

# Inhibitory effect of dietary monoglucosylceramide 1-O- $\beta$ -glucosyl-*N*-2'-hydroxyarachidoyl-4,8-sphingadienine on two different categories of colon preneoplastic lesions induced by 1,2-dimethylhydrazine in F344 rats

Morihiko Inamine,<sup>1</sup> Masumi Suzui,<sup>1,3</sup> Takamitsu Morioka,<sup>1</sup> Tatsuya Kinjo,<sup>1</sup> Tatsuya Kaneshiro,<sup>1</sup> Tomoko Sugishita,<sup>2</sup> Tadashi Okada<sup>2</sup> and Naoki Yoshimi<sup>1</sup>

<sup>1</sup>Tumor Pathology, University of the Ryukyus Faculty of Medicine, 207 Uehara Nishihara-cho, Okinawa, 903-0215; and <sup>2</sup>Oryza Oil and Fat Chemical Company, Research and Development, 1 Numata Kitagata-cho Ichinomiya-city, Aichi, 493-8001, Japan

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Sphingolipids display a wide spectrum of biological activities, including cell growth, differentiation and apoptosis. However, precise mechanisms by which these compounds exert anticancer or cancer-preventive effects are not known. In the present study, we evaluated the preventive efficacy of enriched dietary monoglucosylceramide 1-O- $\beta$ -glucosyl-*N*-2'-hydroxyarachidoyl-4,8-sphingadienine (G<sub>1</sub>CM) on 1,2-dimethylhydrazine (DMH)-induced aberrant crypt foci (ACF) and  $\beta$ -catenin-accumulated crypt (BCAC) formation in F344 rats during initiation stage. We also examined whether G<sub>1</sub>CM affects cell proliferation and apoptosis in these lesions. Pure G<sub>1</sub>CM was isolated from rice bran. Forty-two rats were divided randomly into five experimental groups. Rats in groups 1–3 were given subcutaneous injections of DMH (40 mg/kg body weight) once a week for 2 weeks. One week before the first injection of DMH, rats in groups 2 and 3 were fed a diet containing 200 and 1000 p.p.m. G<sub>1</sub>CM, respectively, for 5 weeks. Rats in group 4 were fed a diet containing 1000 p.p.m. G<sub>1</sub>CM. Rats in group 5 were given the basal diet alone and served as untreated controls. The experiment was terminated 5 weeks after the start. Dietary G<sub>1</sub>CM at both doses (groups 2 and 3) significantly inhibited the induction of ACF and BCAC ( $P < 0.001$ ) when compared to group 1 treated with DMH alone. In groups 2 and 3, the proliferating cell nuclear antigen labeling indices of epithelial cells in ACF and BCAC were also lower than in group 1 ( $P < 0.0001$  for ACF,  $P < 0.05$  for BCAC). These results, that dietary G<sub>1</sub>CM has possible chemopreventive effects in the present short-term colon carcinogenesis bioassays, suggest that longer exposure may cause suppression of tumor development. (*Cancer Sci* 2005; 96: 876–881)

