

## A Ferulic Acid Derivative, Ethyl 3-(4'-Geranyloxy-3-methoxyphenyl)-2-propenoate, as a New Candidate Chemopreventive Agent for Colon Carcinogenesis in the Rat

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The inhibitory influence of ferulic acid (FA), a rice germ component, and its geranylated derivative 3-(4'-geranyloxy-3-methoxyphenyl)-2-propenoate (EGMP) on the post-initiation stage of azoxymethane (AOM)-induced colon carcinogenesis was studied in male F344 rats given two s.c. injections of AOM (15 mg/kg body weight) during week 1. Diets containing EGMP or FA at doses of 0.1 or 0.2% were then fed for 3 weeks from week 2 to 5, when the animals were sacrificed. The numbers of aberrant crypt foci (ACF) and aberrant crypts (AC) per rat in the group given 0.2% FA were significantly decreased ( $P < 0.001$ ) as compared to the AOM alone group. Furthermore, the numbers of ACF and AC per rat fed the 0.2% and 0.1% EGMP were significantly reduced ( $P < 0.001$  and  $P < 0.01$ , respectively). Colonic epithelial cells in S-phase, as measured by bromodeoxyuridine (BrdU) labeling, in rats fed EGMP were significantly decreased in the 0.2 and 0.1% EGMP groups as compared to the AOM alone group ( $P < 0.05$ ). BrdU labeling indices in rats fed FA and EGMP assessed by a test using a coefficient for linear contrast were also significantly decreased as compared to the AOM alone value ( $P < 0.05$ ,  $P < 0.01$ , respectively). The results indicate that FA and EGMP have inhibitory effects on ACF and AC development, EGMP being more potent, possibly due to stronger suppressive effects on cell proliferation. No toxic effects were observed in rats given either compound in terms of body and organ weights, and liver or kidney histology. The findings thus suggest that EGMP and FA, especially the former, might have potential as chemopreventive agents against colon tumor development.

Key words: EGMP — Ferulic acid — ACF — Colon carcinogenesis

Colorectal cancer is well known to be associated with dietary factors. A large number of epidemiological studies and animal experiments have indicated that vegetables and fruits and their constituents may act to prevent colorectal cancer development.<sup>1–5)</sup>

Ferulic acid (FA), widely found in bran from rice, wheat and barley, vegetables, and several other species of plants,<sup>6–8)</sup> is a naturally occurring phenolic compound that has been reported to inhibit benzo[*a*]pyrene-induced forestomach tumor induction in mice.<sup>9)</sup> FA has also been shown to be active against mouse lung carcinogenesis.<sup>10)</sup> Furthermore, it is reported to inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-promoted mouse skin<sup>11)</sup> and 4-nitroquinoline-1-oxide-induced rat tongue tumor development.<sup>12)</sup> While FA was found to enhance development of preneoplastic glutathione *S*-transferase placental form-positive liver cell foci induced by heterocyclic amine,<sup>13)</sup> it was recently reported to reduce numbers of aberrant

crypt foci (ACF) and the incidence of adenocarcinomas in the colon.<sup>14)</sup>

Ethyl 3-(4'-geranyloxy-3-methoxyphenyl)-2-propenoate (EGMP), possessing a geranyl chain, might on theoretical grounds possess more potent antioxidant activity than FA. Auraptene, a geranyl ether derivative of 7-hydroxycoumarin, found in orange (*Unshu mikan*) peel, has been shown to be more effective at inhibition of mouse skin tumor development than the parent compound, umbelliferone.<sup>15)</sup> More importantly, it is reported to remarkably inhibit azoxymethane (AOM)-induced ACF and tumor development.<sup>16, 17)</sup>

Although the mechanisms have yet to be elucidated, the geranyl moiety might play some role in increasing affinity for cell membranes and therefore improving intestinal absorbance. Unfortunately, auraptene is present in only small amounts in *Unshu mikan* peel and its extraction is costly. Therefore, we synthesized EGMP by adding a geranyl chain to parent FA, as in auraptene.

In our previous study to determine an appropriate protocol for screening, EGMP inhibited ACF development most

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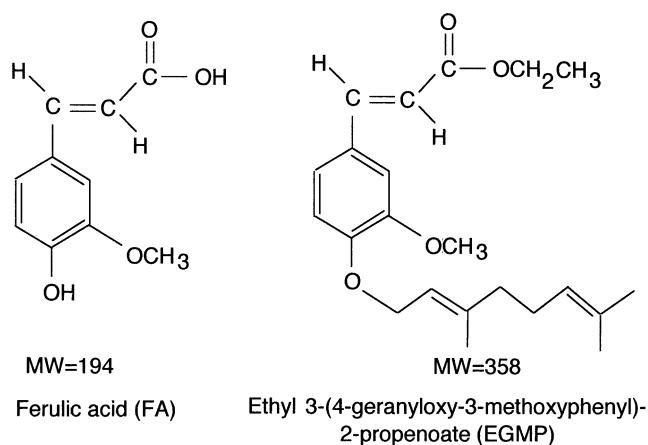


Fig. 1. Chemical structures of FA and EGMP featuring a geranyl chain and an ethyl radical added to the hydroxy group of FA.

clearly when given during the post-initiation stage.<sup>18)</sup> Therefore, the purpose of the present study was to evaluate the effects of FA and EGMP using ACF and a cell proliferation marker (bromodeoxyuridine (BrdU) labeling) in the post-initiation stage.

