Effects of synthetic and natural *in vivo* inhibitors of ß-glucuronidase on azoxymethane-induced colon carcinogenesis in rats

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Abstract. D-Glucaric acid is a non-toxic natural compound found in many fruits and vegetables. Our previous studies have shown that the ß-glucuronidase inhibitor D-glucaro-1,4-lactone, an active metabolite of D-glucaric acid, inhibits chemically-induced tumorigenesis in rodents. D-Glucaro-1,4-lactone has a synthetic precursor, 2,5-di-*O*-acetyl-D-glucaro-1,4:6,3-dilactone or aceglatone (ACE), known as a postoperative prophylactic agent, and a natural precursor, D-glucurono-γ-lactone (GL). In the present study, we first examined the effect of ACE on the initiation phase of rat colon carcinogenesis induced by 15 mg/kg azoxymethane (AOM) administered 3 times by subcutaneous (s.c.) injection at weeks 1, 2, and 3 of a 5-week short-term experiment. ACE (0.5 or 2%) was administered as a dietary supplement for 5 weeks. At 5 weeks after the initiation of treatment, the formation of aberrant crypt foci (ACF) in the rat groups treated with AOM plus a 0.5 or 2% ACE diet was significantly reduced by 48.6 and 55.3%, respectively, compared to the group administered AOM alone. In a previous study, 0.5 and 2% ACE diets dispensed during AOM treatment had a tendency to decrease AOM-induced colonic tumor incidence. In the present long-term 36-week colon tumorigenesis experiment, GL (0.5 or 2%) administered via the diet during the initiation phase (starting 1 week before the first dose of AOM and ending 1 week after the 3rd dose) did not have any significant effects on tumor incidence. On the other hand, continued post-initiation treatment with ACE (0.5 and 2%) markedly reduced colonic tumor incidence by 70 and 80%, respectively. GL was effective to a similar extent (70% inhibition), but only at a concentration of 2%. We conclude that ACE inhibits the initiation and post-initiation stages of AOM-induced colon carcinogenesis, while GL affects only the post-initiation stages.

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Introduction

Colorectal cancer is one of the most common forms of cancer, occurring worldwide. In Japan, colon cancer incidence has shown a marked increase (1). Therefore, research aimed at the prevention of colon cancer, in particular by the identification of cancer chemopreventive agents, is urgently needed (2,3). Our laboratory has examined natural and synthetic compounds, and has provided evidence that newly-extracted and/or previously known products have the potential to prevent the development of chemically-induced tumors in animal models (4-8). It is known that the body detoxifies xenobiotics, i.e., lipid-soluble toxins including certain carcinogenic compounds, by conjugation with glutathione, by sulfation and by glucuronidation, a principal conjugation pathway in all tissues (9). Carcinogenic compounds are conjugated with glucuronic acid in the liver or kidney, then excreted as glucuronides in bile or urine (9). As a result of this process, many carcinogens and tumor promoters are eliminated from the body. However, following the excretion of bile into the gastrointestinal tract, ß-glucuronidase, a bacterial enzyme which resides in the gut, may hydrolyze the glucuronic acid conjugate and liberate toxins such as active carcinogens and tumor promoters, reabsorbing them back into the body instead of allowing them to be excreted. The final rate of elimination of toxic chemicals is therefore determined not only by glucuronidation, but also by de-glucuronidation carried out by ß-glucuronidase (9,10).

Elevated ß-glucuronidase activity is associated with an increased risk of various cancers (11). In the 1950s, Boyland *et al* reported for the first time on the preventive effect of the ß-glucuronidase inhibitor D-glucaro-1,4-lactone on bladder carcinogenesis (12). It is widely accepted that ß-glucuronidase inhibitors, such as D-glucaro-1,4-lactone or its precursors, exert a preventive action on breast, prostate, colon, lung and skin

carcinogenesis (13-17). Ito *et al* reported that 2,5-di-*O*-acetyl-D-glucaro-1,4:6,3-dilactone or aceglatone (ACE), a synthetic precursor of D-glucaro-1,4-lactone, significantly controls the growth of bladder pre-cancerous lesions such as pachmucosa and mamillation, deemed to be early markers for the risk of bladder cancer (17). ACE has been used as a postoperative prophylactic agent for bladder cancer in humans. Additionally, another study has demonstrated that ACE inhibits carcinogen-induced mammary tumorigenesis in rats (13).

