

## Anti-tumor Effect of Chemically Synthesized Sulfolipids Based on Sea Urchin's Natural Sulfoquinovosylmonoacylglycerols

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We recently reported that 3'-sulfoquinovosyl-1'-monoacylglycerol (designated A-5) extracted from sea urchin intestine was effective in suppressing the growth of solid tumors. Although the major fatty acid component of A-5 was a saturated C<sub>16</sub> acid, there were five other fatty acids, 14:0, 18:0, 14:1, 16:1, and 18:1, which constitute minor components of A-5. Therefore, it remains unclear as to which of these six fatty acid components of A-5 has the anti-tumor effect. In this study, we synthesized sulfolipids each containing only one of these six fatty acids and tested their cytotoxicity against tumor cells and *in vivo* anti-tumor effects on nude-mice bearing solid tumors of human lung adenocarcinoma cell line A-549. The IC<sub>50</sub> values of all products against tumor cells were more than 10<sup>-5</sup> M, suggesting weak cytotoxic activity compared with other chemotherapeutic compounds for cancer. On the other hand, *in vivo* anti-tumor assay showed that sulfoquinovosylmonoacylglycerols (SQMG) composed of 14:1 and 18:1 (designated SQMG(14:1) and SQMG(18:1), respectively) were significantly effective in suppressing the growth of solid tumors. Our data suggested that these two SQMGs had a substantial anti-tumor effect *in vivo*, and they are of interest as candidate drugs for anti-cancer treatment.

Key words: Glycolipid — Sulfoglycolipid — Anti-tumor effect — Cancer chemotherapy

Recently, bioactive substances derived from marine invertebrates have shown particular promise as a new source of anti-tumor drugs. Many researchers have been investigating toxic substances produced by marine invertebrates that could be applicable for destroying tumors. The successful extraction of anti-tumor agents such as didemnin,<sup>1)</sup> bryostatin<sup>2)</sup> and dolasstatin<sup>3)</sup> has been reported and these compounds have reached clinical trial. Gustafson and colleagues reported that D-sulfoquinovosylglycerol from blue-green algae possessed anti-viral activity against the human immunodeficiency virus (HIV-1) and cytotoxicity against human lymphoid cells.<sup>4)</sup> Thus, sulfolipids from marine invertebrates merit further investigation.

We recently reported that sulfoquinovosylmonoacylglycerol (designated A-5) extracted from sea urchin intestine was effective in suppressing the growth of solid tumors.<sup>5)</sup> This was the first paper to report that sulfolipid possesses anti-tumor activity *in vivo*. Pathologically, the solid tumors showed hemorrhagic necrosis after treatment with A-5. Although the major fatty acid component of A-5 was a saturated C<sub>16</sub> acid, there were five other fatty acids,

14:0, 18:0, 14:1, 16:1, and 18:1, which constitute minor components of A-5. Therefore, it remains unclear which of these six fatty acids component(s) has the anti-tumor effect. In this study, we chemically synthesized sulfolipids each containing only one of these six fatty acids, and determined their anti-tumor effects *in vivo*. Our data indicated that sulfoquinovosylmonoacylglycerols (SQMGs) containing 14:1 and 18:1 were active as anti-tumor agents.

### MATERIALS AND METHODS

**Synthesis of sulfolipids** The chemical structures of the synthesized compounds are summarized in Fig. 1 and the synthetic procedure is shown in Fig. 2. The synthetic SQMG containing the fatty acids 14:0 (myristic acid), 16:0 (palmitic acid), 18:0 (stearic acid), 14:1 (myristoleic acid), 16:1 (palmitoleic acid), or 18:1 (oleic acid) were designated as SQMG(14:0), SQMG(16:0), SQMG(18:0), SQMG(14:1), SQMG(16:1) and SQMG(18:1), respectively (Fig. 1). Carbon chain lengths of fatty acid (*n*) with saturation (0) or mono-unsaturation (1) are abbreviated as *n*:0 or *n*:1.

**Cell line** Human adenocarcinoma A-549 cells from lung cancer were provided by the Japanese Cancer Research

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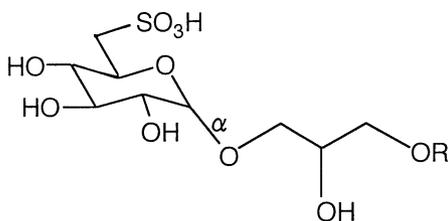


Fig. 1. Structures of sulfolipids. Synthetic sulfoquinovosylmonoacylglycerols (SQMGs) contain a single fatty acid, R= 14:0, 16:0, 18:0, 14:1, 16:1 or 18:1. These are designated as SQMG(14:0), SQMG(16:0), SQMG(18:0), SQMG(14:1), SQMG(16:1) or SQMG(18:1), respectively.

Resources Bank. Cells were cultured in Eagle's minimum essential medium (MEM) (Nissui Co., Tokyo) supplemented with 5% fetal calf serum (FCS) and 2 mM L-glutamine (Gibco, Grand Island, NY).