## MATERIALS AND METHODS

**Chemicals** The carcinogen AOM (CAS: 25843-45-2) was obtained from Sigma (St. Louis, MO). Chemical structures of FA and EGMP are shown in Fig. 1. EGMP was synthesized from FA. Briefly, ethyl ferulate was reacted with geranyl bromide and sodium hydride in tetrahydrofuran to give EGMP as a solid in 96% yield (m.p. 30°C).<sup>19)</sup>

**Animals** A total of 125 male F344 rats (Charles River Japan Co., Ltd., Atsugi) at 5 weeks of age were maintained in plastic cages in an air-conditioned room at 22±2°C and 55±10% humidity. The animals were allowed free access to basal diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) and tap water.

**Animal treatments** The experimental design is illustrated in Fig. 2. Group 1 received subcutaneous injections of 15 mg/kg AOM twice during the first week, then were placed on powdered basal diet containing 0.2 or 0.1% FA or EGMP for 3 weeks from weeks 2 to 5 (20 rats each). Group 2 was given AOM alone (20 rats). Group 3 received 0.2% or 0.1% FA or EGMP without prior carcinogen treatment (5 rats each). Group 4 was untreated (5 rats). All animals were sacrificed under ether anesthesia at the end of week 5 for quantitative analysis of ACF and aberrant crypt (AC). Body, liver, and kidney weights were also recorded. The livers and kidneys were fixed in formalin and routinely processed for histological examination.

**ACF analysis** For quantitation of ACF, the colons were

removed, inflated with 10% buffered formalin, cut longitudinally from cecum to anus, spread on filter papers, fixed in 10% buffered formalin and then stained with 0.2% methylene blue dissolved in saline for 2 min according to the procedures described by Bird.<sup>20)</sup> The numbers of ACF and AC per colon were counted under a microscope at the magnification of ×40. The criteria used to identify ACF topographically included (a) increase in size, (b) protrusion from the epithelium and (c) an increased pericryptal zone relative to normal crypts. Since it has been indicated that large ACF (composed of 4 or more crypts) are more closely related to the occurrence of colon tumors,<sup>21)</sup> the lesions were divided into two size-based categories, 1–3 crypts/focus and 4 or more crypts/focus.

**Immunohistochemical staining of BrdU** For the measurement of BrdU (Sigma-Aldrich Co., Ltd., Tokyo) incorporation into nuclei, the animals (5 rats in each group) were given 100 mg/kg body weight intraperitoneally 1 h prior to sacrifice. Then, segments of the proximal, middle, and distal colons, 2 from each region, were excised and processed for histological sections. Immunostaining was accomplished using an antibody to BrdU (Becton Dickinson, San Jose, CA) by the avidin biotin complex (ABC) method. For determination of labeling indices, BrdU-positive cells in 15–20 full-length crypts were counted in 5 view areas for each section with the aid of a microscope eye-piece grid without reference to the treatment group. Labeling indices were calculated as numbers of labeled cells per 100 cells counted.

**Statistics** The data for body and organ weights, food consumption, numbers of ACF, and BrdU labeling indices were analyzed using the JMP software package (Version 3.1, SAS Institute Japan) on a Macintosh computer. A trend test for BrdU labeling indices was performed using the linear contrast coefficient method.

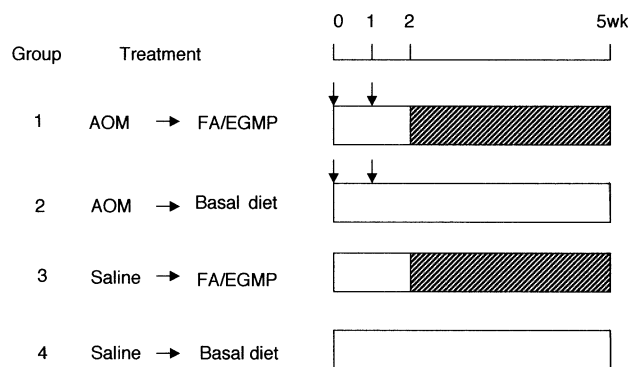


Fig. 2. Experimental protocol. ↓, AOM 15 mg/kg body weight s.c. injection; ▨, FA or EGMP (0.2% or 0.1%) in basal diet; □, basal diet.

