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Research Article

The potential of sphingomyelin as a chemopreventive agent in AOM-induced colon cancer model: *wild-type* and *p53*$^{+/-}$ mice

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A protective effect of sphingolipids on colorectal cancer (CRC) has been reported in certain mouse strains. It is unknown if sphingolipids are protective in a *p53* deficiency mouse model of CRC. This study investigated the effect of sphingomyelin (SM) on intestinal sphingomyelinase (SMase) activity, colonic epithelial biology and azoxymethane (AOM)-induced CRC. Groups of *wild-type* (C57BL/6J) and *p53*$^{+/-}$ mice were fed 0.1% SM diet for 4 wk, administered a single AOM injection and then killed 6 h later to measure apoptosis and proliferation. Separately, both mouse types were fed 0.05% SM diet, administered three AOM injections and killed 33–38 wk later to measure tumour formation. SM significantly increased SMase activity and reduced proliferation ($p < 0.05$) in *wild-type* and *p53*$^{+/-}$ mice. SM did not regulate baseline apoptosis, apoptotic response to AOM or apoptosis in tumours, nor did it restore defective apoptosis in *p53*$^{+/-}$ mice. There was a nonsignificant trend to reduced tumour incidence with SM in *wild-type* ($p = 0.15$) and *p53*$^{+/-}$ ($p = 0.12$) mice. In conclusion, while increasing intestinal SMase activity and suppressing proliferation, SM did not promote any form of apoptosis and failed to achieve significant protection in these mice. Further investigation to understand the variable effect of SM in preventing CRC is warranted.

Keywords: Apoptosis / Carcinogen / Cell proliferation / Colorectal cancer / Sphingomyelin

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1 Introduction

There is an increasing interest to find dietary agents that protect against colorectal cancer (CRC), as this has been suggested as the most cost-effective long-term approach to protect against this disease [1]. Recently, dietary intake of sphingolipids inhibited early and late stages of colon carcinogenesis in mice treated with 1,2-dimethylhydrazine (DMH) [2–5] and reduced the number of tumours in all regions of intestine in *APC*$^{Min/+}$ mice (an inherited genetic defect) [6, 7]. As sphingolipids are found in commonly consumed food products, which include milk, egg, cheese, meat and other food [8–10], it is important to determine whether they are protective against CRC.

Intestinal epithelial cells are regularly exposed to sphingolipid metabolites as a consequence of their hydrolysis/digestion by sphingomyelinase (SMase) [11]. Metabolites, such as ceramide, sphingosine and sphingosine-1-phosphate are bioactive molecules, and may play important roles in colon tumorigenesis. For example, a significant decrease of SMase activity is found in sporadic CRC patients and in both adenomas and flat mucosa of patients with familial adenomatous polyposis [12–14]. Mutation of intestinal alkaline SMase is reported in colon cancer HT-29 cells [15, 16]. In addition, feeding ceramide analogues mimics the preventive effect of sphingomyelin (SM) [7, 17–19].

Sphingolipid metabolites have been proposed as important messengers in cellular functions for events as diverse as proliferation, differentiation and apoptosis [10, 20, 21]. The involvement of sphingolipids in cellular function and behaviour is well documented in many *in vitro* studies [22–25]. However, *in vivo* studies are limited and the underlying mechanisms of sphingolipids effect are unclear. The association of sphingolipids intake and reduced CRC risk has

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Abbreviations: AARGC, acute apoptotic response to genotoxic carcinogen; **ACF,** aberrant crypt foci; **AOM,** azoxymethane; **CRC,** colorectal cancer; **DMH,** 1,2-dimethylhydrazine; **PCNA,** proliferating cell nuclear antigen; **SM,** sphingomyelin; **SMase,** sphingomyelinase

been so far only based on few animal studies, using carcinogen azoxymethane (AOM) or DMH induced-rat/mouse and *APC*$^{Min/+}$ mouse models [2 – 7]. Thus more animal studies and additional animal models are necessary before the role of sphingolipids on CRC prevention is fully understood across a range of different molecular pathways.

