Inhibition of N-Methyl-N-nitrosourea-Induced Mammary Tumors in Rats by the Soybean Isoflavones

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Abstract. Soy-based diets, rich in the isoflavones genistein and daidzein, are thought to protect against breast and prostate cancer. We used the N-methyl-N-nitrosourea (MNU)-induced mammary carcinogenesis animal model to test the effectiveness of these two isoflavones as chemopreventive agents. Each isoflavone was injected daily into 35-day-old rats for six months while we monitored the animals' body weight and mammary tumor appearance. Genistein was effective in reducing tumor multiplicity, but it reduced tumor incidence only marginally. Daidzein was less effective in reducing both tumor incidence and multiplicity. To investigate genistein's mechanism of action, we determined the topoisomerase II (topo II) activity and detected the phosphotyrosine-containing peptides in the extracts of mammary tissues isolated from control and isoflavone-treated animals. Mammary tumors contained over 60-fold higher topo II enzymatic activity than the mammary glands. Similarly, more tyrosine phosphopeptides were detectable in mammary tumors than in mammary glands. Tissue samples from genistein treated animals contained similar topo II and protein tyrosine kinase (PTK) activities as the control group. These data suggest that mammary tumorigenesis is accompanied by an extensive increase in topo II and PTK activities. The mechanism of chemoprevention by genistein, however, is independent of topo II or PTK inhibition.

Consumption of soybeans in China and Japan has been linked to low incidence of breast, prostate, and colon cancer (1). It has been estimated that in Japan and some regions of China, 120 mg of isoflavones are consumed daily by each individual in the form of tofu, soy-milk, and other soy products (2). The average non-vegetarian diet in Western countries, where the incidence of breast, prostate, and colon cancer is high, provides only 1-5 mg of isoflavones daily. Genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-trihydroxyisoflavone) together represent over 90% of the soybean isoflavone content.

Appropriately designed animal studies can scrutinize epidemiological observations and can help to identify the dietary component(s) that provide chemopreventive action. Soy feeding in animal models of carcinogenesis was protective against experimentally induced mammary tumors and other organ cancers (2,3). An earlier study indicated that the isoflavones genistein and daidzein may be responsible for the chemopreventive action of soy products (4). More recently, it was reported that genistein, when injected into neonatal rats (5mg/rat), provided lifelong protection against dimethylbenz(a)anthracene (DMBA)-induced mammary carcinogenesis (5,6).

The cellular mechanisms of genistein's anti-proliferative and anticarcinogenic effects are still largely unexplored. Besides genistein's well documented role in the induction of differentiation of a variety of tumor cells (7, reviewed in 8) there are reports of its involvement in cell-cycle progression and induction of apoptosis (9,10,11,12). Genistein inhibits purified epidermal growth factor-receptor (EGFR) and pp60v-src protein tyrosine kinase (PTK) activities with a half maximal inhibitory concentration (Kᵢ) of about 25 μM (13,14). The PTK activity of several oncoproteins such as Lyn, Fyn, Blk, Gag-fes, Lck, and SYK are also inhibited by genistein (13-15). Higher isoflavone concentrations were necessary to inhibit PTK in intact human A431 cells (Kᵢ of about 150 μM) (13,14). Genistein has also been shown to inhibit eukaryotic DNA topoisomerase (topo) II (7, 16,17).

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involved in chromosomal segregation and other cellular functions requiring changes in DNA conformation. Due to its phenol ring structure, genistein has been shown to act as an antioxidant (18, 19). Angiogenesis in epithelial cells, which is essential for tumor growth, has been shown to be inhibited by genistein at 150 μM (20). Other activities reported to be inhibited by genistein include: S6 kinase, phosphatidylinositol turnover, alcohol dehydrogenase, RNA polymerase, reverse transcriptase, insulin-stimulated glucose transport, and 6-galactosidase (reviewed in 2).

In the present study, we demonstrate that genistein is somewhat effective in inhibiting MNU-induced mammary tumors in female rats. As part of our effort to identify genistein's molecular mechanisms of action, we tested its effects on top II and PTK activities in rat mammary glands and tumors.

Materials and Methods

Experimental animals. Virgin female Sprague-Dawley rats were received from Harlan/Sprague-Dawley (Madison, WI) at 25 days of age and maintained in isolation for one week. Animals were housed in groups of 2-3 in polycarbonate cages containing hardwood bedding. The animal rooms were illuminated for 12 h each day and maintained at a temperature of 22°C and 50% relative humidity. Animals were allowed free access to food and water throughout the study.

