

Inhibition of intestinal carcinogenesis by a new flavone derivative, chafuroside, in oolong tea

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A new flavone derivative, chafuroside, has been isolated as a strong anti-inflammatory compound from oolong tea leaves, and its structure determined to be (2*R*,3*S*,4*S*,4*aS*,11*bS*)-3,4,11-trihydroxy-2-(hydroxymethyl)-8-(4-hydroxyphenyl)-3,4,4*a*,11*b*-tetrahydro-2*H*,10*H*-pyrano[2',3':4,5]furo[3,2-*g*]chromen-10-one. To assess its potential to inhibit intestinal carcinogenesis, 2.5, 5 and 10 p.p.m. chafuroside was given in the diet to *Apc*-deficient Min mice for 14 weeks from 6 weeks of age. Total numbers of polyps were reduced to 83, 73 and 56% of the control value, respectively. Moreover, dietary administration at 10 and 20 p.p.m. reduced azoxymethane (AOM)-induced colon aberrant crypt foci (ACF) development in rats to 69% of the AOM-treated control value with the higher dose. Chafuroside-associated toxicity was not observed at 2.5–10 p.p.m. in Min mice and 10–20 p.p.m. in AOM-treated rats. These results suggest that chafuroside might be a good chemopreventive agent for colon cancer. (*Cancer Sci* 2006; 97: 248–251)

Colon cancer is one of the most common cancers in developed countries⁽¹⁾ and epidemiological studies have shown that a Western-style diet, high in fat and red meat as well as low in fruits and vegetables, increases the risk.^(2,3) Thus, foodstuff is a major focus for research, particularly with regard to identification of effective chemopreventive agents.

Epidemiological evidence suggests that drinking green tea (*Camellia sinensis*) is beneficial for cancer prevention.^(4–6) Many animal studies also have shown that tea and its components have anticancer properties.^(7,8) The major characteristic constituents of green tea are catechins, including EGCG.⁽⁹⁾ In black tea, a large proportion of the catechins are converted into theaflavins and thearubigins through oxidation and polymerization. Another tea, oolong tea, is widely consumed in Asia, especially in China and Japan. The difference among green, black and oolong teas lies in fermentation: green tea is unfermented, black tea is completely fermented, and oolong tea is partially fermented.⁽⁷⁾

Recently, a strong anti-inflammatory compound named chafuroside, (2*R*,3*S*,4*S*,4*aS*,11*bS*)-3,4,11-trihydroxy-2-(hydroxymethyl)-8-(4-hydroxyphenyl)-3,4,4*a*,11*b*-tetrahydro-2*H*,10*H*-pyrano[2',3':4,5]furo[3,2-*g*]chromen-10-one, was isolated from oolong tea leaves with the aid of an inhibition test with DNFB-induced contact hypersensitivity in mice, and its total synthesis reported.^(10,11) The compound was presumed to be produced during the partial fermentation process and

showed strong anti-inflammatory activity in DNFB and 2,4,6-trinitro-1-chlorobenzene-induced contact hypersensitivity models.^(10,11) Moreover, a preliminary study demonstrated the effective dose in the DNFB-induced contact hypersensitivity model to be approximately equal to that of indomethacin, a NSAID, which has been proven to have a cancer-chemopreventive influence.⁽¹²⁾

Although several reports have documented antioxidant, antiallergic and antiobesity activities of oolong tea extracts,^(13–15) effects of individual constituents on colon carcinogenesis have hitherto not been described. In the present study, we therefore investigated the impact of chafuroside on intestinal polyp formation in *Apc*-deficient Min mice, an animal model of human familial adenomatous polyposis that develops numerous polyps in the intestinal tract.⁽¹⁶⁾ We also investigated the impact of chafuroside on the formation of AOM-induced aberrant crypt foci, which are putative preneoplastic lesions in the F344 rat colon. In both cases chafuroside reduced the number of lesions, pointing to possible application as a chemopreventive agent for intestinal cancer.

Materials and Methods

Animals and chemicals

Female C57BL/6J-*Apc*^{Min/+} mice (Min mice) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 5 weeks of age and genotyped using a method reported previously.⁽¹⁶⁾ Heterozygotes, as well as wild-type (C57BL/6J) mice, were acclimated to laboratory conditions for 1 week, along with male F344 rats obtained from Charles River Japan (Atsugi, Japan) at 5 weeks of age. Three to five animals were housed per plastic cage, with sterilized softwood chips as bedding, in a barrier-sustained animal room, air-conditioned at 24 ± 2°C and 55% humidity, on a 12:12 h light:dark cycle. Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality. Bodyweights and food consumption were measured weekly. The experiments were conducted according to the 'Guidelines for Animal Experiments in the National Cancer Center' of the Committee for Ethics of Animal Experimentation of the National Cancer Center.

