Dose Effects of Flaxseed and Its Lignan on N-Methyl-N-Nitrosourea-Induced Mammary Tumorigenesis in Rats

Sharon E. Rickard, Yvonne V. Yuan, Jianmin Chen, and Lilian U. Thompson

Abstract: Dietary supplementation with flaxseed or its lignan secoisolariciresinol diglycoside (SDG) has reduced di-methylbenz[a]anthracene-induced mammary tumor size and number in rats. The objective of this study was to determine whether flaxseed has a dose-dependent effect on N-methyl-N-nitrosourea (MNU)-induced mammary tumor promotion and whether this effect can be attributed to its SDG. Two days after injection with MNU (50 mg/kg body wt ip), female Sprague-Dawley rats were fed a high-fat (20% soybean oil) AIN-93G basal diet alone (BD) or supplemented with flaxseed (2.5%F and 5%F) or SDG by gavage (SDG in 2.5%F (LSDG) and SDG in 5%F (HSDG)) for 22 weeks. Although tumors tended to be smallest in the 5%F group throughout the experimental period, flaxseed feeding did not significantly affect tumor size, multiplicity, or incidence in comparison to BD. However, there was a dose-dependent effect of SDG on tumor multiplicity. Tumor multiplicity was lowest in the HSDG group and highest in the LSDG group throughout treatment (p < 0.05), indicating that HSDG inhibited, whereas LSDG promoted, MNU-induced mammary tumor development. Tumor invasiveness and grade were decreased in all treatment groups compared with the BD (p < 0.032). Thus, although flaxseed feeding had no significant effect on tumor growth indexes, flaxseed and SDG treatment, regardless of dose, appeared to delay the progression of MNU-induced mammary tumorigenesis. Disparities between this study and previous studies on flaxseed may be related to differences in experimental design, the use and dose of a different carcinogen, and protective effects by the α-linolenic acid present in the BD.

Introduction

Mammalian lignans, formed by colonic bacterial action on dietary precursors (1–3), have exhibited anticarcinogenic activity in vitro. Enterolactone (EL), more biologically active than its mammalian lignan counterpart enterodiol (ED), has been shown to reduce the growth of human breast cancer cell lines (4–6) and to inhibit in vitro angiogenesis, the generation of new capillaries necessary for aggressive tumor growth (7).

Some of the anticancer effects of lignans have been partly attributed to their weak antiestrogenic properties. EL and ED have inhibited the binding of steroidal estrogens to the type II rat uterine estrogen receptor (8) or rat α-fetoprotein, an oncofetal plasma protein with high affinity for estrogen (9). In addition to inhibiting the enzyme aromatase, which converts androgens to estrogens (10,11), EL has been shown to stimulate sex hormone-binding globulin synthesis in Hep G2 human liver cancer cells (8), potentially reducing the levels of biologically active estrogen available.

Consumption of foods rich in mammalian lignin precursors has been associated with reduced cancer risk. A small epidemiological study found that vegetarians women have much higher urinary levels of mammalian lignans than women who eat omnivorous diets or have breast cancer (12,13). A recent larger study found that the odds ratio for breast cancer risk decreased significantly with increasing urinary levels of EL (14). Nonhuman primates, shown to be resistant to induction of mammary carcinogenesis by treatment with known carcinogens and/or high levels of estrogens (15–17), excrete high urinary levels of lignans and other phytoestrogens when consuming their regular diet (18,19).

Flaxseed has been identified in vitro (20) and in vivo (21) as a particularly rich source of mammalian lignan precursors, the major precursor being secoisolariciresinol diglycoside (SDG) (21). Flaxseed and SDG feeding have resulted in reductions in mammary tumor size and number at early promotion and late promotion, early progression stages of carcinogenesis (22–24), suggesting that SDG is in part responsible for flaxseed’s anticancer effects. However, the oil of flaxseed is also rich in the n–3 fatty acid α-linolenic acid (α-LA) and has also been found to reduce mammary tumor development in rats (23,25).

Previous studies with flaxseed and SDG have used the chemical carcinogen dimethylbenz[a]anthracene (DMBA). The effect of flaxseed on tumors induced by N-methyl-N-nitrosourea (MNU), a model that appears to be more related to human breast cancer histologically, in endocrine responsiveness, and in metastatic behavior (26), has not been ex-
amined. In addition, to our knowledge, the effect of SDG dose on carcinogenesis has not been tested in any animal model. Thus the objective of this study was to determine the dose-dependent effect of flaxseed and SDG, at levels equivalent to that present in flaxseed, on MNU-induced mammary tumorigenesis.

