SHORT COMMUNICATION

Inhibition of azoxymethane-induced colon carcinogenesis in male F344 rats by the citrus limonoids obacunone and limonin

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The modifying effects of dietary administration of the citrus limonoids obacunone and limonin on azoxymethane (AOM)-induced colon tumorigenesis were investigated in two experiments in male F344 rats. In a pilot study, we examined the modifying effects of obacunone and limonin on AOM-induced (20 mg/kg body wt, once a week for 2 weeks) formation of aberrant crypt foci (ACF). Dietary feeding of both compounds at dose levels of 200 and 500 p.p.m. during AOM exposure for 4 weeks ('initiation' feeding) or after AOM treatment for 4 weeks ('post-initiation' feeding) significantly inhibited ACF formation (55–65% reduction by 'initiation' feeding, P < 0.001; 28–42% reduction by 'post-initiation' feeding, P < 0.05–0.002).

In a long-term study designed to confirm the protective effects of obacunone and limonin on ACF development, one group was treated with AOM alone and another four groups received the carcinogen treatment plus diets containing 500 p.p.m. test compounds for 3 weeks (initiation phase) or 29 weeks (post-initiation phase). Two groups were treated with obacunone or limonin alone (500 p.p.m. in diet) and one group was maintained on the basal diet. At the termination of the study, dietary exposure to obacunone or limonin during the initiation phase was found to have significantly reduced the incidence of colonic adenocarcinoma (72 versus 25 or 6%, P = 0.004 or 0.00003). Obacunone or limonin feeding during the post-initiation phase also reduced the frequency of colonic adenocarcinoma (72 versus 13%, P = 0.0002). Our results suggest that the citrus limonoids obacunone and limonin might be useful for the prevention of human colon cancers.

Dietary factors play an important role in prevention of various human diseases, including cancers (1–3). Experimental and epidemiological evidence suggests that increased dietary fiber is associated with reduced risk of colon cancer, which is the third most malignant neoplasm in the world (4) and the second leading cause of cancer deaths in the USA. An estimated 875,000 new cases were reported in 1996, accounting for 8.5% of all new cases of cancer in the world. In Japan, the progressive introduction of Western dietary habits, especially increased fat intake and reduced carbohydrate and dietary fiber intake, has increased incidence of colon cancer and related deaths (5). Since an inverse relationship has been suggested between the intake of fruits/vegetables and human colon cancer (6–8), primary prevention, including chemoprevention utilizing the active compounds in edible plants, is important for reducing this malignancy (9–11).

Limonoids are a group of triterpene derivatives present in the Rutaceae and Malvacaceae families. Limonoids, including obacunone (Figure 1a) and limonin (Figure 1b), are also found in citrus seeds (12), commercial citrus juice (13) and Philodendron amurense (Kihada) (14). For example, commercial orange juice contains an average of 320 p.p.m. limonoid glucosides (13). These glucosides are responsible for delayed bitterness in citrus juices and processed products (15). Obacunone and limonin have been reported to enhance glutathione-S-transferase (GST) activity in various organs of mice (16,17). Limonin and nomilin are reported to inhibit forestomach, buccal pouch, lung and skin carcinogenesis in rodents (18). However, the modifying effects of the citrus limonoids obacunone and limonin on large bowel carcinogenesis have not been reported.

We have previously reported the chemopreventive ability of natural compounds from edible plants against colon carcinogenesis using aberrant crypt foci (ACF) as a biomarker (19,20). ACF in the colon of rodents (exposed to colonic carcinogens) and humans are regarded as possible precursor lesions for colon cancers (21,22) and as useful biomarkers for detecting the modulatory effects of xenobiotics on colon carcinogenesis (23). We have identified several natural agents from edible plants using this ACF assay model (19). These include inducers of GST and quinone reductase (QR) activities in liver and colon (24–27). Obacunone and limonin could induce GST activity in certain organs (18) and limonin could inhibit carcinogen–DNA adduct formation (18). We also recently found that dietary administration of both compounds increase GST and QR activities in liver and colon of rats (T.Tanaka et al., manuscript in preparation). Therefore, it is possible that these compounds inhibit colon carcinogenesis.

