Inhibition of Oral Carcinogenesis by Citrus Flavonoids

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Six citrus flavonoids were tested for antineoplastic activity. The hamster cheek pouch model was utilized, and the solutions of the flavonoids (2.0–2.5%) and the solution of the carcinogen, 7,12-dimethylbenz[a]anthracene (0.5%), were applied topically to the pouches. The pouches of the positive controls were treated with the solvent used to dissolve the flavonoids and the solution of the carcinogen. The data show that 4 flavonoids (hesperetin, neohesperidin, tangeretin, and nobiletin) were inactive. The results with naringin and naringenin show that both of these flavonoids significantly lowered tumor number [5.00 (control group), 2.53 (naringin group), and 3.25 (naringenin group)]. Naringin also significantly reduced tumor burden [269 mm³ (control group) and 77.1 mm³ (naringin group)]. The data suggest that naringin and naringenin, 2 flavonoids found in high concentrations in grapefruit, may be able to inhibit the development of cancer.

INTRODUCTION

Flavonoids comprise a large group of naturally occurring organic compounds found in a wide variety of plants including fruits, vegetables, nuts, seeds, grains, tea, and wine. It is estimated that in the Western diet, approximately 1 g of mixed flavonoids are consumed on a daily basis. Based primarily on experimental animal studies, a number of health-promoting properties have been ascribed to flavonoids. Some of these effects include antiviral, antiallergic, anti-inflammatory, and anticancer (1). In addition, some flavonoids also possess antioxidant, vitamin C sparing activity. At this time, flavonoids are classified as xenobiotics, nonessential dietary factors. As more is learned about these unique compounds, this classification may need to be modified.

A number of factors contributed to this research. One, data from 24 separate epidemiological studies has consistently shown that the consumption of citrus is protective against a wide variety of cancers. Significant reductions in risk were found for cancers of the oral cavity, larynx, esophagus, stomach, pancreas, lung, colon, and rectum. The results have also suggested that this activity is due to vitamin C plus 1 or more as yet unidentified cancer chemopreventive agents. A review covering these epidemiological studies has been published (2). Two, previous research has shown that citrus limonoids—a group of chemically related, highly oxygenated triterpenoids—can inhibit the development of carcinogen-induced neoplasia in a wide variety of different animal models for cancer (3–13). Limonoids, primarily as glucose derivatives (14,15), are found in high concentrations in the mature fruit and juice (16). One glass of orange juice contains 65 mg of mixed limonoid glucosides (17,18). Three, very little work has been done on the antineoplastic activity of citrus flavonoids. Because some of the citrus flavonoids are also found at high concentrations in citrus products, we decided to test these compounds to see if they might be contributing to the anticancer activity associated with citrus consumption. Like our earlier studies with limonoids (4,5,7,9,10,13), the hamster cheek pouch model for oral carcinogenesis was utilized.

The flavonoids tested included tangeretin, nobiletin, hesperetin, neohesperidin, naringenin, and naringin. Tangeretin and nobiletin are polymethoxylated flavonoids that are found primarily in the peel. The 4 remaining flavonoids are found in high concentrations in the fruit. Neohesperidin and the aglycone hesperetin are major secondary metabolites in oranges and lemons. Naringin and the aglycone naringenin are found in high concentrations in grapefruit.

MATERIALS AND METHODS

Animal Care

A total of 60 hamsters were used in the first experiment with tangeretin and nobiletin and 100 in the second experiment...
with neohesperidin, hesperetin, naringin, and naringenin. The female Syrian golden hamsters (Lak:LVG) were purchased from the Charles River Breeding Laboratories (Wilmington, MA). At the time of arrival, the hamsters were approximately 6 wk old, weighing 90–100 g. The animals were immediately placed in wire-mesh cages of stainless steel in a temperature-controlled room (22°C) with a 12:12 light–dark cycle. After arriving, the hamsters were given 1 wk to adjust to their new surroundings. During this time and throughout the rest of the experiment, food (Rodent Diet 7002 by Harlan Teklad in Madison, WI) and water were provided ad libitum.