Materials and Methods

Animals and Diets

Female Sprague-Dawley rats (n = 155, 42–45 days of age; Charles River, Montreal, PQ, Canada) were doubly housed in plastic cages, with sawdust bedding, in a room with a 12:12-hour light-dark cycle at 22–24°C and 50% humidity. Animal care and use conformed to the Guide to the Care and Use of Experimental Animals (27), and the experimental protocol was approved by the University of Toronto Animal Care Committee. Animals were given free access to a semisynthetic high-fat basal diet alone (BD) or supplemented with 2.5% or 5% (wt/wt) ground, full-fat flaxseed (Linott variety, Omega Products, Melfort, SK, Canada). The BD, prepared by Dyets (Bethlehem, PA), was based on the American Institute of Nutrition AIN-93G formulation (28), except a higher fat content (20% soybean oil) was used at the expense of cornstarch. Proximate analysis of flaxseed has shown that it contains 36.5% fat, 22% protein, and 24% fiber. For the diets containing flaxseed, Dyets prepared the flaxseed-free base so that the freshly ground flaxseed could be added before feeding, with corrections having been made for flaxseed’s contribution to these dietary components. Fresh diet was provided every two days. Prepared diets were stored at 4°C. Food intake and body weights were monitored weekly and biweekly, respectively.

Experimental Treatment Groups, Tumor Induction, and Tumor Measurement

During a three-day acclimatization period, the rats were given free access to water and the BD. At 50 days of age, all rats were injected with MNU (50 mg/kg body wt ip) dissolved in 0.9% NaCl solution containing 0.05% acetic acid. The mean body weight at the time of the MNU injections was 182.4 ± 0.7 g. To ensure that flaxseed and SDG were introduced during the tumor promotion phase only, dietary treatments were initiated two days after MNU injection. Rats were randomly allocated to one of five treatment groups: 1) BD (n = 30), 2) BD supplemented with 2.5% flaxseed (2.5%F, n = 31), 3) BD supplemented with 5% flaxseed (5%F, n = 31), 4) BD plus a daily gavage of SDG equivalent to that consumed by the 2.5%F group (LSDG, n = 31), and 5) BD plus a daily gavage of SDG equivalent to that consumed by the 5%F group (HSDG, n = 32). The SDG (686 g/mol) dose, dissolved in 1 ml of distilled water, was calculated using the value of 2.93 μmol SDG/g flaxseed determined using the high-performance liquid chromatography method described by Obermeyer and co-workers (29). Rats not gavaged with SDG were gavaged with 1 ml of distilled water.

Starting 6 weeks after MNU injection, the rats were palpated weekly, and the mammary tumor number, size, and location were recorded until 22 weeks post-MNU. Tumor size was measured in two perpendicular dimensions, width (smaller diameter, cm) and length (cm), with digital calipers (Canadawide Scientific, Ottawa, ON, Canada). Weekly tumor volume (cm³) was calculated using the following formula: width² × length/2 (30). At sacrifice, tumors and major organs (heart, liver, kidney, spleen, ovaries, uterus, cecum, and colon) were excised and weighed. The volume of the excised tumors was calculated using the following formula: width × height × length/2. Sections of the tumors were fixed in 10% neutral buffered formalin for histological analyses. The remaining tumor samples were quick frozen in liquid nitrogen and stored at −70°C for future analyses.

Statistical Analysis

Weekly and final tumor multiplicity (number of tumors/tumor-bearing rat) were analyzed by Armitage’s trend in proportions (31), as suggested by Constantinou and colleagues (32). Tumor invasiveness and grade were also analyzed by Armitage’s trend in proportions. Tumor incidence was analyzed by a modified log-rank test that adjusts for crossing survival curves described by Le (33). Weekly and final tumor volume, final tumor weight, mean food and SDG consumption, body weight gain, and organ weights were analyzed by one-way analysis of variance using SigmaStat version 2.0 (Jandel Scientific, San Rafael, CA). Post hoc analyses used were Tukey’s test (parametric) and Dunn’s method (nonparametric). For all analyses, the acceptable level of significance was p ≤ 0.05. Values are means ± SE.