The tumour suppressor gene *p53* plays an important role in intestinal homeostatic control by regulating DNA repair and apoptosis, thus maintaining genomic stability. Although evidence is emerging which suggests that cellular response to ceramide may be dependent or independent on *p53* status [18, 26, 27], the role of *p53* in ceramide-induced apoptosis is still not clear. We showed in our previous studies that administration of AOM to rodents is followed rapidly by an acute apoptotic response (termed acute apoptotic response to genotoxic carcinogen (AARGC)) in the distal colon which is *p53*-dependent [28 – 29]. We also showed that decreased AARGC in *p53* knockout mice is associated with increased susceptibility to AOM-induced colorectal oncogenesis demonstrating the importance of AARGC for controlling DNA damage. Moreover, protection against CRC by some agents is associated with their ability to upregulate AARGC. As such, the *p53* knockout mouse presents the opportunity to determine whether SM can also overcome defects in homeostatic control mechanisms and genomic instability subsequent upon *p53* dysfunction [28 – 31]. Therefore, the aim of this study was to test the protective effect of SM on intestinal SMase activity, colonic epithelial biology (homeostatic response to DNA damage) and tumour incidence using AOM-treated *wild-type* as well as *p53*$^{+/-}$ mice models.

2 Materials and methods

2.1 Reagents

AOM was purchased from Sigma (St. Louis, MO). SM (milk, bovine) was purchased from Avanti Polar Lipids (Alabaster, AL Avantilipids, USA).

2.2 Animals

Wild-type C57BL/6J male mice were obtained from Animal Resource Centre, Adelaide, South Australia. *p53*$^{+/-}$ male mice were bred at the Flinders Medical Centre using the original breeding pair (C57BL/6J strain) from the Jackson Laboratory (Bar Harbor, Maine, USA). Mice tail was processed for genotyping using PCR-based assays to determine *p53* status as described previously [32]. Mice were housed in cages (four *per* cage) and maintained in a temperature and humidity-controlled animal facility with a 12 h light – dark cycle. Mice were given free access to water, weighed weekly and were monitored closely for clinical signs of ill health throughout the study. Mice appearing sick were euthanased immediately. All protocols involving animals were approved by the Animal Welfare Committee at Flinders University and conducted according to their guidelines.

2.3 Diets

The experimental diets fed to the animals were based on the modified AIN-76A standard diet for rats and mice [33]. It contained 20% casein, 5% alpha cellulose, 20% sunflower oil, 20% sucrose, 30% corn starch, 3.5% mineral mix, 1% vitamin mix, 0.3% DL-methionine and 0.1% choline. The SM diet was made by adding 0.05 – 0.1% of SM to the control diet which was sphingolipid-free [2]. The diets were made fresh and kept in a sealed container at 4°C. SM (0.1%) was used for experiment 1 to study the effect of SM on colonic epithelial biology, as previous studies have found that SM at this level is protective in rodent models [5, 34]. However, 0.05% SM was used for experiment 2 to study the effect of SM on oncogenesis, as a trend towards weight loss was observed when feeding 0.1% of SM to *p53*$^{+/-}$ mice. The control groups were fed an AIN 76A diet without SM supplementation throughout the study.

2.4 Experimental procedure

2.4.1 Experiment 1: Effect of SM on AOM-induced DNA damage

Ten-wk old *wild-type* mice were randomized into four groups (n = 12/group) according to diet and AOM treatment. Mice that did not receive SM supplementation or AOM were used as a control group to examine baseline apoptosis and cell proliferation. The mice that received SM supplementation without AOM treatment were used to examine the effect of SM on baseline apoptosis and cell proliferation. The mice that received both SM supplementation and AOM treatment were used to examine the effect of SM on the AARGC. After 4 wks on SM or control diets, two groups of mice were given a single subcutaneous injection of AOM (10 mg/kg) to induce acute apoptosis in response to AOM-induced DNA damage, two groups did not receive AOM injection. Six hours following AOM, animals were killed by CO_2 induced asphyxiation, this being the time of maximal apoptotic response to AOM in the rodent [35]. Immediately following death, 2 cm of distal colon was rapidly removed, then placed in 10% paraformaldehyde overnight, before being changed to 70% ethanol. Tissue was embedded in paraffin. Transaxial sections of 5 μm thickness were taken for histological and immunohistological examination. Small intestine and the remaining colon were immediately frozen in liquid nitrogen for SMase activity assay.