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Abbreviations: AC, aberrant crypt; ACF, aberrant crypt foci; AOM, azoxymethane; COX, cyclooxygenase; DNFB, 2,4-dinitrofluorobenzene; EGCG, (-)-epigallocatechin-3-gallate; NSAID, non-steroidal anti-inflammatory drug.

AOM was purchased from Sigma Chemical Co. (St Louis, MO, USA). Chafuroside was synthesized chemically at the University of Shizuoka (Shizuoka, Japan).⁽¹¹⁾ Its chemical structure is shown in Fig. 1. The purity of the compound was examined by ¹H nuclear magnetic resonance and high-performance liquid chromatography, and showed no concomitant peaks. The compound was pure enough, estimated to be above 99% (melting point of the compound was 229–232°C). Chafuroside concentrations of 2.5, 5, 10 and 20 p.p.m. were mixed into the powdered basal diet AIN-76 A (CLEA Japan, Tokyo, Japan) and confirmed to be stable in the diet under the experimental conditions used in the present study. The doses of chafuroside were selected according to our preliminary study in which chafuroside suppressed intestinal polyp formation in *Apc* gene-deficient mice.

Intestinal polyp formation in Min mice

Female Min mice ($n = 9$ –10/group) were fed diets containing 0 (control), 2.5, 5 or 10 p.p.m. chafuroside for 14 weeks from 6 weeks of age. All animals were anesthetized with ether before they were killed. The liver, kidneys and spleen were removed and weighed and the intestinal tract was resected, filled with 10% buffered formalin, and divided into four sections: three segments of small intestine: (1) proximal (4 cm in length from the pylorus ring of the stomach); (2) middle and (3) distal halves of the remainder; and (4) the colon. These segments were opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. Polyp numbers and sizes, and their distributions in the intestine, were determined under a stereoscopic microscope.⁽¹⁷⁾

AOM-induced ACF development in rats

Male F344 rats, 6 weeks of age, were treated subcutaneously with either AOM in sterile saline at a dose of 15 mg/kg

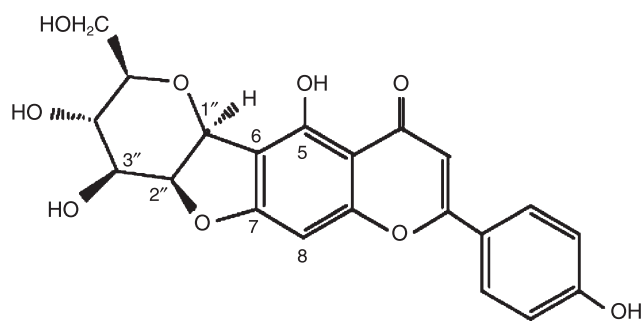


Fig. 1. Structure of chafuroside.

bodyweight or with the saline vehicle, once a week for 2 weeks from 6 weeks of age. From 1 day before the first treatment with AOM, rats were fed control or experimental diets containing chafuroside at 10 or 20 p.p.m. for 4 weeks. At 10 weeks of age, they were killed under ether euthanasia and complete necropsies were carried out. The liver, kidneys and spleen were removed and weighed. The entire colon was resected, filled with 10% buffered formalin, opened longitudinally, and fixed flat between sheets of filter paper in 10% buffered formalin. The colon was then stained with 0.2% methylene blue in saline, and scored under a light microscope for the number of ACF per colon and the mean number of AC per focus.⁽¹⁸⁾

Statistical analysis

The results were expressed as mean \pm SD, and statistical analysis was carried out using Dunnett's multiple comparison test. In addition, the linear regression test was also used. Differences were considered to be statistically significant with P -values less than 0.05.