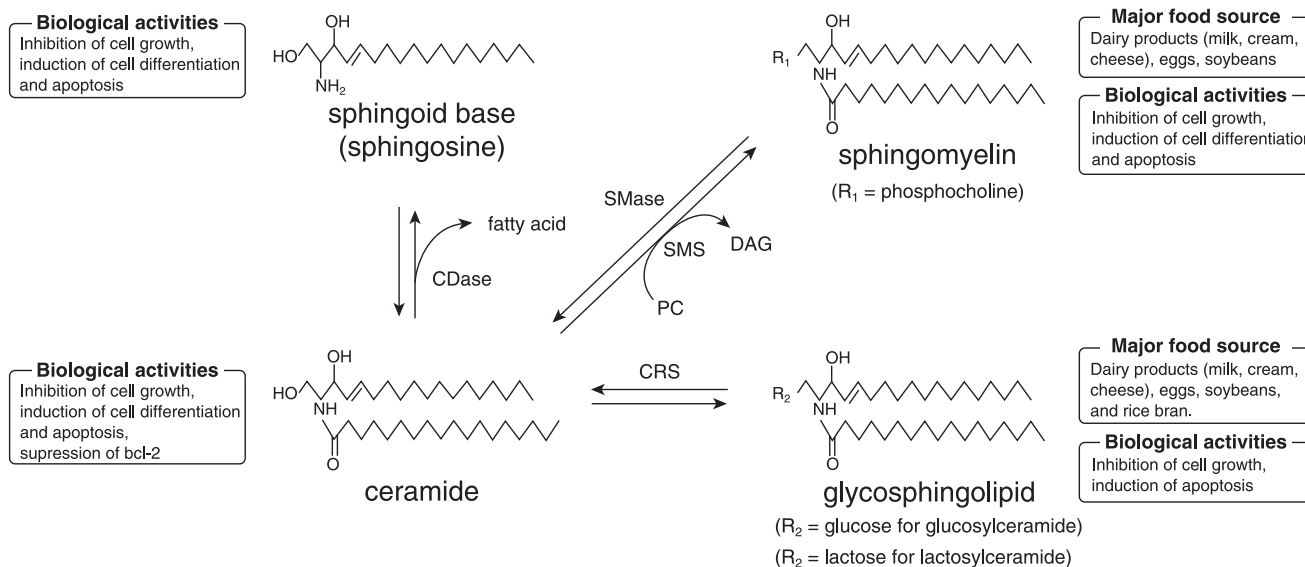
Sphingolipids are a group of structural and functional derivatives that have a long-chain (sphingoid) base backbone and exhibit a variety of biological activities.<sup>(1–3)</sup> In most sphingolipids, the amino group is substituted with a long-chain fatty acid (these compounds are termed ceramide) and there is a group at position 1, such as phosphocholine in sphingomyelin (Fig. 1).<sup>(1,4)</sup> Milk, eggs and soybeans appear to be rich sources although a limited number of foods have been analyzed for the amount and type of sphingolipids.<sup>(1,4)</sup> Fujino

*et al.* reported that ceramide and glucosylceramide (cerebroside) are included in the bran and endoderm of rice grains.<sup>(5)</sup> Because some sphingolipids, including ceramide and glucosylceramide, display a broad range of biological activities related to cell growth, differentiation and apoptosis,<sup>(3)</sup> they may play an important role in all steps of carcinogenesis.<sup>(6,7)</sup> In fact, colonic cell proliferation and aberrant crypt foci (ACF) formation are inhibited by dietary glycosphingolipids in 1,2-dimethylhydrazine (DMH)-treated CF1 mice.<sup>(8,9)</sup> In addition, synthetic sphingomyelins inhibit DMH-induced ACF formation<sup>(10)</sup> and dietary sphingomyelin suppresses colon carcinogenesis induced by DMH in CF1 mice.<sup>(11–13)</sup> It has also been shown that sphingoid bases and ceramides enhance retinoic acid-induced differentiation in the HL60 human leukemia cell line,<sup>(14)</sup> and induce apoptosis in HT29 and HCT116 human colon carcinoma cell lines.<sup>(15)</sup> Although the above-described findings obtained with sphingolipids in experimental studies are promising, the precise mechanisms by which this complex causes tumor suppression are not known.

Aberrant crypt foci are putative preneoplastic lesions of the colonic mucosa in mice, rats and humans.<sup>(16–21)</sup> In the rat colonic mucosa, focal lesions that display accumulation of  $\beta$ -catenin protein are termed  $\beta$ -catenin accumulated crypts (BCAC).<sup>(22,23)</sup> Also, these lesions have been shown to have frequent  $\beta$ -catenin gene mutations.<sup>(22)</sup> Thus, BCAC are thought to be independent precursor lesions. Both ACF and BCAC have been used as valuable biomarkers for short-term colon carcinogenesis bioassays and are also useful to examine possible chemopreventive effects of a wide variety of candidate agents.<sup>(17,24)</sup>

In the present study, we examined whether monoglucosylceramide 1-O- $\beta$ -glucosyl-*N*-2'-hydroxyarachidoyl-4,8-sphingadienine (G<sub>1</sub>CM) has short-term chemopreventive effects on two different categories of putative preneoplastic lesions such as ACF and BCAC involved in rat colon carcinogenesis. We

<sup>3</sup>To whom correspondence should be addressed.  
E-mail: suzui@med-u.ryukyu.ac.jp



**Fig. 1.** General structures, metabolism, biological activities and food sources of major sphingolipids. CDase, ceramidase; CRS, cerebrosidase; DAG, diacylglycerol; PC, phosphatidylcholine; SMase, sphingomyelinase; SMS, sphingomyelin synthase.

have also examined whether this compound affects cell growth and apoptosis by measuring proliferating cell nuclear antigen (PCNA)-positive cells and apoptotic bodies in epithelial cells consisting of the crypts of ACF and BCAC.