Aberrant crypt foci (ACF) are putative pre-neoplastic lesions of colon cancer (18). In short-term chemopreventive studies, the ability to prevent azoxymethane (AOM)-induced ACF has been used as an assay method to evaluate the chemopreventive effects of different test compounds on colon carcinogenesis (19). Carcinogenesis is a multi-stage process characterized by the accumulation of genetic alterations. Accordingly, targets for chemoprevention could be multiple and vary between the initiation, promotion and progression phases (20,21). Suppression of carcinogen-induced mutations during tumor initiation by increasing detoxification is therefore a possible mechanism for cancer prevention (22). On the other hand, so is the suppression of cell proliferation and the induction of differentiation and apoptosis during the promotion and progression stages (23). Proliferating cell nuclear antigen (PCNA) is a co-factor for DNA polymerase δ that participates in DNA synthesis and repair. The PCNA assay used in the present study is a popular method for measuring cell proliferation. It is based on the labeling and then counting of the labeled cells during the late G_1, S, and early G_2 phases (24,25).

In this study, we investigated the chemopreventive effects of ACE and D-glucurono-γ-lactone (GL), synthetic and natural precursors of D-glucaro-1,4-lactone, respectively, on colon carcinogenesis. A short-term assay was first conducted to determine whether ACE might inhibit aberrant crypt foci formation. Then, we demonstrated the inhibition of colon tumorigenesis in rats by ACE and GL in a long-term experiment. Additionally, we investigated whether ACE and GL reduce the rate of cellular proliferation.

Materials and methods

Chemicals. GL and ACE (Fig. 1; molecular structures) were supplied by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). AOM was purchased from Wako Chemical Co. (Tokyo, Japan). A basal diet (CE-2) for rats was purchased from Clea Japan Inc. (Tokyo, Japan).

Animals. Male Fischer 344 rats (F344; 4 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were housed in wire cages (3 or 4 rats per cage) and kept in a temperature (23±2˚C) and humidity (50±10%) controlled aseptic room with a 12 h light/dark cycle.

Experimental procedures. In the first short-term experiment (Fig. 2A), 25 4-week-old male F344 rats were randomly assigned to 3 groups. After one week, the rats in Group 1 were fed a plain basal diet, while the rats in Groups 2 and 3 were fed diets containing 0.5 or 2% ACE for the subsequent 5 weeks. At 6, 7 and 8 weeks of age (weeks 1, 2 and 3 of dietary treatment, respectively), all rats were administered 15 mg AOM per kilogram of body weight (BW) by subcutaneous (s.c.) injection. Rats were sacrificed at 10 weeks of age, following the end of treatment. Colons were fixed in 10% buffered formalin and stained with a 0.2% methylene blue solution for analysis of the ACF.

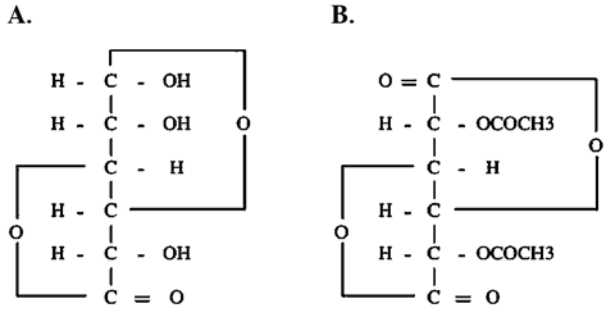

Figure 1. Molecular structure of D-glucurono-γ-lactone (A) and aceglatone (B).

In the second and long-term experiment (Fig. 2B), 4-week-old F344 rats were randomly divided into 8 groups. Intestinal tumors were induced by the injection of AOM in saline. Rats in Groups 1-7 were injected with AOM (15 mg/kg BW, s.c.) at 6, 7 and 8 weeks of age. Rats in Groups 2 and 3 were continually fed diets containing 2 and 0.5% ACE starting one week after the last injection of AOM until the end of the experiment. Rats in Groups 4 and 5 were fed diets containing 0.5 and 2% GL from 5 to 9 weeks of age. Rats in Groups 6 and 7 were continually fed diets containing 0.5 and 2% GL starting one week after the last injection of AOM until the end of the experiment. Group 8 was used as an untreated control. BW was measured weekly during the first month and then monthly until the end of the experiment. All rats were sacrificed at 36 weeks - 35 weeks after the first injection of AOM. Intestines were examined for the presence of tumors, and the size and location of tumors found were recorded. After fixation in 10% buffered formalin, the intestines were histopathologically examined by conventional methods. Intestinal neoplasms were diagnosed according to the criteria of Ward (26).