10 wk old *p53*$^{+/-}$ mice were randomized into two groups (n = 12/group) according to diet treatments. After 4 wk on SM or control diets, all mice were given a single AOM injection. Animal euthanasia, tissue processing and section preparation were the same as for the method used for *wild-type* mice.

2.4.2 Experiment 2: Effect of SM on AOM-induced colon tumour formation

At 4 wk of age, *wild-type* and *p53*$^{+/-}$ mice were each randomized into four groups according to diet treatments (n = 19–25), all commencing at 4 wks of age. All mice received AOM s/c injections (10 mg/kg) once a week for 3 wk commencing at age 6 wk after 10 wk of SM or control diet. All mice remained on the same diet throughout the experiment until killed 30 wk after the last AOM injection for *wild-type* mice and 25 wk for *p53*$^{+/-}$ mice. Our previous studies showed that *p53*$^{+/-}$ mice are more susceptible to AOM-induced tumour formation in colon than *wild-type* mice [28]. Colon was immediately removed, opened longitudinally, flattered on hibond C paper and fixed in 10% paraformaldehyde overnight and examined for tumour outcome. Tumours were further examined histologically by H&E staining and histology assessed as previously [35].

2.5 Assay of SMase activity

The AmplexTM SMase Assay kit (Molecular Probes, Eugene, OR) was used for measuring total neutral and alkaline SMase activity in the mucosa of small intestine and colon [36]. Twelve small intestinal and colon samples from *wild-type* and *p53*$^{+/-}$ mice fed with SM or control diet were measured for SMase activity. In brief, the mucosa was obtained by scraping a 50 mm length of intestine using a glass slide. The mucosa was then homogenized in a buffer containing 0.25 M sucrose, 5 mM $MgCl_2$, 0.15 M KCl, 50 mM K_2HPO_4, 1 mM benzamidine and 6 mM taurocholate (pH 7.4) and centrifuged at 5000 rpm for 20 min at 4°C. The protein concentration in the supernatant fractions was measured using the BioRad Protein assay. The enzymatic hydrolysis of SM to ceramide by neutral SMase was measured in buffer containing 50 mM Tris, 0.15 M NaCl and 2 mM MgCl2 pH 7.4 for 30 min at 37°C, which involved a series of reactions. First, SMase hydrolyses the SM to yield cetrimide and phosphocholine. After the action of alkaline phosphatase, which hydrolyses phosphocholine, choline is oxidized by choline oxidized to betaine and H_2O_2. Finally, H_2O_2 in the presence of horseradish peroxidase reacts with Amplex Red to generate the highly fluorescent product, resorufin. The fluorescence intensity was measured immediately at 590 nm (excitation at 560 nm) using Fluostar Galaxy (BMG Labtechnologies). The activity of alkaline SMase was measured by the similar procedure as neutral SMase with higher pH buffer (containing 100 mM Tris, 0.15 M NaCl and 2 mM EDTA pH 9.0).

2.6 Detection and measurement of apoptosis

The frequency of epithelial cells undergoing apoptosis was determined on paraffin-embedded sections stained with haematoxylin [35]. Apoptotic cells were identified by characteristic morphology, which included cell shrinkage, nuclear condensation and formation of apoptotic bodies and/or a halo observed around apoptotic cells. Twenty complete crypts from distal colonic segments of each mouse were chosen and assessed by an independent observer who was unaware of the experimental condition at a magnification of 100×. The apoptotic index was calculated as the number of apoptotic cells *per* crypt column divided by the total number of cells in the column and multiplied by 100.

Apoptotic cells in tumours were quantified as described by us previously [28]. Briefly, a grid added to the ocular lens was used to count apoptosis in epithelial cells. Ten random grid fields were chosen for each tumour and counted by an independent observer who was unaware of the experimental condition. The total number of apoptotic cells from ten grid fields was summed, then the total number of apoptotic cells was divided by the total number of epithelial cells to give the apoptotic index. Observers were blinded to the experimental intervention.