Diets, chemopreventive agents, and protocol for tumor induction. The basal diet was modified AIN-76A semipurified diet (Teklad, Madison, WI). At 35 days of age, 90 rats were randomized by weight into six groups and began receiving basal diet as shown in Table I. One week later, one group of 20 rats and another group of 10 rats were injected (i.p.) with 0.2 ml of a solution of 4 mg/ml of genistein (Indofine Chem. Co., Somerville, NJ) in 40% ethanol/saline. Another group of 20 rats and a group of 10 rats were injected (i.p.) with 0.2 ml of a solution of 4 mg/ml of daidzein (Indofine Chem. Co.) in 40% ethanol/saline. A third group of 20 rats and a group of 10 rats were injected with 0.2 ml of 40% ethanol/saline. Injections continued daily for 180 days. One week after the beginning of isoflavone treatment (day 50), the three groups of 20 rats each were injected (i.p.) with MNU (50 mg/kg body weight). Cystathione MNU (Ash-Stevens, Detroit MI) was dissolved to a concentration of 12.5 mg/ml in 0.85% NaCl solution acidified to pH 5.0 with glacial acetic acid.

Control animals (groups of 10) received an i.p. injection of the acidified NaCl solution only (vehicle).

Commencing four weeks after receiving MNU, animals were palpated weekly to monitor mammary tumor appearance. The date of appearance and location of every palpable tumor were recorded. Animals were weighed once a week, and their body weights were recorded for the duration of the study. At the end of the study, surviving rats were sacrificed by CO2 asphyxiation. All rats that were killed or found dead were given thorough postmortem examinations. Mammary tumors were coded by location, removed, and weighed when applicable. Mammary tumors, mammary glands or liver were snap-frozen in liquid nitrogen and stored in the freezer at -70°C for use in biochemical analysis.

Statistical analysis. The statistical significance of differences between mean tumor multiplicities was assessed using one-way ANOVA. Armitage's test (for trend in proportions) was used to compare tumor multiplicity between the MNU and the MNU + isoflavone groups during the course of the study. Tumor incidence curves were generated by the life table method and compared by log rank analysis (21).

Western blotting of phosphotyrosine-containing proteins. Tissue homogenates containing 100 μg protein, determined by the Bradford method (22), were analyzed in 10% SDS polyacrylamide gels and immunoblotted as previously described (23). We used the phosphotyrosine-specific PY-20 antibody (Transduction Laboratories, Lexington, KY) as a primary antibody.

Topo II activity. For the determination of topo II catalytic activity, knotted DNA that had been isolated from the tailless capsids of the bacteriophage P4 Vircell was used as the substrate, as described previously (23). Tissue homogenate was prepared, protein concentration was determined, and serial dilutions containing 25 ng to 25 μg of protein were tested for their topo II activity in reactions of 20 μl total volume. Reaction mixtures contained 50 mM Tris-Cl, pH 8.0, 100 mM KCl, 10 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM EDTA, 40 μg/ml bovine serum albumin (nuclease free), and 1 mM ATP. Reactions were started by the addition of 0.6 μg of knotted DNA and terminated by the addition of 5 μl of a stop solution containing 5% SDS, 50 mM EDTA, 25% Ficoll, and 0.05 mg/ml bromophenol blue. Samples were loaded on 0.8% agarose gels and electrophoresed at 4V/cm for 5 h in Tris-borate-EDTA buffer. Gels were stained in 1 μg/ml ethidium bromide, destained, and photographed over a UV light source. To quantify topo II activity, photographic negatives were densitometrically scanned. Knotted DNA, migrating as a single band at the top of the gel, was measured in this manner. By averaging three such experiments, the topo II catalytic activity of each homogenate was determined.

Table I. Effect of isoflavones on MNU-induced mammary carcinogenesis in female Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>MNU</th>
<th>Isoflavone</th>
<th>Cancer incidence (%)</th>
<th>Tumors/Rat (± SD)</th>
<th>Terminal survival (%)</th>
<th>Terminal body weight (g)</th>
<th>Tumor weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>+</td>
<td>-</td>
<td>100</td>
<td>6.7 (4.1)</td>
<td>60</td>
<td>262</td>
<td>1.54</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>Genistein</td>
<td>89.0</td>
<td>4.9 (3.3)</td>
<td>78</td>
<td>265</td>
<td>1.57</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>Daidzein</td>
<td>94.4</td>
<td>4.9 (3.4)</td>
<td>22</td>
<td>266</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>vehicle</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>278</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
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<td>Genistein</td>
<td>0</td>
<td>0</td>
<td>70</td>
<td>276</td>
<td>-</td>
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<td>10</td>
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<td>Daidzein</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>274</td>
<td>-</td>
</tr>
</tbody>
</table>