Determination of aberrant crypt foci. In the short-term experiment, colons were longitudinally cut open along the main axis and washed with saline. They were placed between filter papers and fixed by 10% buffered formalin for at least 24 h. After slides had been stained with a 0.5% methylene blue solution for 30 sec and briefly washed with distilled water, ACF were identified by their larger size and wider pericryptal zones using a light microscope at x40 magnification. The number of ACF per colon, number of aberrant crypts per focus, and the location of each focus were recorded.

Immunohistochemistry for cell proliferation (PCNA assay). In order to measure cell proliferation in the epithelium, PCNA assays were carried out on the colonic mucosae of 8 rats from each group. Immunohistochemical staining was performed as previously described (27,28). The embedded tissues were cut into 4-μm sections and then stained using anti-PCNA antibodies and the LSAB Kit (Dako, Carpinteria, CA). The number of PCNA-positive nuclei per section of the crypts was counted as previously described (27,28), then divided by the total number of nuclei to determine the PCNA-positive index (%).

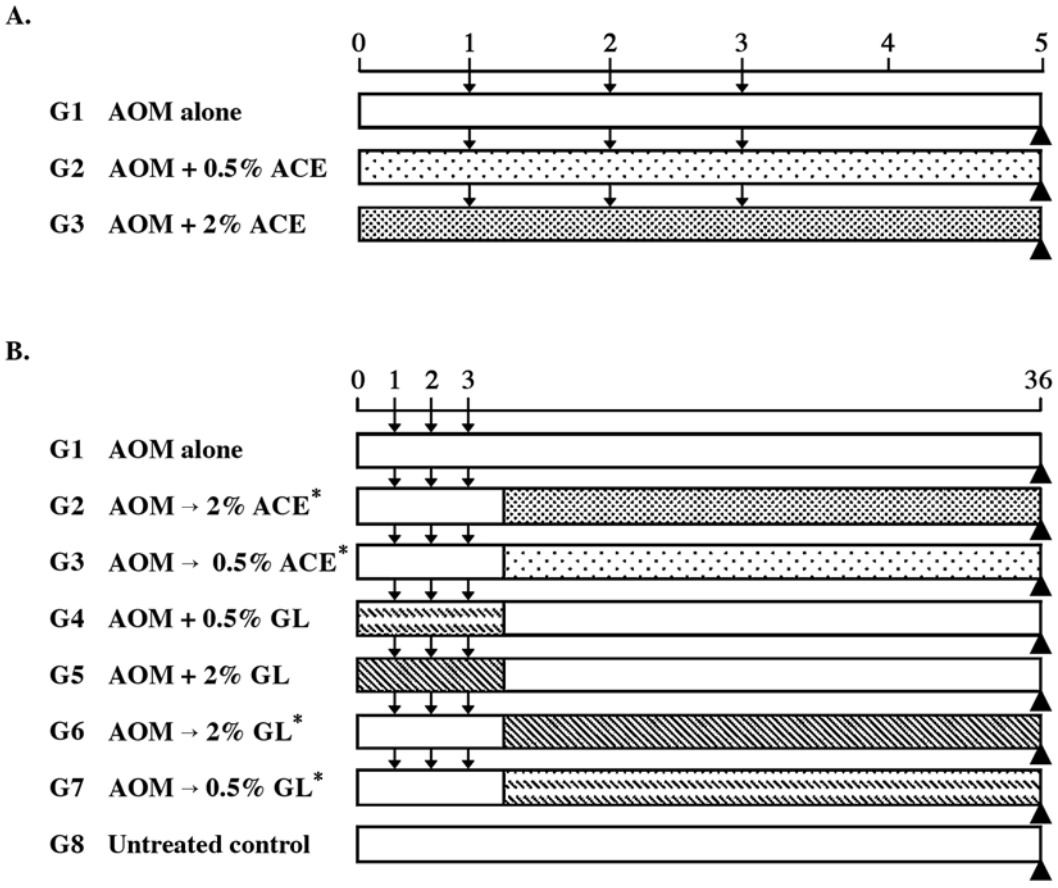

Figure 2. Experimental design of the 5- and 36-week experiments. (A) Aberrant crypt foci bioassay. (B) Long-term experiment. ↓, Administration of azoxymethane (AOM) (15 mg/kg body weight) by s.c. injection; ▲, sacrifice; ACE, aceglatone; GL, D-glucurono-γ-lactone. *Post-initiation.