2.7 Measurement of epithelial proliferation

Proliferative activity of epithelial cells was measured using immunohistochemical staining with antiproliferating cell nuclear antigen (PCNA) mAb (PC-10 clone, Santa Cruz, USA) [28]. In all cases, an independent observer who was unaware of the experimental conditions determined the quantification of PCNA-positive cells. The scoring for cell proliferation was the same as the method used to score apoptosis. PCNA-positive staining was determined by counting 20 separate crypts *per* section. Proliferation was expressed as cell turnover, *i.e.* proportion of cells stained by PCNA. The proliferation index was calculated as the number of 3,3′-diaminobenzidine (DAB)-positive cells stained by PCNA divided by the total number of cells on each crypt multiplied by 100.

Proliferative activity in tumour epithelial cells was measured by staining with PCNA antibody, and the proportion of PCNA-positive cells scored as for apoptosis in tumours.

2.8 Colon tumour analysis

Using a dissecting microscope, the colon was scored for tumour number and location by an independent observer who was unaware of experimental condition and mouse genotype. Tumour size was measured as the tumour size index (TSI) using the formula: Tumour size index = $\log10[\Sigma(\pi(-\text{diameter 1} + \text{diameter 2}))^2/2]$ [37]. Tumours were fixed in formalin, embedded in paraffin and sectioned (5 μm) for histologic examination by H&E staining and morphology assessed based on the criteria described previously [38].

2.9 Statistical analyses

Statistical analyses were performed using SPSS for Windows, version 10.0 (SPSS, Chicago, IL) or State version 8

for Windows. Data are expressed as means with standard errors of mean. Comparisons of apoptosis, PCNA-positive cells and SMase activity between SM and control diet groups were analysed using one-way ANOVA with correction for multiple comparisons by Tukey's *post hoc* test. All incidence data (the proportion of mice with tumours) were analysed with a generalized linear model to compare SM and control diet groups. A two group chi-square test with a 0.05 one-sided significance level has 80% power to detect the difference between a group 1 proportion of 20% and a group 2 proportion of 55% (35% difference) with a sample size in each group of 23. All nonparametric data (tumour multiplicity and tumour size index) were analysed using Mann–Whitney test. A probability value of $p = 0.05$ was used as the critical level for significance.

3 Results

3.1 Effect of the dietary SM on weight gain

In experiment 1, there was a slight but nonsignificant fall in body weight in $p53^{+/-}$ mice (22.5 g ± 2.5) fed the 0.1% SM diet compared to mice (26.4 g ± 2.0) fed control diet. There was no architectural change in the cells within the colonic crypt, such as loss of orientation of cells or nuclear atypia with disturbed cytoplasmic/nuclear ratio. In experiment 2, we changed the dose of SM from 0.1 to 0.05 g/100 g diet due to concern of possible further weight loss during the longer study period. There was no effect of SM (0.05%) intake on weight for either *wild-type* or $p53^{+/-}$ mice during experiment 2.

3.2 Effect of the dietary SM on SMase activity

Dietary SM intake significantly increased intestinal SMase activity in the small intestine and colon; for both neutral and alkaline SMase (Fig. 1A). This effect was found to be independent of genotype, as the changes in SMase activity were observed in both *wild-type* and $p53^{+/-}$ mice (Fig. 1B). Enzymes activity was significantly higher in the small intestine than in the colon, which is in agreement with other previous studies in animals and humans [39, 40].

3.3 Effect of the dietary SM on apoptosis

Dietary SM intake had no significant effect on baseline apoptosis in *wild-type* mice, *i.e.* in mice not given AOM, the baseline apoptotic index was 1.7% in mice fed SM diet, compared to 1.2% in mice fed control diet (Fig. 2A). Similarly, AARGC was not affected by SM in *wild-type* mice, it was 15.2% compared to 13.5% in mice fed the control diet (Fig. 2A). SM intake also did not affect apoptosis in tumours in *wild-type* mice (Fig. 3A).