In the present study, two experiments were conducted to investigate the modifying effects of obacunone and limonin on large bowel tumorigenesis induced by azoxymethane (AOM) in rats. In a pilot study, dietary obacunone and limonin were given to rats in order to determine whether these compounds could modulate the occurrence of ACF. Subsequently, a long-term bioassay was performed to confirm and evaluate the preventive effects of dietary obacunone and limonin on AOM-induced colon carcinogenesis.

Four-week-old male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used. All animals were housed in wire cages (three or four rats per cage) with free access to drinking water and basal diet (CE-2; CLEA Japan, Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), lighting (12 h light/dark cycle) and temperature (23 ± 2°C). They were quarantined for 7 days and randomized into

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; GST, glutathione S-transferase; QR, quinone reductase.
experimental and control groups for a pilot study and a long-term bioassay. AOM was obtained from Sigma Chemical Co. (St Louis, MO). Obacunone (99.9% pure) and limonin (99.9% pure) were isolated from the bark of *P. amurense* (Kihada). AOM (20 mg/kg body wt) was administered by s.c. injection between 10:00 and 11:00 a.m. Powdered CE-2 diet (345.2 cal) was used as the basal diet throughout the study. Obacunone and limonin were mixed in the powdered basal diet CE-2 at a concentration of 200 or 500 p.p.m. (w/w). These experimental diets were prepared on a weekly basis and stored in a cold room (<4°C) until use.

In a pilot study, 104 rats were divided into 12 groups as shown in Figure 2a. Groups 1–9 received two weekly s.c. injections of AOM at a dose of 20 mg/kg body wt. Rats of group 1 were fed the basal diet alone while those of groups 2 and 3 were fed diets containing 200 p.p.m. obacunone and limonin, respectively, for 4 weeks, starting 1 week before the first dose of AOM. Similarly, groups 4 and 5 were given the experimental diets containing 500 p.p.m. obacunone and limonin, respectively, for 4 weeks, starting 1 week before the first injection of AOM. Groups 6 and 7 were fed diets containing 200 p.p.m. obacunone and limonin, respectively, for 4 weeks, commencing 2 weeks after the last dose of AOM. Groups 8 and 9 were fed diets containing 500 p.p.m. obacunone and limonin, respectively, for 4 weeks, starting 2 weeks after the last injection of AOM. Groups 10 and 11 were fed the experimental diet containing 500 p.p.m. obacunone or limonin alone for 8 weeks and did not receive AOM. Group 12 was an untreated control. At weeks 4 and 8, eight rats from each of groups 1–9 and four rats from each of groups 10–12 were killed to count colonic ACF.

The colons of all rats in the pilot study were used for scoring of ACF. At autopsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis and then washed again with saline. The colons were cut into three sections (~4 cm each) starting from the anus, placed between filter papers to reduce mucosal folding and fixed in 10% buffered formalin for at least 24 h. Fixed colonic sections were dipped in a 0.2% solution of methylene blue in distilled water for 30 s and briefly rinsed with distilled water. Using a light microscope at a magnification of ×40, ACF were distinguished from surrounding normal crypts by their large size, more prominent epithelial cells and increased pericryptal space. The number of ACF observed per colon, number of aberrant crypts observed in each focus and location of each focus were recorded.

For the long-term study (Figure 2b), a total of 113 rats were randomly divided into eight groups. Groups 1–5 received two weekly s.c. injections of AOM (20 mg/kg body wt). Rats in groups 2 and 3 were fed diets containing 500 p.p.m. obacunone and limonin for 3 weeks, respectively, commencing 1 week before the first dose of AOM. Groups 4 and 5 were fed the diet mixed with 500 p.p.m. obacunone and limonin, respectively, for 29 weeks of the post-initiation phase, starting 1 week after the last administration of AOM. Groups 6 and 7 did not receive AOM and were fed diets mixed with 500 p.p.m. obacunone or limonin for the duration of the study (32 weeks). Group 8 served as an untreated control. All rats were carefully observed daily, weighed weekly until they reached 14 weeks of age and every 4 weeks thereafter. Consumption of the experimental diets was also recorded to estimate the intake of test compounds. The experiment was terminated 32 weeks after commencement and all animals were killed by an ether overdose to assess the incidences of large bowel tumors, kidney mesenchymal tumors and altered liver cell foci. At autopsy, the intestine was excised, opened longitudinally, flushed clean with saline and examined for the presence of tumors. Colons, kidney and liver were fixed in 10% buffered formalin and then processed for histopathological examination by conventional methods. Neoplasms in the intestine were diagnosed according to the criteria described by Ward (28). If
Inhibition of colon carcinogenesis by limonoids

