Studies on Cancer Chemoprevention by Traditional Folk Medicines XXV.¹⁾ Inhibitory Effect of Isoliquiritigenin on Azoxymethane-Induced Murine Colon Aberrant Crypt Focus Formation and Carcinogenesis

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Isoliquiritigenin is a natural pigment with the simple chalcone structure, 4,2',4'-trihydroxychalcone. The effect of this compound on azoxymethane (AOM)-induced colonic aberrant crypt focus and tumor formation in ddY mice was examined. Administration of 15 ppm of isoliquiritigenin in drinking water, significantly suppressed AOM-induced aberrant crypt focus formation ($p < 0.01$), with an inhibitory ratio of 37.3%. Isoliquiritigenin also inhibited AOM-induced colon carcinogenesis by administration in a mixed diet. The average number of tumors was 14.6 ± 8.9 items in the control group and were 7.3 ± 7.3 , 3.9 ± 5.6 , 4.7 ± 6.5 items in the 10, 100 and 250 ppm in the isoliquiritigenin treated groups, respectively. In histopathological studies, the tumors were identified as adenoma and adenocarcinoma, however, significant differences were not observed between the control group and isoliquiritigenin treated groups. These results indicated that isoliquiritigenin might be a potential chemopreventive agent against colon cancer.

Key words isoliquiritigenin; cancerprevention; colon cancer; aberrant crypt foci

Licorice, the roots and long-stalks of various species of *Glycyrrhiza* genus, has been used as a traditional folk medicine and foodstuff all over the world.²⁾ Isoliquiritigenin is one of the plant pigments of licorice, and is a simple chalcone derivative, 4,2',4'-trihydroxy-chalcone (see Fig. 1). We have reported the isolation of this compound from Egyptian licorice (*Glycyrrhiza grabla*), along with new and known 3-arylcoumarins.³⁾ Shibata reported the anti-tumor promoter effects of various chalcone derivatives *in vitro*, and isoliquiritigenin exhibited potential activity.⁴⁾ Other biological activities for this compound have also been reported. For example, antitumor promoting activity on two-stage mouse skin carcinogenesis,⁵⁾ inhibitory effect on aldose reductase activity,⁶⁾ antiplatelet aggregation effect,⁷⁾ antioxidative and superoxide scavenging activities,⁸⁾ *etc.* However, all these experiments were *in vitro* or topical application *in vivo*, and there is no reported data of the effect by oral administration *in vivo*.

Repetitive treatment with the organotropic colon carcinogen, azoxymethane (AOM), produces colon tumors in rodents exhibiting pathological features that are similar to sporadic forms in human colon cancer.⁹⁾ The AOM-induced colon cancer model in rat has been used to evaluate the potency of chemopreventive agents against colon cancer.¹⁰⁾ Aberrant crypt foci (ACF) were first identified in methylene blue stained, whole-mount preparations of colonic mucosa from carcinogen-treated rodents,¹¹⁾ and ACF have also been found in the colons of patients both with or without colorectal cancer.^{12,13)} McLellan *et al.* reported that ACF may be a significant biological lesion in the development of colon tumors, and aberrant crypt (AC) formation is specific for exposure to colon carcinogens.¹⁴⁾ From these findings, the colon-specific, carcinogen-induced ACF model in rodents has been used as a short term screening to identify chemopreventive agents for colon cancer.^{15,16)} In the present studies, the effect of isoliquiritigenin on the AOM-induced ACF formation

model in mice was studied. Based on the data obtained, AOM-induced murine colon carcinogenesis experiment was also studied.

MATERIALS AND METHODS

Chemicals AOM was purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.). Isoliquiritigenin (ILG) was synthesized by the methods of Hulle *et al.*¹⁷⁾ and purified by column chromatography (silica gel with a mixture of *n*-hexane and ethyl acetate). After recrystallization, the purity was quantified by HPLC (octadecyl silica (ODS) with a gradient of acetonitrile and water) as over 99.8%. The yield of this material was 38.6%.

Animals Five-weeks old ddY male mice were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka, Japan).

Effect of Isoliquiritigenin on Murine Colon Aberrant Crypt Foci Five-weeks old ddY mice were housed in temperature- and humidity-controlled animal quarters with a 12 h light/dark cycle and fed CE-2 diet. (CLEA Japan Co., Ltd.) On the 1st and 8th days, AOM (10 mg/kg b.w.) was given by subcutaneous (s.c.) injection. ILG was dissolved in the drinking water at a final concentration of 15 ppm and given *ad libitum* for all experimental days. As standard control, piroxicam was given by the same method above. Four weeks after the first AOM treatment, the animals were sacrificed and the colons were removed and fixed in 10% phos-

Fig. 1. The Structure of Isoliquiritigenin

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phate buffered formaldehyde solution. The procedures for the determination of aberrant crypt foci (ACF) were those reported by Bird *et al.*¹¹⁾ The fixed colons were stained with 0.2% methylene blue, and the aberrant crypts (ACs) and ACF were counted under a light microscope.