Results

Weight Gain, SDG Consumption, and Organ Weights

No significant differences in weight gain were observed between treatment groups (data not shown). On the basis of the mean food intake over the experimental period, the daily SDG consumption was significantly higher in the 5%F (1.409 ± 0.019 mg) and HSDG (1.430 ± 0.004 mg) groups than in the 2.5%F (0.705 ± 0.011 mg) and LSDG (0.704 ± 0.001) groups (p < 0.05) and not significantly different in the 5%F vs. HSDG and 2.5%F vs. LSDG groups. No differences were observed in final organ weights adjusted for body weight (data not shown).

Weekly and Final MNU Tumor Data

Most of the MNU-induced tumors were malignant; benign neoplasms represented only 3% of the total number of classified tumors (Table 1). The number of malignant tumors
Table 1. Classification of MNU-Induced Tumors in Rats Fed the BD Alone or Supplemented With Flaxseed or Its Lignan Precursor SDG

<table>
<thead>
<tr>
<th>Dietary Group&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total No. of Tumors</th>
<th>No. of Benign Tumors&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Level of Invasion&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Grade&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>30</td>
<td>142</td>
<td>1</td>
<td>9.0</td>
</tr>
<tr>
<td>2.5%F</td>
<td>31</td>
<td>144</td>
<td>8</td>
<td>19.7</td>
</tr>
<tr>
<td>5%F</td>
<td>31</td>
<td>173</td>
<td>1</td>
<td>15.1</td>
</tr>
<tr>
<td>LSDG</td>
<td>31</td>
<td>213</td>
<td>10</td>
<td>14.4</td>
</tr>
<tr>
<td>HSDG</td>
<td>32</td>
<td>127</td>
<td>4</td>
<td>14.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Abbreviations are as follows: MNU, N-methyl-N-nitrosourea; BD, basal diet; F, flaxseed; SDG, secoisolariciresinol.
<sup>b</sup> LSDG and HSDG = BD + dose of SDG consumed in the 2.5%F and 5%F groups, respectively.
<sup>c</sup> Fibroadenomas, papillary cyst-adenomas, and adenomas.
<sup>d</sup> None, no invasion; I, microinvasive; II, invasive to neighboring tissues, e.g., lymph nodes, skeletal muscle, and parotid gland. Significantly higher tumor invasiveness for BD vs. 2.5%F (p < 0.01), vs. 5%F (p < 0.03), vs. LSDG (p = 0.019), and vs. HSDG (p = 0.006) by Armitage’s trend in proportions (31).
<sup>e</sup> I, histologically well differentiated; II, moderately differentiated; III, poorly differentiated. Significantly higher grade for BD vs. 2.5%F (p = 0.0006), vs. 5%F (p = 0.025), vs. LSDG (p = 0.017), and vs. HSDG (p = 0.017) by Armitage’s trend in proportions (31).

followed the pattern of LSDG > 5%F > BD > 2.5%F > HSDG. The proportion of tumors with a higher level of invasiveness was greater in the BD group than in all treatment groups (p ≤ 0.025) (Table 1). In addition, more tumors were of higher grade (i.e., lower level of differentiation) in the BD group (p < 0.032) than in all treatment groups (Table 1).

Although dietary treatment had no effect on the weekly tumor volume, the volume in the 5%F group was generally lowest throughout the experimental period (Figure 1). The weekly tumor multiplicity was highest for LSDG group and lowest for HSDG group throughout the experimental period (p < 0.05) (Figure 2).

Final tumor incidence (percentage of rats with ≥ 1 tumor) and total tumor load (total weight of tumors) were not affected by dietary treatment, but the final tumor multiplicity was significantly lower in the HSDG group than in all other groups (p < 0.05) except 2.5%F (Table 2). In addition to being higher than in the HSDG group, final tumor multiplicity was significantly higher in the LSDG than in the 2.5%F group (p < 0.05) (Table 2). As seen for the weekly tumor volume (Figure 1), the 5%F group had the smallest final tumor weight, but this was not significantly different from BD (Table 2).