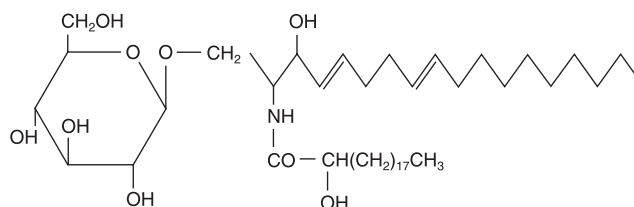
## Materials and Methods

### Animals

Four-week-old male F344 rats were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). All rats were housed 3–4 per wire cage with free access to tap water and basal diet (CE2; CLEA Japan, Tokyo, Japan) under controlled conditions of humidity ( $50 \pm 10\%$ ), lighting (12:12 h L:D cycle) and temperature ( $23 \pm 2^\circ\text{C}$ ). The animals were maintained in the Animal Facility of the University of the Ryukyus according to the Institutional Animal Care Guidelines.

### Treatment

Pure monoglucosylceramide 1-O- $\beta$ -glucosyl-N-2'-hydroxyarachidoyl-4,8-sphingadienine (Fig. 2) obtained from rice bran was provided by the Oryza Oil and Fat Chemical Company, (Aichi, Japan). Purity ( $> 93\%$ ) was determined by carrying out high-performance liquid chromatographic (HPLC) analysis, and three major bands were detected. Of these, the highest peak contained  $G_1\text{CM}$ . The remaining two major bands contained other sphingoid compounds that have different chemical structures from  $G_1\text{CM}$ . Mass spectrometric analysis was then used to evaluate purity and provide detailed information about the molecular structure of  $G_1\text{CM}$ . We used 200 and 1000 p.p.m. dose levels of  $G_1\text{CM}$  in the diet because at this dose range there were obvious inhibitory effects of dietary sphingomyelin or glucosylceramide on colonic ACF formation and colon tumor development.<sup>(8,12,13)</sup> Forty-two rats were assigned randomly to the experimental groups (Fig. 3). Rats in groups 1–3 were given



**Fig. 2.** Chemical structure of 1-O- $\beta$ -glucosyl-N-2'-hydroxyarachidoyl-4,8-sphingadienine ( $G_1\text{CM}$ ).

subcutaneous injections of DMH (40 mg/kg body weight) (Sigma Chemical Company, St Louis, MO, USA) once a week for 2 weeks. One week before the first injection of DMH, rats in groups 2 and 3 were fed a diet containing 200 and 1000 p.p.m.  $G_1\text{CM}$ , respectively, for 5 weeks. Rats in group 4 were fed a diet containing 1000 p.p.m.  $G_1\text{CM}$ . Rats in group 5 were given the basal diet alone and served as untreated controls. The rats were observed carefully and weighed weekly during the experiment. The experiment was terminated at 5 weeks after the start of the experiment. All animals were then killed under  $\text{CO}_2$  anesthesia. Colons were removed carefully, washed with saline, opened longitudinally, and then fixed with 10% buffered formalin.

### Identification of aberrant crypt foci and $\beta$ -catenin-accumulated crypts

Colon tissues were stained in a 0.5% methylene blue solution for 30 s, immediately washed with distilled water and then placed on a glass plate with the mucosal surface up. Using a light microscope at a magnification of  $\times 40$ , ACF were counted according to the criteria described earlier.<sup>(25)</sup> After ACF counting, colon tissues were embedded in paraffin and processed for immunohistochemistry of BCAC and PCNA.<sup>(26)</sup> Briefly, distal and middle segments of the colon were used for immunostaining. Serial sections ( $4\text{-}\mu\text{m}$  thick) were

prepared to include the middle portion between the surface and the bottom of the crypt. These sections were treated in 3% H<sub>2</sub>O<sub>2</sub> for 20 min to block the endogenous peroxidase activity and then incubated with a primary antibody of the  $\beta$ -catenin protein (1:100 dilution) (Transduction Laboratories, Lexington, KY, USA) at room temperature for 60 min. Sections were stained with a Simple Stain kit (Nichirei, Tokyo, Japan).