Results

In Min mice, most polyps were located in the small intestine, with a preponderance in the distal parts, and only a few polyps were observed in the colons (Table 1). Treatment with chafuroside at 2.5, 5 and 10 p.p.m. for 14 weeks clearly decreased the total numbers of polyps to 83, 73 and 56% ($P < 0.01$) of the untreated control value, respectively (Table 1). The numbers of polyps in the proximal, middle and distal parts of small intestine in the 10 p.p.m. group were 54, 78 and 46% of the untreated control values, respectively (Table 1). Dose-dependent inhibition was observed in the number of polyps in the proximal ($r = -0.9958$, $P < 0.0005$) and distal parts ($r = -0.9129$, $P < 0.01$) of the small intestine, and in the total number of polyps ($r = -0.9863$, $P < 0.02$). As shown in Fig. 2, administration of chafuroside reduced the number of polyps mainly less than 1.0 mm in diameter. However, the number of polyps measuring ≥ 1.0 mm in diameter was not affected by chafuroside treatment. Survival rate, general conditions, food consumption and organ weights did not differ among the groups. No significant macroscopic changes were noted in the liver, kidney or spleen. Final body weights in the groups treated with 2.5, 5 and 10 p.p.m. were 103, 105 and 125% of the untreated control value, respectively.

In AOM-treated rats, administration of chafuroside at 10 and 20 p.p.m. in the diet for 4 weeks again did not affect

Table 1. Suppression of intestinal polyp development in Min mice by chafuroside, shown by the number of polyps per mouse

Group (p.p.m)	No. mice	Small intestine			Colon	Total
		Proximal	Middle	Distal		
0	9	17.7 \pm 9.8	39.7 \pm 11.6	86.8 \pm 27.1	0.78 \pm 0.67	144.9 \pm 37.7
2.5	7	15.3 \pm 7.0 (86)	34.4 \pm 7.7 (87)	70.0 \pm 9.9 (81)	0.86 \pm 0.69 (110)	120.6 \pm 21.1 (83)
5.0	9	13.0 \pm 4.3 (73)	33.4 \pm 8.6 (84)	59.2 \pm 18.9 (68)*	0.56 \pm 0.53 (72)	106.2 \pm 26.5 (73)
10.0	8	9.6 \pm 3.1 (54)*	31.0 \pm 19.9 (78)	40.0 \pm 21.8 (46)**	0.75 \pm 0.71 (96)	81.4 \pm 41.9 (56)**

Data are mean \pm SD. Numbers in parentheses are percentages of the control basal diet values. *Significantly different from the basal diet group at $P < 0.05$. **Significantly different from the basal diet group at $P < 0.01$.

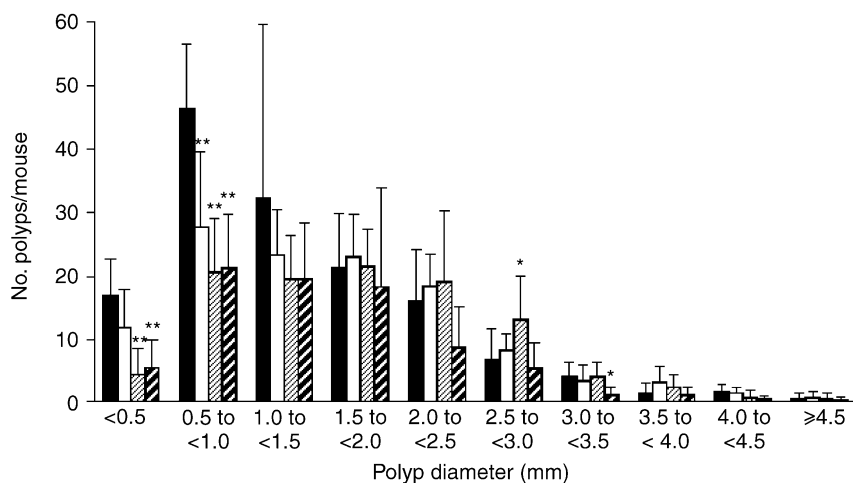


Fig. 2. Effects of chafuroside on the size distribution of intestinal polyps in Min mice. Min mice were fed a basal diet (■) or a diet containing 2.5 (□), 5 (▨) or 10 p.p.m. (▩) chafuroside for 14 weeks. The number of polyps/mouse in each size class is given as a mean value (bars represent SD). * $P < 0.05$, ** $P < 0.01$.

general conditions, body weights, food consumption or organ weights. No significant macroscopic changes were observed in the liver, kidney or spleen. ACF were observed in all rats treated with AOM, mainly located in the distal colon. Administration of 10 and 20 p.p.m. chafuroside reduced the total numbers of ACF per colon to 79 ($P < 0.05$) and 69% ($P < 0.01$) of the AOM-treated control value, respectively (Table 2). The total number of AC per colon was also decreased by 16 and 30%, respectively (Table 2). However, treatment with chafuroside did not decrease the mean number of AC per focus (Table 2).