Discussion

In contrast to the growth-inhibitory effect observed for flaxseed on DMBA-induced tumors (22–24), feeding flax-

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Figure 1. Weekly mammary tumor volume in rats fed basal diet (BD) alone or supplemented with flaxseed (F) or its lignan precursor secoisolariciresinol diglycoside (SDG). Values are means ± SE; n = 30–32 rats. * Significant difference by Armitage’s trend in proportions (31) in that week (p < 0.05): BD vs. HSDG (Weeks 12–15 and 22); LSDG vs. BD (Weeks 10, 15–17, and 19–21); LSDG vs. HSDG (Weeks 10–22), LSDG vs. 2.5%F (Weeks 9, 10, 13, 14, 16, and 19–22); LSDG vs. 5%F (Weeks 9, 10, 13, and 14), and 5%F vs. HSDG (Weeks 16, 17, and 22).

Figure 2. Weekly mammary tumor multiplicity (no. of tumors/tumor-bearing rat) in rats fed BD alone or supplemented with flaxseed or SDG. Values are means ± SE; n = 30–32 rats. * Significant difference by Armitage’s trend in proportions (31) in that week (p < 0.05): BD vs. HSDG (Weeks 12–15 and 22); LSDG vs. BD (Weeks 10, 15–17, and 19–21); LSDG vs. HSDG (Weeks 10–22); LSDG vs. 2.5%F (Weeks 9, 10, 13, 14, 16, and 19–22); LSDG vs. 5%F (Weeks 9, 10, 13, and 14), and 5%F vs. HSDG (Weeks 16, 17, and 22).
Table 2. Final Tumor Incidence, Multiplicity, Weight, and Load in MNU-Treated Rats Fed BD Alone or Supplemented With Flaxseed or Its Lignan Precursor (SDG).\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>Tumor Incidence,\textsuperscript{c} %</th>
<th>Tumor Multiplicity\textsuperscript{d}</th>
<th>Tumor Weight, g</th>
<th>Total Tumor Load,\textsuperscript{1} g</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>93.3</td>
<td>5.1 ± 0.6\textsuperscript{1}</td>
<td>1.34 ± 0.23</td>
<td>6.19 ± 1.18</td>
</tr>
<tr>
<td>2.5%F</td>
<td>90.3</td>
<td>4.9 ± 0.8\textsuperscript{1,2}</td>
<td>1.30 ± 0.23</td>
<td>6.07 ± 1.34</td>
</tr>
<tr>
<td>5%F</td>
<td>87.1</td>
<td>6.3 ± 0.9\textsuperscript{1,3}</td>
<td>0.96 ± 0.14</td>
<td>5.40 ± 1.06</td>
</tr>
<tr>
<td>LSDG</td>
<td>93.5</td>
<td>7.0 ± 0.8\textsuperscript{1}</td>
<td>1.14 ± 0.16</td>
<td>7.88 ± 1.10</td>
</tr>
<tr>
<td>HSDG</td>
<td>100</td>
<td>3.8 ± 0.4\textsuperscript{1}</td>
<td>1.34 ± 0.26</td>
<td>5.31 ± 0.99</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values are means ± SE; \textit{n} = 30–32 rats/group. See Table 1 footnote for definition of abbreviations and explanation of dietary groups.
\textsuperscript{b} Values within column with different symbols (\textsuperscript{*}, \textsuperscript{†}, \textsuperscript{‡}) are significantly different by Armitage's trend in proportions (31) (\textit{p} < 0.05).
\textsuperscript{c} Tumor incidence = percentage of rats in each treatment group with \textgeq 1 tumor.
\textsuperscript{d} Tumor multiplicity = number of tumors/tumor-bearing rat.
\textsuperscript{1} Total tumor load = total tumor weight/\textit{rat}.

seed at the 2.5% and 5% levels had no effect on MNU-induced mammary tumor size, multiplicity, or incidence in comparison to control (BD) rats. Although the 5%F group tended to have the smallest tumors throughout the experimental period, the lack of a significant effect for flaxseed in comparison to our previous studies may be related to differences in experimental design, type and dose of carcinogen, and type of BD. Although SDG also had no effect on mammary tumor size and incidence, there was a dose-dependent effect of SDG on tumor multiplicity in contrast to the flaxseed groups. Feeding SDG at a lower dose (0.7 mg/day, LSDG) increased tumor multiplicity, whereas SDG at a higher dose (1.4 mg/day, HSDG) lowered tumor multiplicity throughout the experimental period. Nevertheless, flaxseed and SDG, regardless of dose, decreased the level of invasion and grade of tumors in comparison to the BD group, suggesting that the BD tumors were at a more advanced stage of carcinogenesis.