### Immunohistochemical staining and measurement of proliferating cell nuclear antigen labeling index

Proliferating activity of cells in the colonic crypts was evaluated by measuring PCNA-positive nuclei as described in a previous paper.<sup>(26)</sup> In brief, after staining 4- $\mu$ m thick sections with an anti-PCNA antibody (1:50 dilution) (Dako, Kyoto, Japan), at least 300 epithelial cells in ACF and BCAC were counted by a single reader who was blind to the treatment groups. PCNA labeling indices were calculated as the percentage of positive cells with respect to the total number of cells counted.

### Detection of apoptosis

Apoptotic cells in ACF and BCAC were evaluated in sections stained with hematoxylin and eosin using light microscopy. Apoptotic cells were identified by cell shrinkage, nuclear condensation and apoptotic body formation.<sup>(27)</sup> Apoptotic index was calculated as the percentage of apoptotic cells with respect to the total number of cells counted, as describe in a recent work.<sup>(28)</sup>

### Statistical analysis

Statistical analyses by Student's or Welch's *t*-test were carried out to determine the significance of the differences between groups. All statements of significance are  $P < 0.05$ .

## Results

### General observations

All rats survived to the end of the experiment and none of them developed colon tumors. To ascertain that dietary feeding of G<sub>1</sub>CM caused no toxic side-effects on body weight gain, the rats were monitored on a routine basis. No significant effects of DMH or G<sub>1</sub>CM were seen on body, liver or kidney weight (Table 1). Food consumption of animals in the experimental groups did not vary. G<sub>1</sub>CM at 200 and 1000 p.p.m. caused no adverse side-effects in any of the rats.

### Inhibitory effect of G<sub>1</sub>CM on aberrant crypt foci and $\beta$ -catenin-accumulated crypts

In the current study, we used the well-established and short-term protocol of the rat colon carcinogenesis model system to examine the potential chemopreventive effect of G<sub>1</sub>CM. We chose ACF and BCAC as end points in this bioassay because ACF and BCAC are preneoplastic lesions and these lesions are regarded as useful biomarkers. All rats in groups 1–3 developed ACF and BCAC in the colonic mucosa. No ACF or BCAC were seen in any of the rats of groups 4 and 5. The number of ACF was significantly lower in DMH-treated rats fed 200 and 1000 p.p.m. G<sub>1</sub>CM diet than that in controls ( $P < 0.0001$ ; Table 2). The number of ACF containing one to three or more than four aberrant crypts was also decreased significantly in DMH-treated rats fed both G<sub>1</sub>CM doses ( $P < 0.0001$  and  $P < 0.05$ , respectively, Table 2). In groups 2 and 3, treatment of rats with both dose levels of G<sub>1</sub>CM caused a significant decrease in mean number of BCAC/cm<sup>2</sup> colon, when compared with the control rats treated with DMH alone (group 1) ( $P < 0.05$ ; Table 3). These results indicate that dietary G<sub>1</sub>CM significantly inhibited ACF and BCAC formation induced by DMH in F344 rats.

Table 1. Body, liver and kidney weights of each experimental group at the end of the experiment

Group	Treatment	Body weight (g)	Liver weight (g)	Kidney weight (g)	Relative liver weight (g)	Relative kidney weight (g)
1	DMH	233 ± 14.6	8.53 ± 0.53	1.82 ± 0.13	3.65 ± 0.11	0.78 ± 0.04
2	DMH + 200 p.p.m. G <sub>1</sub> CM	232 ± 13.4	8.21 ± 0.39	1.74 ± 0.07	3.63 ± 0.15	0.75 ± 0.04
3	DMH + 1000 p.p.m. G <sub>1</sub> CM	235 ± 12.9	8.50 ± 0.70	1.79 ± 0.10	3.62 ± 0.20	0.76 ± 0.05
4	1000 p.p.m. G <sub>1</sub> CM	237 ± 16.5	8.30 ± 0.75	1.79 ± 0.06	3.50 ± 0.09	0.76 ± 0.03
5	Basal diet	253 ± 9.1	9.34 ± 0.18	1.99 ± 0.5	3.74 ± 0.08	0.79 ± 0.07

Values are mean ± SD. DMH, 1,2-dimethylhydrazine; G<sub>1</sub>CM, 1-O- $\beta$ -glucosyl-N-2'-hydroxyarachidoyl-4,8-sphingadienine.