In $p53^{+/-}$ mice, AARGC was 7.5% in mice fed SM diet compared to 9.2% in mice fed control diet, but again this

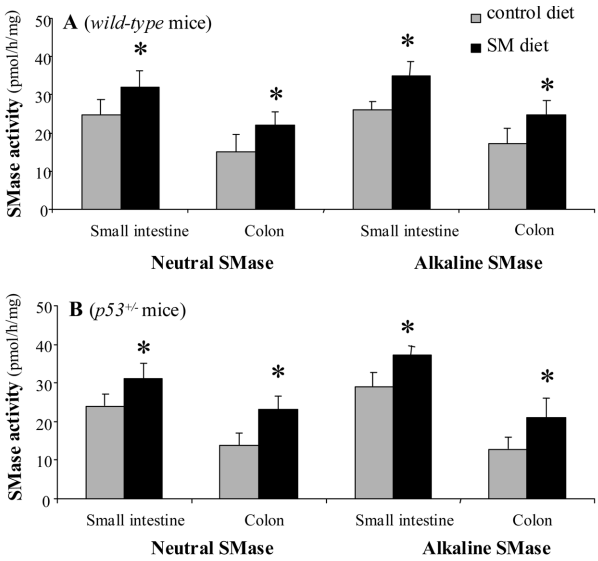

Figure 1. Increase of intestinal neutral and alkaline SMase activity by 0.1% dietary SM intake (4 wk). Data are mean ± SEM, $n = 12$. The increase of small intestine and colon SMase activity relative to control were significant at $^*p < 0.05$ for both *wild-type* (A) or $p53^{+/-}$ mice (B).

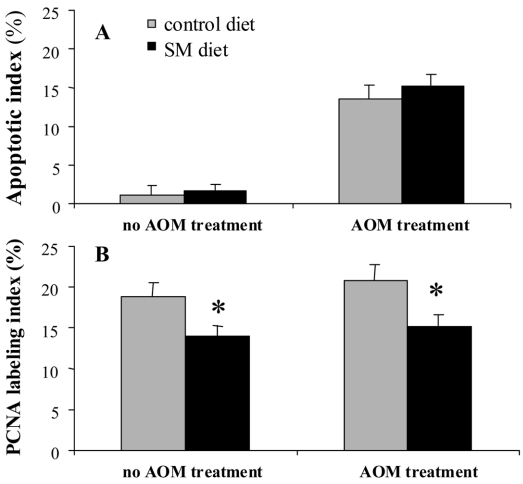

Figure 2. Apoptotic (baseline apoptosis and AARGC) and PCNA labelling index in distal colon of *wild-type* mice fed 0.1% SM or control diet. Data are mean ± SEM, $n = 12$. The suppression of cell proliferation in the low half of crypts relative to control was significant at $^*p < 0.05$ (B), but no effect of dietary SM on baseline apoptosis and AARGC was observed (A).

was not significant (Fig. 4). SM did not restore defective AARGC seen in $p53^{+/-}$ mice [39, 40]. There was also no significant effect of SM on rates of apoptosis in tumours in $p53^{+/-}$ mice (Fig. 3A).

3.4 Effect of the dietary SM on cell proliferation

Dietary SM intake significantly reduced the epithelial proliferation rate (PCNA index) in both *wild-type* and $p53^{+/-}$

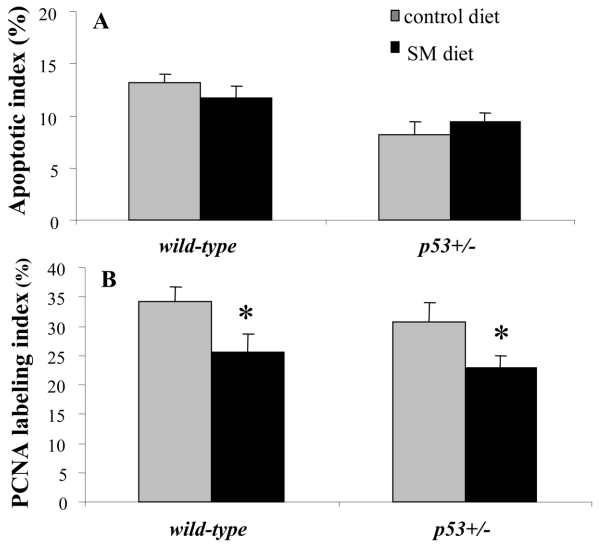

Figure 3. Apoptotic and PCNA labelling index in tumours of *wild-type* and $p53^{+/-}$ mice fed 0.5% SM or control diet. Data are mean ± SEM, $n = 12$. No effect of dietary SM on apoptosis was observed in either *wild-type* or $p53^{+/-}$ mice (A), although SM significantly suppressed cell proliferation. The suppression of cell proliferation in tumours by SM relative to control was significant at $^*p < 0.05$.