Although there were no significant differences in tumor size between the LSDG and HSDG groups, weekly and final tumor multiplicity were higher in the LSDG than in the HSDG group, suggesting a dose-dependent effect of SDG on mammary tumor development. In previous studies, 1.5 mg SDG/day resulted in significant reductions in mammary tumor size and number or multiplicity compared with BD (23,24). The dose of SDG used in the LSDG group had not been tested before.

Dose-dependent effects of EL on DNA synthesis, which is an approximation of cell proliferation and hence tumor growth, have been observed \textit{in vitro} with estrogen-dependent MCF7 human breast cancer cells by Wang and Kurzer (6). They found that \textless 10 \(\mu\)M EL stimulated DNA synthesis, whereas higher doses (half-maximal inhibitory concentration of 82 \(\mu\)M) were inhibitory. In contrast, no stimulatory effects were observed with EL in the estrogen-independent breast cancer cell line MDA-MB-231, but high doses (>100 \(\mu\)M) were found to be inhibitory without cytotoxicity (6). Wang and Kurzer suggested that the inhibition of both types of breast cancer cells at high doses was due to mechanisms independent of the estrogen receptor (6).

In addition to the problem in extrapolating results \textit{in vitro} to what may happen \textit{in vivo}, it is difficult to compare the results of our study with the study by Wang and Kurzer (6) for at least three reasons. First, most of the concentrations used \textit{in vitro} (10–100 \(\mu\)M) were much higher than those fed in this study (1–2 \(\mu\)M SDG). Second, at least in humans, about 80% of the mammalian lignans in plasma are present as glucuronide and sulfoglucuronide conjugates (34), whereas the more bioactive free form of EL was \textit{in vitro}. Third, there may be differences in the action of mammalian lignans on human vs. animal tumor cells. Comparing the effects of various levels of SDG and its mammalian lignans on growth, estrogen-receptor binding, and mRNA expression of estrogen-responsive proteins in estrogen-dependent human and rat mammary tumor cells \textit{in vitro} would provide more information on mechanisms related to estrogen. For comparative \textit{in vivo} human data, preliminary studies examining the role of SDG on human breast cancer risk should first be done using higher-risk women, such as those with benign breast disease (35) or high mammographic density (36). The minimum test dose of SDG should be about 50 mg/day. This is based on the SDG content of flaxseed being 2,000 \(\mu\)g/g and the 5%F diet in rodents being equivalent to a daily human dose of 25 g of flaxseed with use of an approximate dry food weight of 500 g.

The lack of a dose-dependent effect of flaxseed on mammary tumor multiplicity may be due to interactions between its SDG and other components present in flaxseed. In a previous study, we found that flaxseed oil supplementation at the level found in the 5%F diet (1.82%) reduced the size of established but not newly developed tumors and had no effect on tumor number (23). In contrast, a daily gavage of 1.5 mg of SDG (similar to that in 5%F) resulted in the lowest number and size of new tumors in the same study (23). These two anticancer components may interact in a synergistic or antagonistic manner depending on the dose of flaxseed used. Flaxseed also contains approximately 8% soluble fiber by weight (37), but it is not known whether the higher level of soluble fiber in the flaxseed diets played a role.

Differences in the experimental design of studies examining the role of flaxseed and its SDG in mammary tumorigenesis may partly explain some of the discrepancies observed. Serraino and Thompson (22) observed that 5%F
fed during the promotional phase of carcinogenesis significantly reduced mammary tumor size in rats by 67%. We found a similar trend, although not significant, toward smaller tumors with 5%F feeding in the present study. Interestingly, Serraino and Thompson also observed significantly lower tumor multiplicity but not tumor size in rats fed the 5%F diet throughout the experimental period (21 days of age to 20 wk post-DMBA) than in those fed the BD throughout. In a later study by Thompson and co-workers (23), rats were given the treatment diets after 13 weeks on a high-fat BD, testing the role of flaxseed and SDG on the late promotion, early progression stages of mammary cancer. In this study, flaxseed at the 2.5% and 5% levels decreased established tumor size, and 1.5 mg SDG/day reduced the size and average number of new tumors appearing in the group. The present study examined the role of flaxseed and SDG on early tumor promotion, and although flaxseed had no effect on tumor size, we also observed a reduction in tumor multiplicity with a similar dose of SDG (the HSDG group). Furthermore, Thompson and colleagues (24) found that 1.5 mg SDG/day during the early promotion phase significantly reduced mammary tumor multiplicity and tended to reduce tumor size, although the reduction was not significant. Thus a common theme appears, where SDG has a greater effect on mammary tumor multiplicity, whereas flaxseed’s effect on mammary tumorigenesis is determined in part by the time at which it is introduced during cancer development.