Table 2. Inhibitory effect of dietary 1-O- $\beta$ -glucosyl-N-2'-hydroxyarachidoyl-4,8-sphingadienine (G<sub>1</sub>CM) on aberrant crypt foci (ACF) formation induced by 1,2-dimethylhydrazine (DMH)

Group	Treatment	No. ACF/colon	No. ACF containing one to three aberrant crypts/colon	No. ACF containing more than four aberrant crypts/colon
1	DMH	196.6 ± 32.4	186.3 ± 29.2	10.4 ± 4.8
2	DMH + 200 p.p.m. G <sub>1</sub> CM	107.1 ± 15.0 <sup>†</sup>	101.7 ± 14.3 <sup>†</sup>	5.4 ± 4.4 <sup>‡</sup>
3	DMH + 1000 p.p.m. G <sub>1</sub> CM	116.6 ± 21.7 <sup>†</sup>	109.9 ± 22.3 <sup>†</sup>	6.7 ± 3.1 <sup>‡</sup>
4	1000 p.p.m. G <sub>1</sub> CM	0	0	0
5	Basal diet	0	0	0

<sup>†</sup>Significantly different from group 1 by Student's *t*-test,  $P < 0.0001$ . <sup>‡</sup>Significantly different from group 1 by Welch's *t*-test,  $P < 0.0001$ . Values are mean ± SD.

**Table 3. Inhibitory effect of dietary 1-O- $\beta$ -glucosyl-N-2'-hydroxyarachidoyl-4,8-sphingadienine (G<sub>1</sub>CM) on  $\beta$ -catenin-accumulated crypt (BCAC) formation induced by 1,2-dimethylhydrazine (DMH)**

Group	Treatment	Mean no. BCAC/cm <sup>2</sup> colon
1	DMH	0.63 $\pm$ 0.16
2	DMH + 200 p.p.m. G <sub>1</sub> CM	0.21 $\pm$ 0.05 <sup>†</sup>
3	DMH + 1000 p.p.m. G <sub>1</sub> CM	0.20 $\pm$ 0.06 <sup>†</sup>
4	1000 p.p.m. G <sub>1</sub> CM	0
5	Basal diet	0

<sup>†</sup>Significantly different from group 1 by Welch's *t*-test, *P* < 0.05. Values are mean  $\pm$  SD.

### Inhibition of proliferating cell nuclear antigen labeling index by G<sub>1</sub>CM in aberrant crypt foci and $\beta$ -catenin-accumulated crypts

To determine whether G<sub>1</sub>CM affects cell proliferation in ACF and BCAC, we measured the PCNA labeling index in these lesions by immunohistochemistry. In groups 2 and 3, treatment of rats with both dose levels of G<sub>1</sub>CM resulted in a significant decrease in the labeling index of PCNA in ACF and BCAC when compared to group 1 treated with DMH alone (*P* < 0.0001 for ACF and *P* < 0.05 for BCAC; Table 4). Moreover, the PCNA labeling index in BCAC of rats treated with DMH alone and in normal colonic mucosa of rats without treatment was 10.91 (Table 4) and 5.9 (data not shown), respectively. However, in groups 2 and 3 the PCNA labeling index was 5.44 and 5.56 in DMH-treated rats fed 200 and 1000 p.p.m. G<sub>1</sub>CM, respectively (Table 4). These findings suggest that dietary G<sub>1</sub>CM can normalize elevation of cell proliferation induced by DMH.

### Effect of G<sub>1</sub>CM on apoptosis.

Because G<sub>1</sub>CM is able to normalize elevation of cell proliferation in ACF and BCAC, we investigated whether this inhibition is due to the induction of apoptosis in these lesions. There was a clear tendency that an increase in

apoptotic index of BCAC is dose dependent of G<sub>1</sub>CM compared to the control, but this was not statistically significant (Table 5). Regarding the apoptotic index of ACF, no clear tendency was seen. Apoptotic index in untreated normal mucosa was similar to that of group 3 (data not shown).