Table I. Effects of obacunone and limonin on AOM-induced ACF formation in male F344 rats at 4 weeks

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment (no. of rats examined)</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Total no. of ACF/colon (incidence)</th>
<th>Total no. of aberrant crypts/colon</th>
<th>No. of aberrant crypts/focus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM alone (8)</td>
<td>200 ± 8a</td>
<td>11.8 ± 2.0</td>
<td>143 ± 14 (8/8)</td>
<td>9.36 ± 1.64</td>
<td>335 ± 32</td>
</tr>
<tr>
<td>2</td>
<td>AOM + 0.02% obacunone (8)</td>
<td>190 ± 5b</td>
<td>10.3 ± 0.5</td>
<td>50 ± 8° (8/8)</td>
<td>4.58 ± 0.63c</td>
<td>107 ± 17e</td>
</tr>
<tr>
<td>3</td>
<td>AOM + 0.05% obacunone (8)</td>
<td>186 ± 5e</td>
<td>10.6 ± 0.9</td>
<td>50 ± 11° (8/8)</td>
<td>3.35 ± 1.43c</td>
<td>109 ± 22°</td>
</tr>
<tr>
<td>4</td>
<td>AOM + 0.02% limonin (8)</td>
<td>188 ± 5e</td>
<td>10.4 ± 0.4</td>
<td>65 ± 14° (8/8)</td>
<td>6.44 ± 1.36c</td>
<td>128 ± 27°</td>
</tr>
<tr>
<td>5</td>
<td>AOM + 0.05% limonin (8)</td>
<td>187 ± 7e</td>
<td>10.2 ± 0.7</td>
<td>63 ± 6° (8/8)</td>
<td>5.08 ± 0.47c</td>
<td>123 ± 16°</td>
</tr>
<tr>
<td>10</td>
<td>0.05% obacunone (4)</td>
<td>207 ± 11</td>
<td>10.8 ± 0.8</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
</tr>
<tr>
<td>11</td>
<td>0.05% limonin (4)</td>
<td>195 ± 7</td>
<td>10.0 ± 0.4</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
</tr>
<tr>
<td>12</td>
<td>No treatment (4)</td>
<td>209 ± 14</td>
<td>10.8 ± 1.2</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
</tr>
</tbody>
</table>

*Mean ± SD.

Significantly different from group 1: ±P < 0.01; ²P < 0.001; ³P < 0.02; ⁴P < 0.005; ⁵P < 0.002; ⁶P < 0.05.

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tumor cells of tubular form invaded the submucosa, the tumor was diagnosed as adenocarcinoma. When the tumor cells did not invade the submucosa, the tumor was diagnosed as adenoma. Other organs were also examined histopathologically.

One-factor ANOVA, the Kruskal–Wallis test or Fisher’s exact probability test was used for statistical analyses. A value of \( P < 0.05 \) was considered significant. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kanazawa Medical University.