Effect of Isoliquiritigenin on Murine Colon Carcinogenesis Six-weeks old ddY mice were housed under the same conditions as above. The experimental protocol is shown in Fig. 2. Groups 2—7 were treated with AOM (10 mg/kg b.w.) by s.c. injection once a week for 8 weeks. Vehicle treated groups (Groups 1, 8) were given saline in the same way. Since the compound was difficult to dissolve in water at a dose of more than 15 ppm, sample was mixed into basal diet in this experiment. ILG was mixed in the basal diet at final concentrations of 10, 100 and 250 ppm, respectively, and given *ad libitum* for the experimental term (Groups 3—5, 8). Moreover, Groups 6 and 7 were administrated the 250 ppm mixed diet for only the initiation term or post initiation term. Twenty four weeks after the first AOM-treatment, all animals were sacrificed and the colons were removed and fixed in 10% phosphate buffered formaldehyde solution. After fixation, the colon was examined and the number of macroscopically obvious tumors were counted. The tumors were then measured in two dimensions with the aid of a dissecting microscope. Transverse blocks of the colon were

taken to include each tumor. These blocks were processed routinely to paraffin wax, sectioned at 4 μ m and stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

ILG was isolated from plants associated with *Leguminosae*,¹⁸⁾ *Moraceae*¹⁹⁾ and *Compositae*.²⁰⁾ In our previous study, we first isolated the compound from *Allium chinense* (Liliaceae).²¹⁾ The biological activities of this compound were also studied, *e.g.* inhibitory effect on aldose reductase,⁶⁾ anti-tumor promoting effect on two-stage skin tumor formation,⁵⁾ suppressive effect against 5-lipoxygenase,⁵⁾ antioxidative and superoxide scavenging activities,⁸⁾ *etc.* Topical application of ILG suppress the 12-*O*-tetra-decanoylphorbol-13-acetate (TPA) or bromomethylbenz [a]anthracene-induced skin tumor formation,⁵⁾ and acute inflammation in mouse ear,⁵⁾ however, oral administration of this compound showed weak activity on two-stage lung and skin carcinogenesis in our previous studies (unpublished data). These results indicated that the suppressive effect of ILG might be due to direct action against the organs.

Effect of Isoliquiritigenin on Murine Colon Aberrant Crypt Foci The effects of ILG on AOM-induced murine colon aberrant crypt focus formation were examined. No difference in the mean body weight, diet and water intake was observed in each group, receiving AOM with or without ILG. As shown in Table 1, ILG significantly inhibited ACF formation ($p < 0.01$ by student's *t*-test). The average number of ACF of control mice was 72.4 ± 29.9 items, in contrast, that of sample treated animals was 45.4 ± 16.8 items. Piroxicam, as a standard inhibitor, inhibited ACF formation the same as in our previous studies (unpublished data). In all experimental periods, no animal from any group died.

Effect of Isoliquiritigenin on Murine Colon Carcinogenesis The inhibitory effect of ILG on murine colon carcinogenesis is summarized in Table 2. As shown in Table 2, ILG suppressed colon tumor formation significantly, and tumor incidence was suppressed in a dose dependent manner. The mixed diet administrated groups showed suppressed colonic tumor formation. The average number of tumors were significantly lower in the AOM plus 10 ppm ILG group (7.3 ± 7.3 items), the AOM plus 100 ppm ILG group (3.9 ± 5.6), and the AOM plus 250 ppm ILG group (4.7 ± 6.5), compared with AOM plus basal diet group (14.6 ± 8.9). In the term-limited administration of ILG mixed diet, each group showed an inhibitory effect. These effects were almost the same, and less than the all-term administration group. The

Fig. 2. Experimental Protocol of Colonic Carcinogenesis in ddY Mice

Six weeks old ddY male mice were treated with (Groups 2—7) or without (Groups 1, 8) AOM (10 mg/kg b.w.) by s.c. injection once a week for 8 weeks. ILG were mixed in the basal diet at the final concentration of 10, 100, 250 ppm, respectively, and given *ad libitum* for the whole experimental term (Groups 3—5, 8). However, Groups 6 and 7 were administrated the 250 ppm mixed diet only for the initiation term or post initiation term.