## Discussion

There has been considerable interest in sphingolipids, including ceramide and glucosylceramide, in the prevention or therapy of malignant neoplasms. Despite the reported benefits of these compounds in experimental animal models or cell culture systems, it remains unclear whether these agents have cancer-preventive properties and whether there is a molecular basis to their growth inhibitory effects on carcinoma cells. In parallel studies, we have also found possible cancer-preventive effects of crude glycosphingolipid using a carcinogen-induced rat colon carcinogenesis model (unpublished data). Therefore, we have focused on the effect of rice bran G<sub>1</sub>CM, a purified derivative of sphingolipids, on the earliest morphological changes of colonic cells to neoplasms such as ACF and BCAC, cell proliferation and apoptosis. We carried out the present studies to provide further evidence to support the use of this compound in cancer prevention. In the present study, G<sub>1</sub>CM was extracted from rice bran and purified using HPLC analysis. We found that dietary G<sub>1</sub>CM caused significant inhibition of ACF and BCAC formation in F344 rats. G<sub>1</sub>CM also normalized the elevation of DMH-induced cell proliferation in ACF and BCAC. This result is in accordance with that of a previous report by Lemonnier *et al.*<sup>(11)</sup> DMH is a commonly used carcinogen and enhances proliferation of cells constructing crypts in the rat colon.<sup>(8)</sup> This agent also induces ACF and BCAC formation and tumor development in the rat colon.<sup>(26)</sup> Cell proliferation is more enhanced in ACF, BCAC and tumor than in normal colonic mucosa.<sup>(29)</sup> Taken together, cell proliferation appears to be an important aspect in the occurrence of these lesions. Dietary sphingolipids isolated

**Table 4. Inhibition of proliferating cell nuclear antigen (PCNA) labeling index by dietary 1-O- $\beta$ -glucosyl-N-2'-hydroxyarachidoyl-4,8-sphingadienine (G<sub>1</sub>CM) in the preneoplastic lesions aberrant crypt foci (ACF) and  $\beta$ -catenin-accumulated crypts (BCAC)**

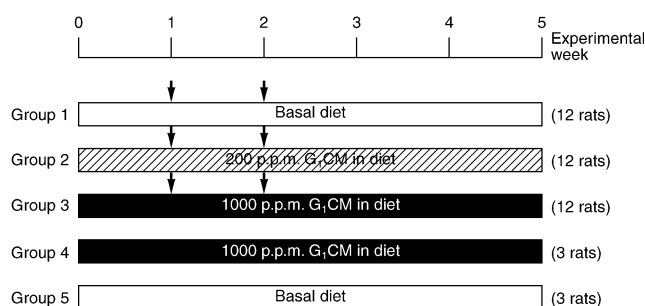
Group	Treatment	PCNA labeling index in ACF		PCNA labeling index in BCAC	
		%	Crypts examined (n)	%	Crypts examined (n)
1	DMH	3.00 $\pm$ 4.73	325	10.91 $\pm$ 9.39	51
2	DMH + 200 p.p.m. G <sub>1</sub> CM	1.22 $\pm$ 2.35 <sup>†</sup>	246	5.44 $\pm$ 4.89 <sup>†</sup>	20
3	DMH + 1000 p.p.m. G <sub>1</sub> CM	1.37 $\pm$ 2.89 <sup>†</sup>	222	5.56 $\pm$ 6.57 <sup>§</sup>	16

Significantly different from group 1 by Welch's *t*-test, <sup>†</sup>*P* < 0.001, <sup>‡</sup>*P* < 0.005. <sup>§</sup>Significantly different from group 1 by Student's *t*-test, *P* < 0.05. Values are mean  $\pm$  SD.

**Table 5. Apoptotic index in aberrant crypt foci (ACF) and  $\beta$ -catenin-accumulated crypts (BCAC)**

Group	Treatment	ACF		BCAC	
		%	Crypts examined (n)	%	Crypts examined (n)
1	DMH	0.08 $\pm$ 0.49	325	0.47 $\pm$ 1.14	51
2	DMH + 200 p.p.m. G <sub>1</sub> CM	0.05 $\pm$ 0.47	246	0.62 $\pm$ 1.28	20
3	DMH + 1000 p.p.m. G <sub>1</sub> CM	0.03 $\pm$ 0.40	222	1.36 $\pm$ 2.19	16

Values are mean  $\pm$  SD.