Table I. Effect of aceglatone on azoxymethane-induced aberrant crypt foci formation in male F344 rats.[a]

Group	Treatment	No. rats	BW (g)	LW (g)	RLW (g/100 g BW)	No. ACF/colon	No. aberrant crypts/colon	No. aberrant crypts/focus	No. ACF with ≥4 crypts/colon
1	AOM alone	8	224±7.2	10.3±1.7	4.6±0.7	133.0±33.1	276±74.5	2.06±0.1	10.0±7.8
2	AOM + 0.5% ACE	8	220±7.4	11.1±1.6	5.0±0.7	68.4±20.6[c]	131±39.0[c]	1.92±0.1[d]	2.6±1.3[e]
3	AOM + 2.0% ACE	9	189±13.8[b]	9.1±1.8	4.8±0.7	59.5±16.9[c]	111±33.1[c]	1.86±0.1[c]	2.5±1.8[e]

[a]Data are the means ± SD. Significantly different from Group 1 according to [b]Welch's t-test ($p<0.01$); [c]Student's t-test ($p<0.01$); [d]Student's t-test ($p=0.014$); [e]Welch's t-test ($p<0.05$). BW, body weight; LW, liver weight; RLW, relative liver weight; ACF, aberrant crypt foci; AOM, azoxymethane; ACE, aceglatone.

Statistical analysis. All data were presented as the means ± SD. Fisher's exact probability test, the Student's t-test and Welch's t-test were used for statistical analysis. A value of $p<0.05$ was considered significant.

Results

Effect of aceglatone on aberrant crypt foci formation. As shown in Table I, the mean BW of rats treated with 2% ACE was significantly lower than in the Group 1 rats, which were administered AOM alone ($p<0.01$). However, there were no significant differences in the mean liver weight (LW) or mean relative liver weight (RLW; g/100 g BW) of the rats. The number of ACF/colon in Group 1 (AOM alone) was 133±33.1. Dietary administration of 0.5 or 2% ACE led to a significant reduction in the number of ACF/colon ($p<0.01$), as well as in the number of aberrant crypts/colon ($p<0.01$) and aberrant crypts/focus (0.5% ACE, $p=0.014$; 2% ACE, $p<0.01$). It also reduced the number of large ACF containing 4 or more aberrant crypts ($p<0.05$).

Summary of body weight, liver weight, and relative ratio of liver to body weight. No statistically significant difference in dietary intake with or without chemicals (on average 15 g/rat/day) was observed. As shown in Table II, the BW and LW of rats treated with AOM was lower than in the controls, but the

Table II. Summary of body weight and relative ratio of liver to body weight in the long-term experiment.[a]

Group	Treatment	No. of rats	BW (g)	LW (g)	RLW (g/100 g BW)
1	AOM alone	27	350±2[b]	13.1±2.5[b]	3.7±0.7
2	AOM → 2.0% ACE	26	333±17[c,f]	13.4±1.8[d]	4.0±0.5
3	AOM → 0.5% ACE	25	339±22[b]	14.4±2.3	4.2±0.5
4	AOM + 2.0% GL	24	360±21[d]	14.3±1.9	4.0±0.5
5	AOM + 0.5% GL	26	349±15[c]	13.7±1.6	3.9±0.4
6	AOM → 2.0% GL	25	342±15[c]	13.3±1.6[e]	3.9±0.4
7	AOM → 0.5% GL	23	347±20[b]	13.7±1.7	3.9±0.4
8	Untreated control	6	377±12	15.0±1.1	4.0±0.3

[a]Data are the means ± SD. Significantly different from corresponding Group 6 according to [b]Welch's t-test ($p<0.01$); [c]Student's t-test ($p<0.01$); [d]Welch's t-test ($p<0.05$); [e]Student's t-test ($p<0.05$). [f]Significantly different from corresponding Group 1 according to the Student's t-test ($p<0.01$). BW, body weight; LW, liver weight; RLW, relative liver weight; AOM, azoxymethane; ACE, aceglatone; GL, D-glucurono-γ-lactone.