Experimental Design
At the start of each experiment, the animals were randomly divided into groups (20 hamsters/group). In each experiment, the animals in Group 1 served as the controls. In the first experiment, the left buccal pouches of the hamsters in the 3 groups were pretreated with 2 separate daily applications of dimethyl sulfoxide (DMSO; Group 1), a 2% solution of tangeretin (Group 2), or a 2% solution of nobiletin (Group 3). The 2 polymethoxylated flavonoids were dissolved in DMSO. In the second experiment, the left buccal pouches were pretreated with 2 separate daily applications of a 50:50 mixture of DMSO and propylene glycol (Group 1), a 2.5% solution of hesperetin (Group 2), or a 2.5% solution of neohesperidin (Group 3). For the second experiment, the pouches were painted with the 50:50 mixture of DMSO and propylene glycol (Group 1), the 2.5% solution of hesperetin (Group 2), the 2.5% solution of neohesperidin (Group 3), the 2.5% solution of naringenin (Group 4), or the 2.5% solution of naringin (Group 5). All of the solutions were painted on the pouches with a No. 5 sable-hair brush. Each application places ≈50 μl of the liquid on the surface of the pouch (19).

Data Collection
In each experiment, there was a total of 71 applications, 34 with either the 0.5% solution of DMBA (experimental animals) or heavy mineral oil (negative controls) and 37 with either one of the solvents used to dissolve the flavonoids or one of the solutions of the citrus flavonoids. At 1 wk after the last treatment, the hamsters were sacrificed, and the left buccal pouches were excised. Tumors, when present, were counted and measured (length, width, and height). Because the tumors are exophytic and tend to be spherical in shape, the sum of the 3 measurements divided by 6 was used to calculate an average radius for each tumor. Using this number and the formula for the volume of a sphere, \( V = \frac{4}{3} \pi r^3 \), an approximate value for the volume of the tumor was calculated. The sum of the volumes of all of the tumors in each pouch was defined to be that animal’s total tumor burden (4,20).

RESULTS
Of the 136 experimental animals used in the 2 experiments, 5 died. These deaths occurred early in the experiments and in each case were due to respiratory problems associated with the anesthetic used to lightly anesthetize the hamsters before each treatment. At the time of death, all of the pouches were free of any apparent tumors. The pouches were removed and processed for histological examination. In each case, some signs of dysplasia were found. These animals were excluded from the study.
TABLE 1
Tangeretin and nobiletin: Effects on oral carcinogenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Total no. of tumors</th>
<th>Avg. no. of tumors(^b)</th>
<th>Avg. tumor radii (mm)</th>
<th>Avg. tumor burden (mm(^3))(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>77</td>
<td>4.53 ± 0.53</td>
<td>2.4</td>
<td>259 ± 57</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>74</td>
<td>4.35 ± 0.51</td>
<td>2.3</td>
<td>226 ± 81</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>67</td>
<td>4.19 ± 0.51</td>
<td>2.2</td>
<td>186 ± 38</td>
</tr>
</tbody>
</table>

\(^a\)Group 1 was the control group, the group that was treated with the solvent used to dissolve the flavonoids. Group 2 was treated with the 2% solutions of tangeretin, and Group 3 was treated with the 2% solutions of nobiletin.

\(^b\)Values are means ± SE.

The data for the experiment with tangeretin and nobiletin are given in Table 1. The number of hamsters in the 3 experimental groups was 17 for Groups 1 and 2 and 16 for Group 3. Multiple tumors (2 to 10) were found in 49 of the 50 pouches. A single tumor was found in one of the pouches from an animal in Group 3. As illustrated, the treatments with tangeretin and nobiletin decreased average tumor number by 5–10%. Using the Student’s t-test, it was found that the differences between Groups 1 and 2 and Groups 1 and 3 were not significant. The average radii for the tumors in the 3 groups ranged from a high of 2.4 mm (Group 1) to a low of 2.2 mm (Group 3). Tumor burden is unique in that it takes into account both tumor number and tumor volume. Comparing Group 2 to Group 1, it can be seen that the treatment with tangeretin reduced average tumor burden by 15%. A similar comparison (Groups 3 and 1) showed that the painting with nobiletin reduced average tumor burden by 30%. These differences were not significant.