**Fig. 3.** Experimental protocol. Arrow indicates 1,2-dimethylhydrazine subcutaneous injection (40 mg/kg bodyweight). G<sub>1</sub>CM, 1-O- $\beta$ -glucosyl-N-2'-hydroxyarachidoyl-4,8-sphingadienine.

from milk or soy suppress DMH-induced ACF formation and cell proliferation of colonic crypts in CF1 mice.<sup>(8–10,12)</sup> Also, dietary sphingomyelin and glucosylceramide inhibit the development of adenomas in multiple intestinal neoplasia (min) mice,<sup>(12)</sup> and DMH-induced adenocarcinomas in CF1 mice.<sup>(11,13)</sup> Sphingosine, sphinganine and C2-ceramide cause dose-dependent growth inhibition and induce apoptosis in HT29 and HCT116 human colon carcinoma cell lines.<sup>(15)</sup> Therefore, cell proliferation may play a critical role in suppression of ACF and BCAC formation by sphingolipids, including G<sub>1</sub>CM. In view of the above-described results and discussions, G<sub>1</sub>CM may have a potent chemopreventive effect in this short-term colon carcinogenesis bioassay system. In the present study, we did not find an appreciable contribution of G<sub>1</sub>CM to the induction of apoptosis but G<sub>1</sub>CM appears to increase the rate of apoptosis to the normal level. This aspect warrants further investigation, including long-term carcinogenesis studies. In general, a 100–250 p.p.m. dose range of potent chemopreventive agents has been used in many experimental animal models.<sup>(30)</sup> However, it is not known whether this is the maximal effect of G<sub>1</sub>CM because our data did not display a clear dose–response relationship in the inhibition of ACF and BCAC formation and cell proliferation. This is presumably due to the maximum tolerant dose of this compound and the small number of animals involved. To address these questions, more extensive dose–response studies should be conducted in a larger number of animals.

Schmelz *et al.* reported that up to approximately 88% of dietary sphingomyelin is degraded to ceramide or sphingoid

bases in the small intestine, and the remaining 12% of dietary sphingomyelin is found in the cecum and colon.<sup>(31)</sup> By this mechanism, colonic epithelial cells could be exposed to ceramide and sphingoid bases. These bioactive metabolites are taken up by cells in the colonic epithelium and affect a broad range of target molecules in cells through regulation of growth, differentiation and apoptosis.<sup>(1,3,6,7)</sup> Also, most sphingolipids are hydrophobic, and are thus insoluble in aqueous solution. There have been no reports investigating the cancer-preventive effects of G<sub>1</sub>CM. Therefore, whether this effect is confined to colon cancer requires further investigation. In the present study, we found that G<sub>1</sub>CM inhibited proliferation of epithelial cells in ACF and BCAC. However, G<sub>1</sub>CM did not inhibit the growth of SW480 human colon carcinoma and HL60 human leukemia cell lines (unpublished data). Therefore, further studies are in progress to examine whether rat colonic tissues are exposed to the effective concentrations of G<sub>1</sub>CM or to examine cell permeability *in vitro*.

The specific targets of dietary sphingolipids have not yet been identified because sphingolipids affect several signaling pathways.<sup>(3,32)</sup> Schmelz *et al.*<sup>(33)</sup> demonstrated that sphingolipids cause a marked redistribution of the  $\beta$ -catenin protein from a diffuse (cytoplasm plus membrane) to a more normal (intercellular junctions) distribution in intestinal epithelial cells of C57BL/6JMin/+mice. We earlier demonstrated that the  $\beta$ -catenin signaling pathway plays a major role in short-term and long-term colon carcinogenesis models induced by a specific carcinogen.<sup>(22,34,35)</sup> In the present study, we found that dietary G<sub>1</sub>CM inhibited DMH-induced colonic BCAC formation. Under microscopic examination in a pilot study, BCAC is likely to display junctional localization of the  $\beta$ -catenin protein in crypt epithelial cells of BCAC exposed with G<sub>1</sub>CM (unpublished data). Thus, more detailed studies are required to further characterize possible effect of G<sub>1</sub>CM on the  $\beta$ -catenin signaling pathway.

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