Discussion

In the present study, we obtained clear evidence that a new flavone derivative, chafuroside, suppresses development of intestinal polyps in Min mice and AOM-induced colon ACF in F344 rats at doses of 5–20 p.p.m. in the diet. Although it is a natural compound found in tea leaves, the doses effective in Min mice were much lower than those reported earlier for well-known, naturally occurring and synthesized chemopreventive agents. Indeed, the effective dose to reduce numbers of polyps in Min mice was 10 p.p.m. for chafuroside. This value is lower than with (+)-catechin at 1000 p.p.m.,⁽¹⁹⁾ genistein at 1000 p.p.m.,⁽²⁰⁾ curcumin at 2000 p.p.m.,⁽²¹⁾ and with synthesized aspirin at 250 p.p.m.,⁽²²⁾

piroxicam at 200 p.p.m.⁽²³⁾ and celecoxib at 1500 p.p.m.⁽²⁴⁾ As treatment with 2.5–10 p.p.m. chafuroside affected only the polyps of smaller size, it might be important to clarify the mechanism by which chafuroside inhibits polyp growth. It has been reported that 100 p.p.m. EGCG is effective for approximately 60% inhibition of AOM-induced ACF formation in F344 rats,⁽²⁵⁾ and our results thus suggest that chafuroside possesses a strong potential to inhibit development of putative preneoplastic lesions in the colon.

Because there were no signs of chafuroside-induced adverse effects in the present study, long-term consumption for cancer prevention in humans is conceivable. The daily estimated consumption level from a diet containing 10 p.p.m. chafuroside in mice corresponds to approximately 120 mg per day for a 60-kg adult man. The average concentration of chafuroside in a commercially available oolong tea in Japan is almost 55 $\mu\text{g/L}$ (unpublished data). An adult human might reach an effective dose of chafuroside with consumption of more than 100 L of oolong tea. Therefore, taking a chafuroside supplement or drinking concentrated oolong tea may be useful for cancer prevention.

The strong anti-inflammatory and chemopreventive effects are presumably related to the two characteristic moieties of chafuroside: the mannose moiety of which the C1 position provides a C-glycoside linkage with the C6 position of apigenin, and the dihydrofuran moiety, obtained

Table 2. Effects of chafuroside on azoxymethane (AOM)-induced aberrant crypt focus (ACF) formation in F344 rats

Group	No. rats with ACF	Total no. ACF/colon (%)	Total no. AC/colon (%)	Mean no. AC/focus
AOM treatment				
Control diet	9/9	278 \pm 51	618 \pm 81	2.25 \pm 0.18
Chafuroside (10 p.p.m.)	9/9	219 \pm 42 (79)*	522 \pm 105 (84)	2.40 \pm 0.27
Chafuroside (20 p.p.m.)	9/9	192 \pm 42 (69)**	435 \pm 69 (70)**	2.29 \pm 0.30
Saline treatment				
Control diet	0/3	0	0	0
Chafuroside (10 p.p.m.)	0/3	0	0	0
Chafuroside (20 p.p.m.)	0/3	0	0	0

Data are mean \pm SD. Numbers in parentheses are percentages of the control basal diet values. *Significantly different from the basal diet group at $P < 0.05$. **Significantly different from the basal diet group at $P < 0.01$. AC, aberrant crypt.

by ring closure via dehydration between hydroxyl groups at the C7 position of apigenin and at the C2 position of mannose.

According to the mechanism of DNFB-induced contact hypersensitivity, several factors moderated by chafuroside treatment could be assumed. DNFB-induced skin immune responses are divided into a distinct sensitization phase and an early and late elicitation phase. In the sensitization phase, prostaglandin E₂-EP₄ receptor (PGE₂-EP₄) signaling pathways are reported to be involved in antigen uptake into Langerhans cells, and in antigen migration to draining lymph nodes.⁽²⁶⁾ In addition, other colon tumor-related pathways and mediators, such as ERK1/2 and p38 MAPK signaling pathways, 5-lipoxygenase and inducible nitric oxide synthase, are also reported to be involved in DNFB-induced contact hypersensitivity.⁽²⁷⁻²⁹⁾ Thus, chafuroside may exhibit its chemopreventive effects by mediating these inflammatory factors. However,

the natural function of chafuroside is not well defined and the anti-inflammatory and chemopreventive mechanisms of this novel flavonoid need to be clarified.

From the present results, we can conclude strong inhibitory effects of chafuroside on intestinal polyp development and ACF formation in *Apc*-deficient mice and AOM-treated rats, respectively, so that chafuroside may be a promising candidate chemopreventive agent for colon cancer.

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