**RESULTS**

No significant inter-group differences in body, liver or kidney weights, or food consumption were noted (Table I). In addition, on gross and histological observation of the liver and kidney, no toxic effects were evident in group 3 rats fed FA or EGMP alone.

The data for ACF and AC counts are summarized in Table II. The numbers of total ACF in group 1 given 0.2% FA and EGMP, and 0.1% EGMP were significantly decreased as compared to group 2 values ( $P<0.001$  or

$P<0.01$ , respectively). Similarly, values for ACF larger than 4 crypts in group 1 given both doses of EGMP and 0.2% FA ( $P<0.001$ ,  $P<0.05$ , respectively) were significantly decreased as compared to group 2. Values for ACF of 1–3 crypts in group 1 given 0.2% FA and EGMP or 0.1% EGMP were significantly decreased as compared to group 2 ( $P<0.001$  or  $P<0.05$ , respectively).

The data for cell proliferation analysis are summarized in Table III. BrdU labeling indices in rats fed 0.2% and 0.1% EGMP were significantly decreased as compared to the AOM alone value ( $P<0.05$ ). BrdU labeling indices in

Table I. Body Weight, Food Consumption and Relative Organ Weights

Group	Treatment (%)	No. of rats	Body weight	Food consumption (g/rat/day)	Relative organ weight (%)		
					Liver	Kidney <sup>a)</sup>	
1	AOM →	FA (0.2)	20	239±10 <sup>b)</sup>	15.1±0.4	3.57±0.14	0.74±0.02
		FA (0.1)	20	242±9	14.2±0.7	3.57±0.14	0.74±0.02
		EGMP (0.2)	20	240±8	14.8±0.5	3.64±0.14	0.73±0.04
		EGMP (0.1)	20	239±10	14.4±0.6	3.68±0.14	0.74±0.03
2	AOM →	Basal diet	20	239±10	15.0±0.3	3.57±0.17	0.74±0.03
3	Saline →	FA (0.2)	5	233±8	14.7±0.2	3.49±0.12	0.75±0.03
		FA (0.1)	5	239±8	14.6±0.2	3.67±0.21	0.75±0.06
		EGMP (0.2)	5	240±8	15.1±0.3	3.98±0.26	0.75±0.02
		EGMP (0.1)	5	239±9	14.3±0.2	3.86±0.16	0.72±0.03
4	Saline →	Basal diet	5	243±8	14.6±0.4	3.72±0.14	0.71±0.01

a) Values are sum of left and right kidney weight.

b) Data are mean±SD value.

Table II. Effects of FA and EGMP on Induction of ACF

Group	Treatment (%)	No. of rats	No. of ACF <sup>a)</sup>		Total ACF	AC	
			1–3 crypts	4–9 crypts			
1	AOM →	FA (0.2)	20	125±51 <sup>***</sup> (65.4)	19±6 <sup>*</sup> (62.1)	143±53 <sup>***</sup> (64.9)	330±108 <sup>***</sup> (67.7)
		FA (0.1)	20	167±53 (74.6)	24±12 (81.9)	191±55 (86.7)	431±166 (88.4)
		EGMP (0.2)	20	115±35 <sup>***</sup> (60.6)	14±1 <sup>***</sup> (46.7)	129±42 <sup>***</sup> (58.5)	280±11 <sup>***</sup> (57.3)
		EGMP (0.1)	20	142±37 <sup>*</sup> (74.6)	15±7 <sup>***</sup> (50.7)	157±42 <sup>**</sup> (71.3)	333±87 <sup>***</sup> (68.2)
2	AOM →	Basal diet	20	190±51 (100)	30±11 (100)	220±58 (100)	488±115 (100)
3	Saline →	FA (0.2)	5	0	0	0	0
		FA (0.1)	5	0	0	0	0
		EGMP (0.2)	5	0	0	0	0
		EGMP (0.1)	5	0	0	0	0
4	Saline →	Basal diet	5	0	0	0	0

a) Data are mean±SD value.

Data in parentheses represent percentages of group 2 values.

\*, \*\*, \*\*\*,  $P<0.05$ ,  $P<0.01$ ,  $P<0.001$ , respectively, as compared to group 2.

Table III. Effects of FA and EGMP on BrdU Labeling Indices

Group	Treatment (%)	No. of rats	BrdU indices (%)
1	AOM → FA (0.2)	5	6.88±2.20 (68.6) <sup>a)</sup>
	FA (0.1)	5	6.90±1.98 (68.8) <sup>a)</sup>
	EGMP (0.2)	5	5.56±1.40 (55.4) <sup>*,b)</sup>
	EGMP (0.1)	5	5.96±1.88 (59.4) <sup>*,b)</sup>
2	AOM → Basal diet	5	10.03±3.28 (100)
3	Saline → FA (0.2)	5	2.25±0.31 (22.4)
	FA (0.1)	5	2.28±0.11 (22.7)
	EGMP (0.2)	5	2.20±0.14 (21.9)
	EGMP (0.1)	5	2.48±0.21 (24.7)
4	Saline → Basal diet	5	2.40±0.41 (23.9)

Data are mean±SD value.