Table III. Incidence and multiplicity of intestinal neoplasms in the long-term experiment.

			Incidence (%)			Multiplicity[a]		
Group	Treatment	No. of rats	Small intestine	Colon	Total	Small intestine	Colon	Total
1	AOM alone	27	3 (11)	10 (37)	13 (44)	0.11±0.32	0.48±0.70	0.59±0.75
2	AOM → 2.0% ACE	26	3 (12)	2 (8)[b]	5 (19)[b]	0.12±0.33	0.08±0.27[d]	0.19±0.41[c]
3	AOM → 0.5% ACE	25	2 (8)	3 (12)[b]	5 (20)	0.08±0.28	0.16±0.47	0.24±0.52
4	AOM + 2.0% GL	24	1 (4)	8 (33)	9 (38)	0.04±0.21	0.38±0.58	0.42±0.58
5	AOM + 0.5% GL	26	5 (19)	11 (42)	16 (54)	0.22±0.51	0.44±0.58	0.67±0.73
6	AOM → 2.0% GL	25	1 (4)	3 (12)[b]	4 (16)[b]	0.08±0.28	0.12±0.33[c]	0.20±0.52[c]
7	AOM → 0.5% GL	23	1 (4)	11 (48)	12 (52)	0.04±0.21	0.57±0.73	0.61±0.72
8	Untreated control	6	0 (0)	0 (0)	0 (0)	-	-	-

[a]Data are shown as the mean ± SD. Significantly different from Group 1 according to [b]Fisher's exact probability test ($p<0.05$); [c]Welch's t-test ($p<0.05$); [d]Welch's t-test ($p<0.01$). AOM, azoxymethane; ACE, aceglatone; GL, D-glucurono-γ-lactone.

relative ratios between groups were not significantly different. Among the groups treated with AOM, the BW of rats in Group 2 was lower than in Group 1 ($p<0.01$); LW and RLW did not differ significantly. Histologically, there were no pathologic changes in the liver or kidney of the majority of rats; only one, treated with GL in Group 6, had a liver tumor.

Incidence and multiplicity of colonic neoplasms. Most tumors had formed in the large intestine; 2/3 of these were mainly located in the middle and distal colon. Tumors were determined to be sessile or pedunculated, and were histologically diagnosed as adenomas, tubular adenocarcinomas or mucinous carcinomas. Tubular adenocarcinomas had the highest incidence. The incidence and multiplicity of intestinal neoplasms are shown in Table III. AOM alone (Group 1) induced a 37% incidence of large intestinal tumors with a multiplicity of 0.48±0.7. The incidence of large intestinal tumors and total incidence of colon and small intestinal tumors in Groups 2, 3, 4 and 6 were lower than in Group 1. Statistically, the incidence of large intestinal tumors in Groups 2, 3 and 6 was significantly lower than in Group 1 ($p<0.05$), as was the total incidence of colon and small intestinal tumors in Groups 2 and 6 ($p<0.05$). As well, the multiplicity of large intestinal tumors and the total multiplicity of colon and small intestinal tumors in Groups 2, 3, 4 and 6 were reduced when compared with Group 1. Statistically, the multiplicity of large intestinal tumors in Groups 2 and 6 was significantly lower ($p<0.01$ and $p<0.05$, respectively) than in Group 1. The total multiplicity of colon and small intestinal tumors in Groups 2 and 6 was significantly lower than in Group 1 ($p<0.05$).

Proliferating cell nuclear antigen-positive index. The results of measurements of the PCNA-positive index in the colonic mucosal epithelium are shown in Fig. 3. PCNA-positive indices in groups treated with ACE during the post-initiation phase or with GL during the initiation or the post initiation phases were significantly lower than in the rats in Group 1 (AOM alone) ($p<0.01$).

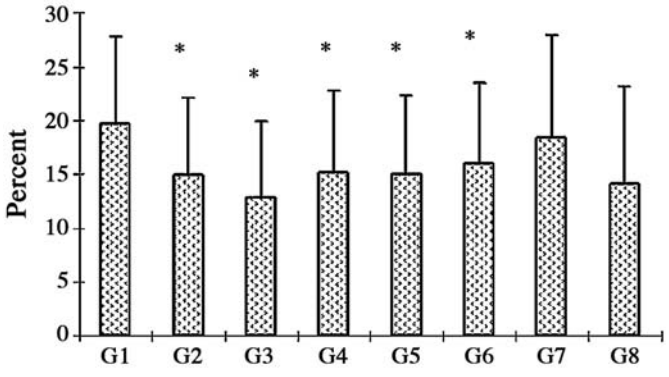

Figure 3. PCNA-positive cell index (%) in the colonic mucosa (*p<0.01). For experiment details see Fig. 2.