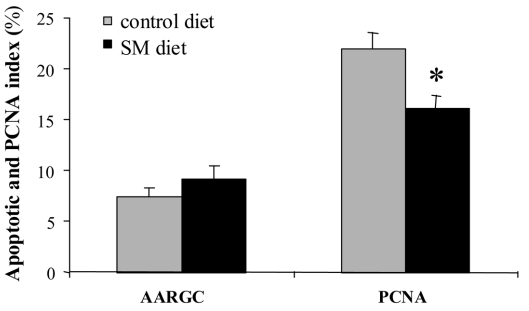

Figure 4. AARGC and PCNA labelling index in distal colon of $p53^{+/-}$ mice 0.1% SM or control diet. Data are mean ± SEM, $n = 12$. The suppression of cell proliferation in the low half of crypts relative to control was significant at $^*p < 0.05$, but the restore of defective AARGC in $p53^{+/-}$ mice relative to control was not significant. See Fig. 4 for the level of AARGC in *wild-type* mice.

mice. The PCNA index in *wild-type* mice on control diet was 18.8% without AOM administration and 20.8% with AOM administration. SM intake significantly decreased the PCNA index from 18.8 to 14% and 20.8 to 15.2%, respectively. The reduction was 24% in mice without AOM administration and 27% in mice with AOM administration, $p < 0.05$ (Fig. 2B). PCNA positive cells were mostly located in the lower half of the colonic crypts, where cells have been shown to divide. A significant effect of SM on proliferation rate was also found in tumours; PCNA index was 25.6% in *wild-type* mice fed SM diet compared to 34.2% in the control group; the reduction was 25%, $p < 0.05$ (Fig. 3B). Loss of *p53* had no effect on intestinal epithelial cell proliferation, and SM intake reduced the PCNA index from 22% in $p53^{+/-}$ mice fed control diet to 16% in $p53^{+/-}$ mice fed SM diet, the reduction being 28% ($p < 0.05$) (Fig. 4). Similarly, a significant reduction in cell proliferation was seen in tumours; it was 22.8% in $p53^{+/-}$ mice fed SM diet and 30.8% in $p53^{+/-}$ mice fed control diet, $p < 0.05$ (Fig. 3B).

3.5 Effect of the dietary SM on colon tumour formation

The effect of dietary SM intake on AOM-induced colon tumour incidence, multiplicity and size in *wild-type* and $p53^{+/-}$ mice is summarized in Table 1. SM administration produced a trend to lower tumour incidence in *wild-type* as well as $p53^{+/-}$ mice but this failed to reach significance. The tumour incidence in *wild-type* and $p53^{+/-}$ mice fed with control diet was 36% and 52.6%, consistent with increased genomic instability and susceptibility to AOM in the $p53^{+/-}$ mice. SM intake reduced the tumour incidence to 24% and 38.1% respectively. *Post-hoc* power calculations, at these levels of difference, showed that group sizes of at least 198 would be needed to achieve significance.

The risk ratio for developing colon tumours in the *wild-type* and $p53^{+/-}$ mice relative to the controls was 0.75 and 0.74 and the corresponding 95% confidence intervals were 0.3 – 1.8 and 0.3 – 1.6. These trends also failed to reach statistical significance ($p = 0.15$ for *wild-type* mice and $p = 0.12$ for $p53^{+/-}$ mice). There were also a trend to reduced tumour multiplicity and tumour size in mice fed

Table 1. Effect of dietary SM intake on AOM-induced colon tumour formation in *wild-type* and $p53^{+/-}$ mice

