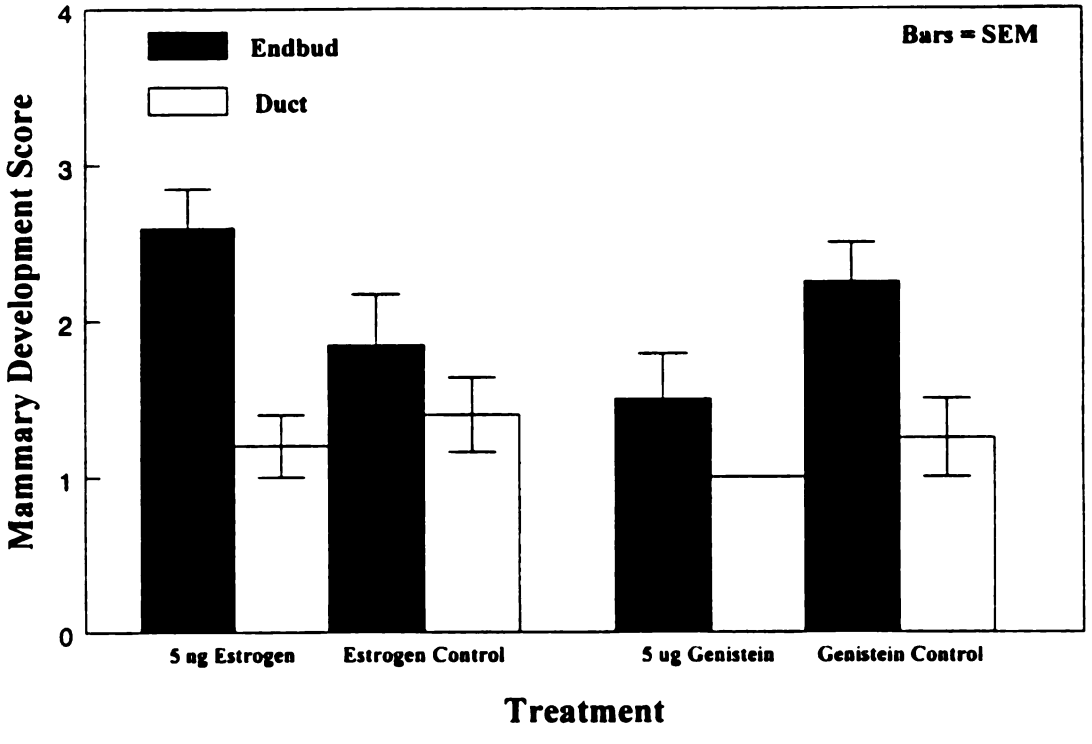

Figure I2. Effect of genistein pellet implant on the mammary gland in intact mice. Plastic pellets containing either 5 ng estradiol or 5 µg of genistein were implanted into the mammary gland of 36 day old female Balb/C mice (n=5) for a period of 5 days. The contralateral mammary gland was implanted with plastic pellets to serve as a control. The mammary glands were removed, stained and analyzed for development by assessing endbud size and number and extent of ductal growth.

Conclusion:

Genistein containing plastic pellets, implanted into the mammary gland of the mouse, does not appear to stimulate development of the mammary gland in ovariectomized or intact mice. Genistein appears to have slightly inhibited development in the intact mouse. These results suggest genistein is potentially capable of inhibiting the growth of mammary tissue *in vivo*.

Appendix J

Genistein/Cholesterol Pellet Implant into Tumor bearing Mice:

Dietary genistein (750 µg genistein/g diet) does not appear to affect the growth of existing tumors and administration of genistein by gavage is not practical; therefore, genistein pellets were made and implanted into the mice at the site of the tumor. Composition of the pellets was 10 mg genistein and 20 mg cholesterol or 30 mg cholesterol. The cholesterol matrix was chosen over elvax because the pellets would have been too large with the elvax.

Experimental Protocol:

3-19-96 received 4 mice at 28 days of age

3-26-96 injected tumor cells

5-16-96 implanted genistein pellets in two mice and cholesterol pellets in 2 mice between the body wall and the tumor at each tumor site

5-31-96 collected blood, measured and collected tumors

Results:

1. The percent increase in tumor area was 64% for the genistein pellet group and 60% for the control.
2. In addition pellet weights were assessed and are presented below. All pellets weighed 30 mg at the beginning of the study.
3. Genistein was not detected in the plasma of the mice implanted with pellets containing genistein.

Genistein pellets	**Cholesterol pellets**
1) 30.4	29.1
2) 27.2 (pellet chipped)	30.2 (pellet discolored)
3) 28.8	29.4
4) 30.5 (pellet discolored)	30.0 (pellet discolored)
5) 26.1 (pellet chipped)	30.5 (pellet discolored)
6) 31.5 (pellet discolored)	28.6
7 and 8 (pellet chipped, part missing)	25.5 (pellet chipped, discolored)
	26.3 (pellet discolored)

Conclusion:

The experimental groups consisted of only two mice. The study was conducted to assess the viability of this approach. There were two tumors on two mice that were necrotic so these mice were separated into the two treatment

groups. As a result beginning tumor area was not equal among the groups. The group receiving the genistein pellets had a lower mean tumor area, 65.89 mm^2 compared to 106.2 mm^2 in the control. Pellet weights changed very little suggesting little release of genistein from the pellet. However it is difficult to gain much information from pellet weights due to a couple of factors. Chipping of the pellet during implantation or removal results in loss of the pellet which could lower the weight. Also while in the mouse the pellet could possibly increase in weight through infiltration of substances from the body as well as encapsulation material on the outside of the pellet which is difficult to remove. The implantation process itself damages the host tumor environment which in itself could affect the growth of tumor. This is a major limitation in employing this approach to study changes in tumor growth with respect to a control.

Appendix K

Genistein Pellet Implanted into Mice:

The previous pellet implantation study did not show an effect on tumor growth. The lack of an effect could have been due to genistein not releasing from the cholesterol matrix therefore the following study was conducted. Pure genistein pellets were made and implanted subcutaneously on the backs of athymic mice. Pellets remained in the mice for a period of 17 days after which time they were removed and weighed.

Results:

INITIAL/FINAL MOUSE WEIGHT	INITIAL/FINAL PELLET WEIGHT
20.2 / 21.6 g	23.8 / 24.5 mg
18.6 / 20.4 g	24.1 / 22.2 mg
18.6 / 20.5 g	37.2 / 36.1 mg
20.6 / 22.5 g	37.7 / 36.1 mg (pieces)

Conclusion:

Pellet weight did not change appreciably over the 17 days they were implanted in the mice. Furthermore some of the pellets actually weighed more. The pellets remained intact

for the most part with the exception of some chipping during the implantation and removal procedure. It appears that genistein is not released from the pellets. This could be due to the compact nature of the pellets. In order to get genistein to remain in pellet form a large amount of force is required to compress the genistein.

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List of References

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