Data in parentheses represent percentages of group 2 value.

\*, Significantly different from the value of group 2 at  $P<0.05$ .

a, b) Significantly different from the value of group 2 assessed by a test using coefficient for linear contrast at  $P<0.05$ ,  $P<0.01$ , respectively.

rats fed FA and EGMP assessed using the coefficient for linear contrast were significantly decreased as compared to the AOM alone value ( $P<0.05$ ,  $P<0.01$ , respectively).

## DISCUSSION

The results of the present study clearly demonstrated that dietary feeding of FA and EGMP during the post-initiation stage can significantly reduce the development of preneoplastic lesions of the colon induced by AOM, EGMP having the more potent action. It should be noted that FA and EGMP in the diet did not exert any toxic effects, in terms of body and organ weights and histological parameters.

It has been reported that development of ACF correlates well with tumor development in experimental colon carcinogenesis in the rat.<sup>22-25</sup> Especially, ACF composed of multiple aberrant crypts were shown to have a high potential for developing into colon carcinomas.<sup>26</sup> Thus, our results for ACF with 4 or more crypts suggest that FA and EGMP, and especially the latter, are candidate chemopreventive agents for colon carcinogenesis. In the cell proliferation analysis using BrdU labeling indices, FA and EGMP showed significant inhibitory effects in a dose-dependent manner, and EGMP showed more potent effects than FA. The result indicates that the geranyl moiety of EGMP increases the inhibitory effect on cell proliferation.<sup>17</sup> This could be due to facilitated intestinal absorbance of the compound owing to an increase of the cellular uptake rate or to hydrophobic interactions with target sites.<sup>15</sup> The flavonoid, auraptene, with a geranyl chain has been reported to exert strong inhibitory effects on skin, colon and oral cavity carcinogenesis,<sup>16,17</sup> which

were explained in terms of reduction in cell proliferation, induction of phase II enzymes such as quinone reductase and glutathione *S*-transferase, and reduced levels of lipid peroxidation products. It also induces apoptosis in metastatic lung tumors.<sup>27</sup> We presume that EGMP has similar functions to auraptene. However, further studies are required to elucidate the mechanisms of action of FA and EGMP.

With regard to effects on the liver, preneoplastic liver lesions, such as glutathione *S*-transferase-positive foci, were not counted because AOM is not a potent liver carcinogen.

As shown in Table III, treatment with FA and more clearly EGMP caused suppression of cell proliferation in non-ACF/AC areas. Such an effect may partly explain the ACF data for auraptene.<sup>28</sup> Future long-term studies should be aimed at clarifying whether the observed suppression of BrdU indices in normal-appearing areas truly reflects inhibition of tumor development. Further comparative studies of different FA-related compounds are also required. Since FA can be obtained in large amounts from rice bran and the method for synthesis of EGMP is well established (unpublished data), production of sufficient quantities of EGMP for clinical studies in humans is readily achievable.

Although there have been some contradictory reports regarding the correlation between ACF and tumors, ACF numbers appear to be dependent on the dose of colorectal carcinogens.<sup>29</sup> Cyclooxygenase inhibitors, such as aspirin and non-steroidal anti-inflammatory drugs are known to impact on ACF and inhibit chemically induced colon tumor development in rodent models.<sup>30,31</sup> Furthermore, recent studies have provided support for ACF being precursors of colon cancers in humans.<sup>32-34</sup> Thus, ACF counts may be appropriate as end-point indicators of possible colon tumor development.

In conclusion, FA and more especially EGMP effectively reduced the number of ACF in the rat colon in the present study. Thus, they might find application as chemopreventive agents against colonic tumor development. We are planning a long-term study to determine effects on tumor development and to elucidate the mechanisms involved, in preparation for practical application.

## ACKNOWLEDGMENTS

This work was supported in part by Special Coordination Funds for Leading Research Utilizing Potential of Regional Science and Technology of the Science and Technology Agency of the Japanese Government and a Grant-in-Aid for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan. Beom Seok Han and Cheol Beom Park were recipients of Foreign Research Fellowships from the Program for Invitation of Foreign Researchers from the Foundation for Promotion of Cancer Research, sup-

ported by the 2nd Term Comprehensive 10-Year Strategy for Cancer Control.

(Received November 17, 2000/Revised February 1, 2001/  
Accepted February 6, 2001)

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