In the pilot study, the mean daily intakes of diets mixed with obacunone and limonin during AOM exposure (groups 2–5) were between 14.1 and 14.7 g/rat, while those in groups 1, 10, 11 and 12 were between 14.6 and 15.1 g/rat (weeks 0–4). The mean daily intakes of diets mixed with obacunone and limonin after AOM exposure (groups 1 and 6–12) were between 16.9 and 17.4 g/rat (weeks 4–8). During the study (8 weeks), no clinical signs of toxicity were observed in any group. Histologically, there were no toxic changes in the liver, kidneys, lung and heart of the rats in any group. At the commencement of the study there was no difference in body weights of the animals among the treatment groups. The ‘initiation’ feeding of obacunone and limonin (groups 2–5) significantly inhibited body weight gains at week 4, when compared with that of group 1 (\( P < 0.01 \) or 0.001), as shown in Table I. The range of liver weight and the liver weight:body weight ratio (%) were 10.0 ± 0.4–11.8 ± 2.0 g and 4.83 ± 0.17–5.82 ± 0.70%, respectively. All animals administered AOM in all treatment groups contained at least one ACF. The incidence of ACF/colon in groups 2–5 was significantly less than that in group 1 (\( P < 0.001 \)). As indicated in Table II, the ‘post-initiation’ feeding of 500 p.p.m. obacunone and limonin significantly decreased the body weight gains in groups 7 and 9 at weeks 8 (\( P < 0.02 \) and 0.05), but did not affect the liver weights (9.9 ± 1.3–11.4 ± 1.2 g) or liver weight:body weight ratio (4.05 ± 0.36–4.45 ± 0.38%). The mean number of aberrant crypts/focus of groups 6–9 was significantly smaller than in group 1 (\( P < 0.001 \)).

In the long-term bioassay, the daily food intake of groups 2–7 did not differ from that of groups 1 and 8, which were fed the basal diet without obacunone or limonin (data not shown). In this study, dietary administration of the two test compounds did not cause any clinical signs of toxicity, low survival or poor clinical condition. Weight gains of rats in all groups were comparable throughout the experimental period. At the end of the study, the mean liver weights and relative liver weights (g/100 g body wt) of all groups except that of group 5, which was fed 500 p.p.m. limonin after AOM treatment, were also comparable (Table III). Both the mean and relative liver weights of group 5 were significantly lower than those of group 1 (\( P < 0.05 \)).

Macroscopically, most tumors developed in the large intestine (mainly the middle and distal colon) and some in the small intestine of rats in groups 1–5. They were sessile or pedunculated tumors histologically diagnosed as tubular adenomas, tubular adenocarcinomas or mucinous carcinomas, with a higher incidence of tubular adenocarcinoma. A few rats

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Table II. Effect of obacunone and limonin on AOM-induced ACF formation in male F344 rats at 8 weeks

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment (no. of rats examined)</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Total no. of ACF/colon (incidence)</th>
<th>Total no. of aberrant crypts/colon</th>
<th>No. of aberrant crypts/focus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM alone (8)</td>
<td>269 ± 13a</td>
<td>11.3 ± 1.7</td>
<td>149 ± 36 (8/8)</td>
<td>11.76 ± 2.75</td>
<td>436 ± 70</td>
</tr>
<tr>
<td>6</td>
<td>AOM +0.02% obacunone (8)</td>
<td>254 ± 15</td>
<td>9.9 ± 1.3</td>
<td>91 ± 10° (8/8)</td>
<td>8.75 ± 1.05</td>
<td>219 ± 38°</td>
</tr>
<tr>
<td>7</td>
<td>AOM +0.05% obacunone (8)</td>
<td>253 ± 11c</td>
<td>11.0 ± 0.7</td>
<td>87 ± 13° (8/8)</td>
<td>7.23 ± 1.04c</td>
<td>220 ± 35°</td>
</tr>
<tr>
<td>8</td>
<td>AOM +0.02% limonin (8)</td>
<td>269 ± 9</td>
<td>10.8 ± 1.3</td>
<td>107 ± 33° (8/8)</td>
<td>8.49 ± 2.15c</td>
<td>216 ± 71°</td>
</tr>
<tr>
<td>9</td>
<td>AOM +0.05% limonin (8)</td>
<td>255 ± 10f</td>
<td>11.4 ± 1.2</td>
<td>102 ± 27° (8/8)</td>
<td>7.82 ± 1.29b</td>
<td>209 ± 55°</td>
</tr>
<tr>
<td>10</td>
<td>0.05% obacunone (4)</td>
<td>262 ± 18</td>
<td>10.1 ± 0.7</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
</tr>
<tr>
<td>11</td>
<td>0.05% limonin (4)</td>
<td>264 ± 20</td>
<td>10.8 ± 0.3</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
</tr>
<tr>
<td>12</td>
<td>No treatment (4)</td>
<td>286 ± 13</td>
<td>12.1 ± 0.9</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
<td>0 ± 0 (n/a)</td>
</tr>
</tbody>
</table>

*Mean ± SD.