The use and dose of a different carcinogen may have influenced the results observed in this study. In contrast to the present study, which used the direct-acting carcinogen MNU, the mammary carcinogen DMBA requires metabolic activation (38) and was used in our previous studies with flaxseed and SDG (22–24). Nevertheless, the difference between a direct- and an indirect-acting carcinogen would be crucial only if the treatment in question was more effective at the initiation stage, where carcinogen metabolism could be affected. Because we have observed protective effects with flaxseed and SDG given 13 weeks after DMBA treatment (23), alteration of carcinogen activation does not appear to be a main mechanism of action for lignans. In terms of carcinogen dose, our previous studies used 5 mg of DMBA per animal (22–24), whereas a dose of 50 mg MNU/kg body wt, which was approximately 9 mg/animal on the basis of a mean weight of 180 g at the time of injection, was used in the present study. Carcinogen dose has been shown to affect dietary modulation of mammary carcinogenesis (38,39). Thus the use of a lower MNU dose, perhaps 35 mg/kg body wt, might have been more appropriate for testing the effect of flaxseed and its components on MNU-induced mammary tumorigenesis.

In addition to the dose of carcinogen used, the type of BD fed during the study may also affect the outcome of mammary tumorigenesis experiments. Cohen and colleagues (39,40) examined the interactions between carcinogen dose (5 vs. 15 mg DMBA) and type of BD (AIN-76A vs. NIH-07) on the effectiveness of different doses of the antioxidant butylated hydroxytoluene (BHT). The AIN-76A diet was a semipurified standard diet of the American Institute of Nutrition (41), whereas the NIH-07 diet was a cereal-based formulation (42). In their first study, where rats were exposed to BHT for four weeks only, significant reductions in tumor multiplicity with the high dose of BHT vs. control were observed in rats fed the AIN-76A diet but not in those fed the NIH-07 diet, regardless of carcinogen dose (40). In their subsequent study with BHT supplementation throughout the experiment, Cohen and colleagues (39) found that the inhibitory effects of BHT with the high dose of DMBA (15 mg) were greater in animals fed the AIN-76A diet than in those fed the NIH-07 diet. Thus, although these studies differ from the design of the present study, where the dietary treatment was introduced before carcinogen administration, the data indicate that the BD and the carcinogen dose can play a role in the results observed in carcinogenesis studies.

Some of the discrepancies that we have observed between the present study and previous studies examining the role of flaxseed in mammary carcinogenesis may involve the type of diet fed during critical developmental periods of the rats. The rats in the study by Serraino and Thompson (22) were fed a high-fat BD from 21 to 50 days of age and then introduced to the flaxseed diet after carcinogen treatment. The rats in the present study, however, were fed a commercial rat chow until they were received at our facility at 42–45 days of age. Analysis of rat chow, which contains soybean and alfalfa meals, for the isoflavone phytoestrogens genistein and daidzein has indicated that combined levels could be as low as 74 to as high as 491 µg/g chow (43–45). Because commercial rat chow has also been shown to contain mammalian lignan precursors (46), the total phytoestrogen exposure with rat chow could be much higher. Phytoestrogen exposure at these levels during this critical period of mammary gland development (21–50 days of age) may have altered the mammary gland structure to types that can potentially influence future susceptibility to mammary carcinogenesis (47; unpublished observations). Investigators have observed differences in mammary tumor development when using rat chow as opposed to purified diets (38). The NIH-07 diet used in the studies by Cohen and colleagues (39, 40) also contained soybean and alfalfa meals and has been found to have a phytoestrogen content of 228 µg/g diet (45). Phytoestrogens have not been detected in the purified American Institute of Nutrition diets (45). Because the NIH-07 diet (42) also contained other dietary components that may influence cancer development (e.g., fish meal and an n-3 fatty acid-rich fat source consisting of a mixture of soybean and fish oils), it is difficult to attribute the modulatory effects observed by Cohen and colleagues (39,40) with the NIH-07 diet to its soybean meal content alone.