The results for the second experiment with hesperetin, neohesperidin, naringenin, and naringin are given in Table 2. The number of animals in the 5 experimental groups ranged from 15 in Group 1 to 16 in Groups 3 and 4 to 17 in Groups 2 and 5. Multiple tumors (2 to 15) were found in 65 of the 81 pouches. Some of the pouches, 5 (1 in Group 2 and 4 in Group 5) were free of visible tumors. A single tumor was found in 11 pouches (2 in Group 1, 2 in Group 2, 1 in Group 3, 2 in Group 4, and 4 in Group 5). The average number of tumors per pouch ranged from a high of 5.00 for Groups 1 and 3 to a low of 2.53 for Group 5. Compared to the control group (Group 1), statistically significant differences (\(P < 0.05\)) were found for Groups 4 and 5, the hamsters treated with naringenin and naringin. The results for average tumor radii were similar for Groups 1 through 4, ranging from a low of 2.2 mm (Group 3) to a high of 2.4 mm (Group 4). In Group 5, the tumors were smaller, with an average tumor radii of only 1.9 mm. All of the treatments with the citrus flavonoids reduced tumor burden. The overall reduction was 20% with neohesperidin, 30% with hesperetin and naringenin, and 70% with naringin. Using the Student’s t-test, it was found that the only statistically significant difference (\(P < 0.05\)) was between Groups 1 and 5.

Histologically, all of the tumors in the 2 experiments were classified as epidermoid carcinomas. Signs of dysplasia were seen in the sections taken from the 5 pouches that were free of visible tumors. Sections taken from the pouches of the negative controls (3-animals group) in the 8 groups were free of any signs of dysplasia and appeared normal. No significant differences were found in the weight-gain profiles for the hamsters in the different experimental groups. Throughout the course of the 2 experiments, the gross appearance of the animals in the different groups appeared to be normal.

TABLE 2
Hesperetin, neohesperidin, naringenin, and naringin: Effects on oral carcinogenesis

<table>
<thead>
<tr>
<th>Group(^a)</th>
<th>No. of animals</th>
<th>Total no. of tumors</th>
<th>Avg. no. of tumors(^b)</th>
<th>Avg. tumor radii (mm)</th>
<th>Avg. tumor burden (mm(^3))(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>75</td>
<td>5.00 ± 0.88</td>
<td>2.3</td>
<td>269 ± 75</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>63</td>
<td>3.71 ± 0.55</td>
<td>2.3</td>
<td>191 ± 47</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>80</td>
<td>5.00 ± 0.85</td>
<td>2.2</td>
<td>222 ± 43</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>52</td>
<td>3.25 ± 0.42(^c)</td>
<td>2.4</td>
<td>188 ± 55</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>43</td>
<td>2.53 ± 0.81(^c)</td>
<td>1.9</td>
<td>77.1 ± 30(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Group 1 was the control group, the group that was treated with the solvent used to dissolve the flavonoids. Groups 2 through 5 were treated with the 2.5% solution of hesperetin (Group 2), the 2.5% solution of neohesperidin (Group 3), the 2.5% solution of naringenin (Group 4), or the 2.5% solution of naringin (Group 5).

\(^b\)Values are means ± SE.

\(^c\)\(P < 0.05\) when these values are compared to the corresponding values for the control group (Group 1).
DISCUSSION

The results with the polymethoxylated flavonoids were unexpected. Earlier research with tangeretin and nobiletin has consistently suggested that these compounds can inhibit the growth of cancer cells and inhibit the formation of carcinogen-DNA adducts. Data from in vitro studies (21–23) have shown that these citrus flavonoids can inhibit the proliferation of a human squamous cell carcinoma (HTB43), a gliosarcoma (9L), and two melanoma cell lines (B16F10 and SK-MEL-1). Tangeretin was also found to inhibit the binding of benzo[a]pyrene and aflatoxin B1 to DNA (24,25).