Discussion

It has been suggested that colorectal cancer is triggered by the presence of carcinogenic bacterial metabolites (29,30). In the case of AOM-induced colonic carcinogenesis in rats, AOM is hydroxylated and conjugated with glucuronic acid in the liver, then excreted in the bile (16). In turn, bacterial ß-glucuronidase hydrolyzes the glucuronic acid conjugate, liberating the active carcinogen methylazoxymethanol in the intestines (16). According to principle, ACE and GL, as *in vivo* precursors of the ß-glucuronidase inhibitor, may prevent the induction of colon cancer by inhibiting the hydrolysis of glucuronides. However, they may also have anti-proliferative effects, as discussed below.

ACF are pre-neoplastic lesions in the colon of rats. They are useful biological markers for the evaluation of the preventive effects of test compounds in rat colon carcinogenesis (31). In this study, we evaluated whether ACE has the ability to prevent ACF development in AOM-induced colon carcinogenesis in rats. Dietary administration of ACE for 5 weeks during AOM exposure significantly decreased the number of ACF/colon and aberrant crypts/colon ($p<0.01$). As well, ACE suppressed the number of ACF with 4 or more crypts ($p<0.05$), an intermediate biomarker that is predictive of tumor incidence (32). The data suggest that during the initiation phase of AOM-induced carcinogenesis, ACE acts as a blocking agent and may inhibit pre-neoplasia.

The results of the long-term experiment indicate that ACE or a high dose of GL (2%), when used during the post-initiation phase, inhibit AOM-induced colon carcinogenesis ($p<0.05$). When administered during the initiation phase, 0.5 and 2% ACE (Yoshimi *et al*, Proc Am Assoc Cancer Res 42: abs. 957, 2001) or a high dose of GL (2%, this study) also had a tendency to reduce colon tumor incidence compared to the control, although without statistical significance. This is the first report to describe the preventive effects of ACE and GL in the initiation and post-initiation phases of colon cancer development in rats. Our results suggest that both ACE and GL are potential chemopreventive agents against colon carcinogenesis. However, as a low dose of GL (0.5%) had no inhibitory effect on colon carcinogenesis in either the initiation or post-initiation phases, further dose-response studies of GL are necessary prior to its being used as a chemopreventive agent.

The marked inhibitory effects of ACE and of a high dose of GL (2%) observed during the post-initiation phase rather than in the initiation phase of AOM-induced colon carcinogenesis indicate that other mechanisms, in addition to detoxification, might be involved. Increased cellular proliferation is thought to play an important role in multi-stage carcinogenesis (33). In the present study, we evaluated the PCNA-positive cell index in colonic mucosa and found it reduced in the groups treated with ACE and GL. Yoshimi *et al* have demonstrated that various ß-glucuronidase inhibitors have antiproliferative effects in mammary gland, colon and skin carcinogenesis *in vivo* (34; Walaszek *et al*, Proc Am Assoc Cancer Res 31: abs. 735, 1990), and that they also inhibit the growth of breast cancer cells *in vitro* (35). In addition, we recently demonstrated *in vitro* that ACE has the potential to reduce cell proliferation, induce apoptosis and inhibit DNA synthesis in human colon cancer cells (COLO 320) (Yoshimi *et al*, unpublished data). Thus, recent studies suggest that the chemopreventive effects of ACE and GL could be explained, in part, by their ability to inhibit the increased proliferation of colonic epithelium cells induced by AOM treatment, as well as by their ability to induce apoptosis.

In conclusion, the results of the present study demonstrate the inhibitory effects of ACE and GL on the initiation and post-initiation phases of AOM-induced colon carcinogenesis in rats. As precursors of the ß-glucuronidase inhibitor D-glucaro-1,4-lactone, ACE and GL might exert their chemopreventive action through multiple mechanisms, including the inhibition of ß-glucuronidase, the reduction of cell proliferation and the induction of apoptosis. Pre-clinical efficacy and other possible mechanisms thus need to be further studied.

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