Another dietary factor that could have contributed to the results observed in the present study was the type of oil used in the BD. In the previous studies with DMBA (22–24), the fat source in the standard AIN-76A diet (41) was corn
oil, which has very low levels of n–3 fatty acids (about 1.2% by weight α-LA). To provide a more adequate level of n–3 fatty acids, the revised American Institute of Nutrition standard diet AIN-93G (28) uses soybean oil, a moderate source of the 18:3n–3 fatty acid (approximately 8% by weight). It was calculated that the BD with 20% soybean oil used in the present study contained 1.60% α-LA, whereas the BD with 20% corn oil used in previous studies (22–24) contained about 0.24% α-LA. Interestingly, the α-LA content of the 5%F with 20% corn oil (which contained 1.82% flaxseed oil, consisting of 55% α-LA) was 1.22% and thus lower than that of the BD with 20% soybean oil.

Thus the moderate α-LA content of the BD with soybean oil may have masked the inhibitory effect of flaxseed on MNU-induced tumor development. The mean palpable tumor size with BD containing 20% corn oil in the study by Thompson and co-workers (23) was twice the size of that found with the BD containing 20% soybean oil in this study, suggesting that the higher α-LA content in the BD with soybean oil may be partly responsible for the smaller tumor size. Fatty acid analysis by the same method described in our previous study (23) showed a higher tumor α-LA content in rats fed BD (0.99 ± 0.21%) and 5%F (1.58 ± 0.30%) with soybean oil as the fat source in this study than in rats fed BD (0.23 ± 0.06%) and 5%F (0.67 ± 0.08%) with corn oil as the fat source in our previous study (23). The tumor α-LA content in the BD with 20% soybean oil in this study was not significantly different from that with 5%F with 20% corn oil in the previous study (23). By use of the data from both of these studies, there was a significant inverse correlation between logistically transformed tumor volume and tumor α-LA content ($y = -0.204x + 0.573$, $r = -0.419$, $p = 0.014$, $n = 34$), suggesting that the α-LA may play a role in reducing tumor growth. These data suggest that the composition of the BD, including the type of oil used (e.g., soybean vs. corn oil), may influence the outcome of mammary tumorigenesis studies.

Many studies have demonstrated the inhibitory effect of α-LA on tumorigenesis. Fritsche and Johnston (25) observed a significant reduction in the growth of transplantable 410.4 mouse mammary tumors in BALB/c mice fed 10% flaxseed oil compared with mice fed 10% corn oil. Thompson and co-workers (23) showed that even low levels of flaxseed oil supplementation (1.82%) could significantly reduce mammary tumor size. Other fats rich in α-LA (e.g., perilla oil and Zizyphus jujube seed oil) have also been shown to be protective against mammary tumorigenesis (48,49). Furthermore, α-LA has exhibited inhibitory effects on the growth of various estrogen-dependent and -independent human breast cancer cell lines in vitro (50,51). Mechanisms proposed for the protective effects of n–3 fatty acids are inhibition of the production of the tumor-promoting eicosanoids, such as prostaglandin E2 and leukotriene B4 from arachidonic acid (52), and cytotoxic action of lipid peroxidation products in tumor, but not normal, cells (50). However, the role of lipid peroxidation in reducing tumor growth is controversial (52).

In conclusion, although the 5%F group tended to have the smallest tumors throughout the experimental period, flaxseed treatment did not have a significant effect on tumor size, multiplicity, or incidence in comparison to BD (control) in the MNU tumor model. SDG, like flaxseed, did not reduce tumor size or incidence, but it had a dose-dependent effect on tumor multiplicity, with LSDG promoting and HSDG inhibiting tumor formation. This may be the result of estrogen-dependent vs. -independent mechanisms and suggests that non-SDG components of flaxseed are also playing a role in its effect on mammary tumorigenesis. However, flaxseed and SDG feeding, regardless of dose, lowered tumor invasiveness and grade, indicating that the tumors in the control group were at a more advanced stage. The discrepancies observed between this and previous studies examining the role of flaxseed and SDG on mammary tumorigenesis may be due to differences in experimental design, to the use and dose of a different carcinogen, and to protective effects from the α-LA present in the BD. This study further underlines the role that the BD composition might play in the outcome of mammary tumorigenesis experiments.

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