Tangeretin, and to a lesser extent nobiletin, also appeared to be able to inhibit the final phase of carcinogenesis, progression. This was primarily seen in a chick heart assay used to measure the potential for invasion and metastasis. Tangeretin partially blocked the movement of mouse MO4 cells (Kirsten murine sarcoma virus transformed fetal mouse cells) and MCF-7/6 cells (human breast cancer cell line) into the chick heart fragments (26–28). Further research (28,29) with the breast cancer cell line showed that tangeretin reactivated cell–cell adhesion by helping to correct a defect in the E-cadherin/catenin complex. Because tamoxifen produces similar effects in vitro, in vivo studies were conducted. In these experiments (29,30), nude mice were injected subcutaneously with MCF-7/6 cells and then treated with tamoxifen, tangeretin, or a combination of tangeretin + tamoxifen. Only the treatment with tamoxifen inhibited the growth of the tumor cells. Tangeretin was ineffective and neutralized tamoxifen’s inhibitory effect. It was also found that both tangeretin and nobiletin reduced the cytotoxic competence of murine natural killer cells on MCF-7/6 cells in vitro.

Compared to the in vitro studies on tumor cell growth (21–23), our data tends to parallel the data with MCF-7/6 cells in nude mice (29,30). One possible reason for this is that this experiment, like the experiment with nude mice, was an in vivo study. A second reason is a characteristic of the hamster cheek pouch model (31). This tumor model is designed to study the early phases of chemical carcinogenesis, initiation and promotion, not progression. The experiments are terminated before the tumors start to invade the underlying normal tissue and before the tumor cells have a chance to metastasize.

A couple of investigators have looked at the potential cancer chemopreventive activity of neohesperidin and hesperetin, two of the flavonoids commonly found in high concentrations in orange juice. Most of the work has focused on neohesperidin. In an early study (32), it was shown that neohesperidin possessed significant alkylperoxyl (ROO) radical-scavenging activity. These free radicals have been found to promote colon carcinogenesis in rats (33). Using a variety of different in vitro assays, 11 citrus chemicals (limonoids, flavonoids, and coumarins) were recently tested for antioxidant activity (34). One of these chemicals, neohesperidin, demonstrated mild to moderate activity in these assays. Recently, it was also reported (35) that hesperetin can efficiently scavenge peroxynitrite (ONOO–). This free radical has been associated with the pathogenesis of a number of diseases including stroke, Alzheimer’s, atherosclerosis, and heart disease. Our results with an in vivo assay suggest that hesperetin and neohesperidin lack significant anticancer activity.

The results from a number of different laboratories suggest that naringin and naringenin, flavonoids found primarily in grapefruits, may be potential cancer chemopreventive agents. Naringenin has been shown to inhibit the growth of a variety of human cancer cell lines in vitro (36,37). Cytotoxicity was seen in human cell lines originating from cancers of the breast (MDA-MB-435, MCF-7, MDA-MB-231), colon (Caco-2), pancreas (PK-1), liver (HepG2, Hep3B, Hu7), cervix (HeLa, HeLa-TG), stomach (KATOIII, MKN-7), and leukemia (HL-60, NALM-6, Jurkat, U937). A series of intraperitoneal or peroral injections of naringenin also inhibited the growth of sarcoma S-180 cells in mice (37). Naringin was only effective in this assay when given by peroral injections.

Other studies have shown that both of these flavonoids can inhibit the microsomal pathways that convert the tobacco-specific nitrosamine, NNK, and heterocyclic amines into ultimate carcinogens (38,39). An epidemiological study (40) that focused on the diets of 582 patients with incident lung cancer and 582 matched controls indicated that there was a statistically significant inverse correlation between the intake of the primary food source for naringin and naringenin (grapefruit) and lung cancer risk. Recently, it was also reported (41) that naringin protects against radiation-induced DNA damage in the bone marrow of mice. The flavonoid was given at various doses (0.5–8.0 mg/kg body weight) 45 min before the exposure to the γ-irradiation. In addition, in one study, evidence was presented suggesting that naringin is an antineoplastic agent (36). In this experiment, it was found that a naringin-supplemented diet (0.5%) delayed the appearance of DMBA-induced mammary tumors in Sprague-Dawley rats. A similar effect was not seen with a naringenin-supplemented diet (0.25%).