Significantly different from group 1: ±P < 0.005; ²P < 0.02; ³P < 0.001; ⁴P < 0.002; ⁵P < 0.05.
in groups 1–5 had renal mesenchymal tumors and/or altered hepatocellular foci, but these lesions were not evident in any other group. Animals of groups 6–8 did not have neoplasms in any organs examined, including the intestines. The incidence and multiplicity of large bowel neoplasms are shown in Table IV. AOM administration was associated with an increased incidence of large intestinal adenocarcinomas (72%, 18 of 25 rats) and with a multiplicity of 0.76 ± 0.51. The incidences and multiplicities of groups 2–5 were significantly lower than those of group 1 (P = 0.004, 0.00003 or 0.0002).

In the pilot study, dietary feeding of obacunone and limonin for 4 weeks either during or after AOM exposure significantly decreased development of ACF, suggesting that the two chemicals tested could inhibit the growth of colonic ACF and suppress the progression of preneoplasia to malignancy. Subsequent long-term experiments confirmed the results of the pilot study and indicated that the suppressing effects of both compounds fed to rats during either the initiation or post-initiation phase were significant. It should be noted, however, that since commercial orange juice contains 320 p.p.m. limonoid glucosides (13), the concentrations necessary to achieve the effects observed in our study would be 12- to 30-fold higher than those obtained from normal dietary ingestion of these limonoids. Our data suggest that obacunone and limonin are possible new dietary preventive agents against colon cancer development. Further investigations of the effects of dietary obacunone and limonin on carcinogenesis are therefore warranted.

The effects of dietary obacunone and limonin on AOM-induced colonic ACF indicate that this biological marker of colon carcinogenesis may be useful for screening agents (19,29) for chemoprevention of colon tumorigenesis. This lesion has been suggested to be the premalignant lesion of chemically induced colon cancer (22). However, it would probably be prudent to use tumor incidence as the end point for definitive investigations as there are many sites at which chemopreventive compounds may affect tumorigenesis (23). Both compounds tested in this study may have blocking and suppressing effects on AOM-induced colon tumorigenesis when fed in the diet during the initiation and post-initiation phases.

Several dietary factors are known to modulate carcinogenesis in humans (30) and rodents (31). The results of the present study confirmed previous epidemiological data suggesting that consumption of vegetables and/or fruits is inversely related to cancer risk, including colon cancer (6,7,32). In the pilot study, we observed no differential dose-response effect on ACF formation between 200 and 500 p.p.m. of the two limonoids. The reason for this is unknown, but it is likely that the dose of 200 p.p.m. is already above the threshold level for the effect. It would be interesting to investigate the effects of lower doses in future experiments.

One possible mechanism for the suppression of colonic tumor development might be through the control of cell proliferation in ACF and/or normal appearing’ crypts of rats exposed to AOM. Increased cell proliferation is suggested to play an important role in multistage carcinogenesis (33,34), including colon tumorigenesis (35). Zheng et al. (36) reported a greater correlation between ACF and
reduction in proliferating cell nuclear antigen labeling index in ACF than between ACF and reduction in size of the proliferative component of ACF in rats. Overexpression of cyclin D1 has been reported in ACF and adenoacarcinomas in the mouse colon (37,38). Overexpression of cyclin D1 plays an important role and is an early event in colon tumorigenesis. Therefore, we suspect that dietary administration of obacunone and limonin post-AOM injections might lower cell proliferation activity in ACF and/or colonic tumors.

In conclusion, the results of our study clearly demonstrate the inhibitory effects of dietary obacunone and limonin on AOM-induced colon tumorigenesis. Further experiments, including pre-clinical efficacy and mechanistic studies, are warranted to fully evaluate these natural compounds for their cancer preventive properties and to understand their mode of action. Additional toxicity studies, such as genotoxicity, reproduction toxicity, acute oral toxicity and 2 year carcinogenicity trials should also be conducted prior to their use as chemopreventive drugs. One advantage of these compounds as chemopreventive agents in human trials is that, unlike synthetic chemopreventive agents, they are naturally occurring compounds that are produced endogenously in edible plants and are present in human foods (15).

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References


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