Genotype	No. of mice	Diet	Incidence	p^c	Tumour multiplicity (Mean ± SEM)	p^c	Tumour size index (Mean ± SEM)	p^c
wild-type	25	Control	9/25 (36%)		0.57 ± 0.23		0.60 ± 0.22	
wild-type	25	SM	6/25 (24%)	0.15	0.42 ± 0.31	0.14	0.35 ± 0.18	0.18
$p53^{+/-}$	19	Control	10/19 (52.6%)		0.74 ± 0.29		0.79 ± 0.15	
$p53^{+/-}$	21	SM	8/21 (38.1%)	0.12	0.52 ± 0.25	0.17	0.52 ± 0.27	0.12

A generalized linear model was used for tumour incidence.Mann-Whitney test was used for tumour multiplicity and tumour size index. p^c, significance *versus* control mice. No significant differences were found in tumour incidence, tumour multiplicity or tumour size index between mice fed SM and control diet.

the SM diet relative to control diet in both *wild-type* and $p53^{+/-}$ mice. Most of the tumours were found in the middle or distal region of the colon, with similar incidence of adenomas and carcinomas in mice fed with either control or SM diet (data not shown).

4 Discussion

In this study, dietary supplementation of SM in *wild-type* and $p53^{+/-}$ mice increased intestinal mucosal neutral and alkaline SMase activity. The activation of alkaline SMase may be important for CRC prevention as it is expressed specifically in human and animal intestinal tract and is the key enzyme responsible for digestion of dietary SM [39–41]. Previous studies suggested that there is a direct correlation between the level of ceramide in the colonic mucosa and the amount of SM intake [9]. Mass spectrometry analysis of dietary sphingolipids hydrolysis in the intestinal mucosa show that most dietary sphingolipids are digested over time in parallel with substantial production of ceramide and sphingosine [5, 39, 42]. Our results indicate that by feeding SM to animals, SM metabolic products are likely to be delivered to the intestinal epithelial cells following activation of SMase.

There is great interest in the role that SM and its metabolites may play in regulating apoptosis, as a mechanism for protecting against CRC [11, 17, 43–46]. Although the link between SM and increased apoptosis has been shown in *in vitro* studies, the results of *in vivo* studies are inconclusive. Lemonnier *et al.* [34] showed that dietary intake of SM normalized apoptosis, but not beyond the level of control group. Other studies have shown no difference between the number of apoptotic cells in the colonic epithelium of control and SM diet [5, 34, 47]. With respect to the potential of dietary SM on preventing tumour initiation, our particular interest was to examine whether SM regulated the apoptotic response to DNA damage (AARGC), which is important for eliminating DNA-damaged cells during tumour initiation [35]. Our previous studies have shown that defective AARGC is associated with increased risk for CRC in $p53^{+/-}$ and $p53^{-/-}$ mice [28, 29], and that up-regulation of AARGC by a variety of dietary agents and drugs is associated with protection for CRC [29, 35, 48–52]. In keeping with earlier studies [5, 34, 47], our study showed that SM intake (0.05–0.1%) did not affect baseline apoptosis. Furthermore, it did not increase AARGC or apoptosis in tumours nor did it restore defective AARGC characteristic of $p53^{+/-}$ mice. The lack of significant effect of SM on AARGC is perhaps surprising, given that an intrinsic function of the SM pathway is to respond to a variety of stress signals, whether environmental or pharmacologic [53, 54]. As we did not observe an effect of SM on any form of apoptosis measured, this suggests that regulation of apoptosis would not seem to be a primary mechanism by which SM might prevent CRC. The reason for a lack of effect on apoptosis is not clear, as metabolites of SM include the proapoptotic ceramide and sphingosine. However, sphingosine-1-phosphate on the other hand has an opposite effect by promoting cell survival and inhibiting apoptosis [10]. It is possible that the balance between SM metabolites in colonic epithelial cells may also be important in this regard and further investigation is needed.