In many ways, the results with naringin and naringenin tend to parallel our earlier results with active limonoids. In these earlier experiments, several limonoids, such as limonin and limonin 17-β-D-glucopyranoside, were classified as having highly significant cancer chemopreventive activity (4,5,10,13). With these chemicals, we consistently saw an effect on both tumor number and tumor burden. The overall reduction in tumor burden ranged from 50–60%; however, the reduction in tumor number was less (20–30%). With naringin, the pattern was similar. The reduction in tumor burden was approximately 70%, and the reduction in tumor number was 50%. Other limonoids, such as nomilin and nomilin 17-β-D-glucopyranoside, were classified as having partial activity. With these chemicals, there was a 20–30% reduction in average tumor number. No effect on average tumor radii was seen. For this reason, the overall reduction in tumor burden was similar to the reduction in tumor number (20–30%). With naringenin, we found a 35% reduction in average tumor number and a 30% reduction in average tumor burden. No reduction in average tumor radii was found.
A variety of other chemicals have been tested in the hamster cheek pouch model for oral carcinogenesis. Out of this group, topical applications of a 2.5% solution of β-carotene reduced tumor burden by 90–95% (42). Similar studies with glutathione, vitamin E, and vitamin C reduced tumor burden by 70%, 50%, and 0%, respectively (43). Again, DMBA was the carcinogen used in these experiments.

The results of our work with naringin and naringenin also tend to parallel the results of the earlier experiments on mammary tumorigenesis (36). In the two studies, naringin was the more effective agent. Also, the data showed that the primary effect in both cases was on tumor number not tumor size or weight. One of the obvious differences in the two sets of experimental results was the overall effectiveness of naringin in inhibiting the appearance of the DMBA-induced tumors. In this study, the treatments with naringin reduced average tumor number by approximately 50%. In the earlier study, the naringin was incorporated into a 5% and a 20% corn oil diet. On average, the overall reduction in average tumor number was approximately 20%. In addition, in our experiment, we found that naringenin also significantly lowered average tumor number by 35%. Additional support for the activity of these two flavonoids can also be found in a recent report from another group of investigators (44).

In this study, it was found that both naringin and naringenin significantly inhibited the development of azoxymethane-induced high multiplicity aberrant crypt foci in male Sprague-Dawley rats. One difference between the two sets of results was overall effectiveness. In this second set of experiments, naringenin was the more active agent.

An interesting aspect of this work is the fact that both of these flavonoids are present in grapefruit juice at extremely high concentrations. The combined average concentration of naringin and naringenin in white and pink grapefruit juice is 1,000 ppm (45). Naringin is by far the more common compound. The ratio of naringin to naringenin usually exceeds 9:1. To start to put this number (1,000 ppm) in perspective, the concentration of vitamin C in grapefruit juice ranges from 200–400 ppm. The 1,000 ppm value also suggests that this work may have relevance for humans. People drinking grapefruit juice are exposing the oral mucosa to a 1% solution of these flavonoids (mainly naringin). It is true that this concentration is less than the 2.5% concentration used in our experiment; however, most individuals bath the oral mucosa not once but several times with this solution as they consume the juice. Multiple exposures at a reduced concentration may yield similar levels of protection. In addition, several servings per day increase the exposure level even more.

At this time, it is impossible to tell how naringin and naringenin might be inhibiting at the molecular level the action of DMBA. The data do indicate that the primary effect is on tumor number and not tumor burden. This suggests in the hamster cheek pouch model that the inhibition is occurring early in the process of chemical carcinogenesis, during initiation and/or promotion. Because the tumors appeared to have grown at the same rate, there is very little likelihood that the flavonoids are inhibiting the final phase of chemical carcinogenesis, progression. In addition, a recent article (46) now suggests that naringenin at low doses (10–80 μmol/l) can significantly stimulate DNA repair following oxidative damage in a human lymph node prostate cancer cell line (LNCaP). The results also showed that the exposure to naringenin led to a significant increase in the concentration of several major enzymes in the DNA base excision repair pathway. Naringin was not tested in this series of experiments; however, the structural similarity in these 2 flavonoids suggests that similar results might have occurred. If this proves to be true, then it is possible that the cancer chemopreventive activity of these flavonoids is due in part to a stimulation of this cellular mechanism to remove carcinogen-induced damage to the genetic information.

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