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Results

MNU-induced rat mammary carcinogenesis. The chemical structures of genistein and daidzein, the two most common isoflavones of soybeans, are shown in Figure 1. Daidzein differs from genistein in that it has no hydroxyl group at the C-5 position (highlighted in genistein). Due to this deficiency, daidzein is not able to inhibit top II or PTK in vitro (13,14,24). It should be noted, however, that both daidzein and genistein show anti-estrogenic activity (25). These differences could serve to reveal the molecular mechanism of chemoprevention of the effective isoflavone.

The effects of genistein and daidzein on MNU-induced rat mammary carcinogenesis, as measured by tumor multiplicity, are shown in Figure 2. Fewer tumors appeared in the genistein (+MNU)-treated group than in the vehicle (+MNU)-treated group. The daidzein treated group also showed lower tumor multiplicity than the vehicle group. Genistein’s effect was evident throughout the study, but that of daidzein became evident only in the second half of the study. Using Armitage’s test for trend in proportions for the statistical analysis of the data points shown in Figure 2, we found that the effectiveness of genistein in reducing tumor multiplicity was nearly significant (P=0.07). The effect of daidzein, however, due to its delayed action, was not significant (P=0.26). The tumor incidence in the genistein-treated group was also lower than the control group throughout the study, but log rank analysis (life table method) of cancer incidence curves showed no statistical significance (P=0.09). Daidzein was ineffective in lowering tumor incidence (P=0.22).

The data summarized in Table I indicate that, at the end of the study, there was an average of 4.9 tumors/rat in the genistein as well as the daidzein treatment groups. This represents a 27% reduction when compared to the average of 6.7 tumors per rat observed in the positive control group (MNU). Animals treated with either genistein or daidzein in the absence of MNU were free of tumors at the end of the study. It can be concluded that these agents are not carcinogenic when injected at 0.8 mg/day for 180 days. Neither genistein nor daidzein caused any adverse effects on the overall health of the animals, as determined by visual examination during necropsy. The intestines, however, were fused in both control and treatment groups, but this effect has been attributed to ethanol, which was used as a vehicle in the
study. No significant differences in weight-gain were evident between the different treatment groups, indicating that the effect of the isoflavones in reducing tumor multiplicity is not due to reduced body weight gain. With the exception of the daidzein (+MNU) treatment group, no significant difference in animal survival was evident between the treatment groups and the control groups. Genistein was also ineffective in reducing tumor size (Table 1) and latency time (data not shown).

PTK-mediated phosphorylation. We assessed the effect of genistein, an in vitro inhibitor of PTK, on tyrosine phosphoproteins in representative tissues of animals treated with genistein. Western blots of tissue lysate from liver, mammary gland, or mammary tumor were analyzed using the PY-20 antibody, which detects phosphorysine-containing peptides. Over 10 tyrosine-containing phosphopeptides were detected in the liver extracts of rats (Figure 3). Treatment of these animals with genistein, however, had no impact on the patterns of tyrosine phosphopeptides. Daidzein was also ineffective. The lack of a reproducible effect on PTK-mediated phosphorylation was evident when we used higher doses of isoflavones (4 mg for 4 days) than those used in the chemoprevention study (0.8 mg for 180 days). Lysate from mammary tumors contained a higher number of tyrosine phosphopeptides than mammary glands, but genistein (or daidzein) treatment had no consistent effect on the patterns of phosphorylation of the glands or tumors (data not shown).

Topo II activity. At the end of the in vivo study, mammary tumors or mammary glands were removed from the animals, and the topo II activity of these tissues was determined using the unknotted assay (as described in "Materials and Methods"). Mammary glands contained on the average 0.65 × 10^7 units/mg, a relatively low level of topo II activity. Mammary tumors, however, contained 43.6 × 10^7 units/mg of topo II, representing over a 60-fold increase over the glands' levels. Genistein treatment for 180 days was not effective in inhibiting the topo II activity in normal mammary glands or mammary tumors (Figure 4). Similar results were obtained in animals treated with 4 mg genistein for 4 days (data not shown).