Sphingolipid metabolites are also considered major antiproliferative molecules that might regulate tumorigenesis [6]. *In vitro* studies using HT-29 colon carcinoma cells have shown that SMase inhibits proliferation in both dose dependent and time-dependent manners without inducing apoptosis [24]. In animal studies, dietary sphingolipids suppress cell proliferation and formation of aberrant crypt foci (ACF) [6, 12, 19, 47]. Lemonnier *et al.* [34] reported that SM reduced proliferation to the normal level but did not decrease it any further. The decrease in proliferation is more evident in the upper half of crypt than in the lower half of the crypt [3–5]. In agreement with these reports, an antiproliferative effect of dietary SM was observed in our study, suggesting the level of SM (0.1%) administrated was sufficient to influence sphingolipid metabolism, epithelial function and behaviour. Importantly, we found inhibition of proliferation was primarily in the base of the crypt, where initiating mutational events have the greatest impact on tumour development [55]. Furthermore, reduced cell proliferation was found in tumours, which may account for the trend to reduced tumour size in mice fed the SM diet (0.05%) relative to control diet. This would be consistent with SM having a continuing antiproliferative effect throughout the processes of oncogenesis. Thus, inhibition of cell proliferation rather than induction of apoptosis might be relevant to any protective effect of SM.

We found a nonsignificant trend to reduced tumour incidence with SM in both *wild-type* ($p = 0.15$) and $p53^{+/-}$ ($p = 0.12$) mice. This was found to be the case for all measures of tumour burden. Several other animal studies have shown protection by sphingolipids. Lemonnier *et al.* [34] report that long-term intake of sphingolipids (0.05%) inhibited colonic tumour incidence by 60%. Approximately 50% inhibitory effect on ACF was found for milk SM and synthetic SM in short-term studies [4, 5]. The reason for a less clear effect of dietary SM on tumour suppression in our studies is not clear, especially as we did see an increase in SMase activity and a reduction in colonic epithelial proliferation. Tumour susceptibility to AOM is strain-dependent; C57BL/6J mice, the background strain for the knockout mice in this study, are susceptible although more susceptible strains such as A/J mice have been described [56, 57]. Whether strain differences confer different susceptibilities to SM, perhaps because active SM metabolites vary between strains, is unclear but possible and should be addressed in future studies. Sphingolipids have been tested in DMH-induced colon cancer models on CF1 mice and

F344 rats for their anticancer effects [2 – 5, 47] while this is the first study to test SM in AOM-induced CRC in C57BL/6J mice. Differences in SM metabolism might account for the difference and this would raise major questions about the broad applicability of SM as a preventive agent.

One important factor that may contribute to the effectiveness of SM in colon cancer risk is the dietary concentration of SM. Previous animal studies have added sphingolipids to the diet at intakes of 0.025 – 0.1% with inconsistent results on ACF and tumour incidence. While some studies showed sphingolipids exhibited a dose dependent inhibition [7, 19, 58], some studies showed no difference between intakes of 0.025 and 0.1% [3, 5]. It is possible that high intakes of SM (0.1%) might produce better protection at least in *wild-type* mice, as at this level SM significantly inhibited cell proliferation without causing weight loss. Factors that affect intestinal epithelial SMase pathway and absorption may also influence the efficacy of sphingolipids. Thus, further studies are required to determine the concentration and efficacy of dietary sphingolipids with regard to their ability to prevent colon tumours.

One of our interests of this study was to determine whether SM was protective in the context of genomic instability associated with *p53* dysfunction. This is the case for $APC^{Min/+}$ mice, where the specific genetic defects seem to be bypassed or normalized by SM [7]. The fact that SM activates SMase activity, and inhibits cell proliferation in $p53^{+/-}$ mice, together with its protection in $APC^{Min/+}$ mice [7, 58, 59] suggest that the protective effect of SM is not limited to the chemically (carcinogen) induced CRC animal model [2 – 5]. Therefore it remains plausible that dietary SM consumption might also have potential to influence human CRC risk.

In conclusion, dietary SM increases intestinal SMase activity and reduces epithelial proliferation but does not enhance apoptosis, a proposed mechanism for protection. While suppressing epithelial proliferation, SM failed to achieve significant protection against CRC in AOM-treated *wild-type* and $p53^{+/-}$ mice, despite showing a trend to protection. Further studies are required to better understand the context in which protection might be achieved and whether varying metabolism of dietary SM is important. Therefore, the efficacy of milk SM and indeed other sources of SM, need further investigation with regard to their ability to prevent colon tumorigenesis.

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The authors have declared no conflict of interest.

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