Table 1. Inhibitory Effect of Isoliquiritigenin on AOM-Induced Aberrant Crypt Foci in Mouse Colon

Condition		No. of mouse with AC	No. of ACF/colon	No. of ACs	Mean No. of ACs/focus
Control	(+AOM)	10/10	72.4 $\pm$ 30.0 (100.0%)	108.6 $\pm$ 54.7 (100.0%)	1.47 $\pm$ 0.14 (100.0%)
Piroxicam (15 ppm in DW)	(+AOM)	10/10	43.5 $\pm$ 30.5* (60.1%)	61.8 $\pm$ 46.3 (56.9%)	1.40 $\pm$ 0.19 (95.1%)
Isoliquiritigenin (15 ppm in DW)	(+AOM)	10/10	45.4 $\pm$ 16.8* (62.7%)	72.1 $\pm$ 33.3 (66.4%)	1.54 $\pm$ 0.17 (104.9%)

Mice were treated with 10 mg/kg bw of AOM once a week for two weeks. Four weeks after the first AOM injection, all mice were sacrificed and the colons were fixed in 10% phosphate buffered formaldehyde solution. Fixed colons were stained by methylene blue and the ACF were counted under light microscopically. *: $p < 0.01$ (student's *t*-test).

Fig. 3. Location of AOM-Induced Colorectal Tumors in Mice with or without Isoliquiritigenin

1, normal (–AOM, basal diet); 2, control (+AOM, basal diet); 3–5, ILG administrated groups (+AOM, 10 or 100 or 250 ppm in basal diet); 8, vehicle+ILG (–AOM, 250 ppm in basal diet). Data are expressed as mean±S.D. values.

Table 2. Effect of Isoliquiritigenin on AOM-Induced Mouse Colon Carcinogenesis

Experimental group	Animal	Condition		No. of mice		Tumor bearing mice (%)	Ave. No. of tumors
		AOM	Diet	Start	Final		
1 Normal	ddY male	–	Basal	8	8	0/8 (0.0)	0.0±0.0 (0.0)
2 Control	ddY male	+	Basal	30	24	24/24 (100.0)	14.6±8.9 (100.0)
3 Isoliquiritigenin	10 ppm	+	10 ppm mix	15	13	12/13 (92.3)	7.3±7.3* (48.2)
4 Isoliquiritigenin	100 ppm	+	100 ppm mix	15	15	10/15** (66.7)	3.9±5.6** (25.5)
5 Isoliquiritigenin	250 ppm	+	250 ppm mix	30	27	19/27** (70.4)	4.7±6.5** (31.0)
6 Isoliquiritigenin	Pre	+	250 ppm mix →basal	15	14	12/14 (85.7)	7.1±7.0** (46.7)
7 Isoliquiritigenin	Post	+	Basal →250 ppm mix	15	14	11/14* (78.6)	6.3±6.6** (41.5)
8 Isoliquiritigenin	250 ppm	–	250 ppm mix	10	9	0/9 (0.0)	0.0±0.0 (0.0)

Twenty four weeks after the first AOM-treatment, all animals were sacrificed and the colons were removed and fixed in 10% phosphate buffered formaldehyde solution. After fixation, the colon was examined and the number of macroscopically obvious tumors were counted. Data are expressed as mean±S.D. *: $p<0.05$, **: $p<0.01$ (student's *t*-test).

location of colonic tumors is expressed in Fig. 3. As shown in this figure, about 70% of colonic tumors were observed in distal colon. No significant difference in the location of tumors was observed between groups.

The mean body weight of mice treated with AOM was significantly reduced compared with vehicle control groups. The groups fed a mixed diet did not show any difference in mean body weight, diet and water intake.

The histopathological appearance of colon and other organs were also studied. The regions of colon were identified as adenoma and adenocarcinoma. The incidence of adenocarcinoma was significantly reduced in ILG-treated group. (21/24 in control group, 11/27 in 250 ppm treated group; $p<0.05$ (χ^2 -test)). All treated animals given AOM showed hepatic anisocytosis. In hepatic observation, there was no difference between the groups treated with or without ILG.

This experiment was performed by a modification of the method of Fukutake *et al.*²²⁾ In our preliminary experiments, ddY mice were more sensitive to AOM than ICR (CD-1) mice (Charles River Japan Inc., Kanagawa, Japan), and exhibited a not so larger deviation compared to ICR mice. The

ddY strain used was closed colony mouse, which is the same as ICR (CD-1), and is used for toxicity evaluations. This model may be useful to evaluate the potency of anti-colonic-tumor effects.

The non-steroidal anti-inflammatory drugs (NSAIDs) have been studied for cancer-chemopreventive effects against colonic tumor epidemiologically.²³⁾ NSAIDs exhibit their pharmacological effect by inhibiting cyclooxygenase (COX) which performs the critical initial reaction in the arachidonic metabolic cascade leading to the formation of prostaglandins. Isoliquiritigenin does not inhibit COX activity, however, it does suppress 5-lipoxygenase (LOX) activity.^{24,25)} These facts suggest that both COX and LOX may be involved in colorectal tumorigenesis.

The concept of cancer-chemoprevention requires that agents non-toxic synthetic chemicals or naturally occurring substances in foodstuffs with an easy of intake. ILG could fulfill these requirements. Further studies are required to understand the mechanisms of anti-carcinogenic activity of ILG.

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