Discussion

The data presented in this study show that genistein effectively inhibits chemically-induced mammary carcino-
genesis in female rats, as determined by reduced tumor multiplicity. Using statistical analysis which takes into consideration the rate of tumor appearance (Armitage's test for trend in proportions), the difference between the genistein and control groups was found to be significant with a 93% confidence limit. The difference between the control and the daidzein-reagent group was non-significant (P=0.26), although daidzein reduced the final tumor multiplicity to the same extent as genistein. Recently, Lamartiniere et al. found genistein to be very effective in suppressing not only rat mammary tumor multiplicity but also tumor incidence, and effective in increasing tumor latency (5,6). Differences in the design of the two studies may account for the different outcomes. In the Lamartiniere study neonatal rats received three injections at an age of less than one week old; moreover, the genistein dose in that experiment was about six-fold higher than in the present study. The early age of the animals at which genistein was introduced may have provided a window of opportunity during which interference with the differentiation program may have given a long-lasting effect. Although the above animal study furnished a strong expectation that genistein can be useful in cancer chemoprevention, the study is of limited value because neonatal rats were used. In terms of clinical application, the study design is limited to only infants that can be benefited from genistein. Injecting infants, however, with genistein to reduce the future risk of breast cancer may not be practical. Our study was designed to apply to the dietary modification of the adult individual. Unfortunately, the soybean isolavones were less effective in preventing mammary tumors when injected into 30-day-old rats. We also had to compromise, due to the high cost of genistein, and introduced it through injections (a more economical approach) instead of providing it as a dietary supplement.

Another objective was to identify genistein's in vivo molecular target whose inhibition mediates the anti-proliferative and antitumor activity of the agent. Part of genistein's antitumor activity may rely on its ability to block the metabolic activation of carcinogens. This quality may explain why it was more effective in the DMBA carcinogenesis model, which requires activation, than the MNU model, which is a direct-acting carcinogen. Although a multitude of enzymes and activities are inhibited by genistein, in cell-free experiments, there is little documentation for the isolavone's effectiveness in cultured cells or animal systems. For example, genistein was reported to inhibit PTK with a Kᵢ value of about 25 μM in cell-free assays, but a Kᵢ of about 150 μM was necessary for intact human A431 PTK inhibition (13,14). Similar discrepancies between the in vitro and in vivo inhibitory effects on topo II were reported (7). Data presented here show clearly that topo II activity increases dramatically during mammary tumorgenesis but genistein does not inhibit topo II activity in the tissues of the animals treated chronically with the isolavone. The induction of DNA breaks (another measure of topo II inhibition by genistein) has not been determined in these studies. Genistein was equally ineffective in inhibiting substrate phosphorylation on tyrosine residues—a property of increased PTK activity. It can be concluded then that genistein's mechanism of chemoprevention is independent of both topo II and PTK inhibition.

Genistein is generated when the flavonoid glycoside genistin undergoes hydrolysis by intestinal flora. The resulting aglycone, genistein, and related isoflavonoids are efficiently absorbed from the gastrointestinal tract and reach measurable levels in the plasma and urine (26,27). Predictably, plasma and urine levels are much higher in consumers of soy-rich diets (28). Even in these individuals, measured genistein or daidzein plasma levels remain below 600 nM. This plasma level is at least 40 times lower than the concentration required to inhibit PTKs or topo II in vitro. The induction of apoptosis or inhibition of angiogenesis also requires 200 to 300 times greater concentrations of genistein. Furthermore, genistein is known to undergo conjugation in the liver with glucuronic acid and sulfate shortly after entering circulation. The conjugates may not be effective inhibitors of PTKs or topo II.

Genistein was identified initially as a phytoestrogen (25) shown to compete with estradiol in MCF-7 cells for the estrogen receptor, and to elucidate other typical estrogen-induced responses such as a decrease in the expression of estrogen receptors and increase in the expression of progesterone receptors in epithelial cells (29). Genistein's binding affinity for the estrogen receptor is approximately 200 times lower than that of estradiol. Daidzein, which does not inhibit PTK or topo II, binds to ER, but its binding affinity is 1,000 times less than that of estradiol (30,31). It is not clear at present which of the multitude of possible enzymes are inhibited physiologically by the soybean isolavones. The possibility that these isolavones exert their chemopreventive action as antiestrogens cannot be excluded at present. The moderate effectiveness of daidzein in inhibiting late-appearing tumors combined with the inhibition of early-appearing tumors by genistein (Figure 2) suggests that the two isolavones exert their antitumor action through different mechanisms. This observation also suggests that the combination of the two, in a chemoprevention study, may provide an additive or synergistic effect. More studies are necessary for a conclusive identification of the physiological targets of soybean isolavones. Future chemoprevention studies should focus initially on identifying the maximum tolerated doses of genistein and daidzein (alone and in combination) when introduced in the animal diet as supplements.

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References


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