**MTT assay** MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed using A-549 cell lines according to the method described previously by Takahashi *et al.*<sup>6)</sup> Briefly, cells ( $5 \times 10^3$ /well) were cultured in 96-well plates for 24 h and then various amounts of samples suspended in phosphate-buffered saline (PBS) were added to the wells. In the control, PBS was added to wells at a volume of 10% of the medium. Following cultivation for 48 h, 50  $\mu$ g of MTT was added to the culture medium and incubation was continued for 3 h. Then, 4% HCl in 2-propanol was added to each well and mixed by pipette to disrupt the cells. The absorbance of each well was measured using a multiwell scanning photometer (Micro ELISA MR600, Dynatech Laboratories, Inc., Alexandria, VA) at a wavelength of 570 nm.  $IC_{50}$ s were calculated by curve fitting.

In this study, we employed six synthetic sulfolipids, SQMG(14:0), (16:0), (18:0), (14:1), (16:1) and (18:1) and prepared three mixtures of sulfolipids, M6, M30 and M31. M6 was a mixture of SQMG(14:0), (16:0), (18:0), (14:1), (16:1) and (18:1) each at equal molecular volume. M30 and M31 were mixtures of SQMG(14:0), (16:0) and (18:0), and SQMG(14:1), (16:1) and (18:1), respectively, each at the same molecular volume.

**In vivo assessment of anti-tumor assay** A-549 cells ( $5 \times 10^5$  cells/mouse) were injected subcutaneously into female BALB/c *nu/nu* mice (20–22 g; 7 weeks of age). At 37 days after implantation, the tumor sizes in all of these mice were measured at 3-day intervals. The mice bearing solid tumors that had grown to 25–35 mm<sup>3</sup> in volume (tumor volume=length  $\times$  (width)<sup>2</sup>  $\times$  0.5) at 43 days after implantation were used for the assessment of anti-tumor effect. They were divided randomly into 18 groups ( $n=4$ /group). One of the 18 groups was a control group injected with 0.1 ml of PBS and 6 groups were injected

with SQMG(14:0), SQMG(16:0) and SQMG(18:0) at a dose of 4 or 20 mg/kg (3 groups each). Another 7 groups comprised 3 groups injected with SQMG(14:1), SQMG(16:1) or SQMG(18:1) at 4 mg/kg; 3 groups injected with the same SQMGs at 20 mg/kg and a control group injected with 0.1 ml of PBS alone. The above administrations all took place between days 43 to 64 subsequent to implantation.

In some experiments, to investigate the synergistic effects of sulfolipids, we employed mixtures of sulfolipids, M6, M30 and M31, and performed an *in vivo* anti-tumor assay using 4 groups ( $n=4$ /group). Three groups were injected with M6, M30 and M31 at a dose of 24, 12 and 12 mg/kg, respectively, and the 4th group with 0.1 ml of PBS alone as a control for a period of 43–64 days after implantation. All the above-mentioned sulfolipid doses correspond to each single application; for example, 4 mg/kg every 3 days for the SQMG(14:0) group.

All mice were injected subcutaneously 8 times at 3-day intervals with these compounds and PBS alone. Tumor growth was measured at 3-day intervals for 70 days after implantation, and the statistics were analyzed by using Student's *t* test. At the end of the assay, some mice treated with sulfolipids and PBS were separately examined to observe pathohistological features of the tumors and of major organs such as lung, heart, spleen, stomach, liver, pancreas, kidney, intestine and brain.

## RESULTS

**Cytotoxicity of sulfolipids *in vitro*** The synthetic sulfolipids, SQMG(14:0), SQMG(16:0), SQMG(18:0), SQMG(14:1), SQMG(16:1) and SQMG(18:1) were added to A-549 cells at doses of 1, 25, 50 and 100  $\mu$ g/ml, and cell viability was measured by means of MTT assay. The experiments on the growth-inhibitory effects of these sulfolipids against A-549 cells were repeated 3 times. As shown in Fig. 3A, the  $IC_{50}$  values of SQMG(16:0) and SQMG(18:0) were 83.5 and 75.5  $\mu$ g/ml, respectively. These values correspond to approximately  $5 \times 10^{-5}$  M. Since the inhibitory activity of SQMG(14:0) was 69.75% at 100  $\mu$ g/ml concentration, its  $IC_{50}$  value was not determined. However, its activity appeared to be dose-dependent. On the other hand, the inhibitory activities of SQMG(14:1), SQMG(16:1) and SQMG(18:1) were rather weak, and not dose-dependent, with percent growth of 76.0, 55.5 and 60.5, respectively, at 100  $\mu$ g/ml concentration (Fig. 3B)

The  $IC_{50}$  values of M6 and M30 were 47.8 and 59.3  $\mu$ g/ml, respectively (Fig. 3C). The growth-inhibitory activity of both compounds appeared to be dose-dependent. The activity of M31 was as weak as those of SQMG(14:1), (16:1) and (18:1), and was not dose-dependent, with 66% growth at 100  $\mu$ g/ml concentration (Fig. 3C). When M6

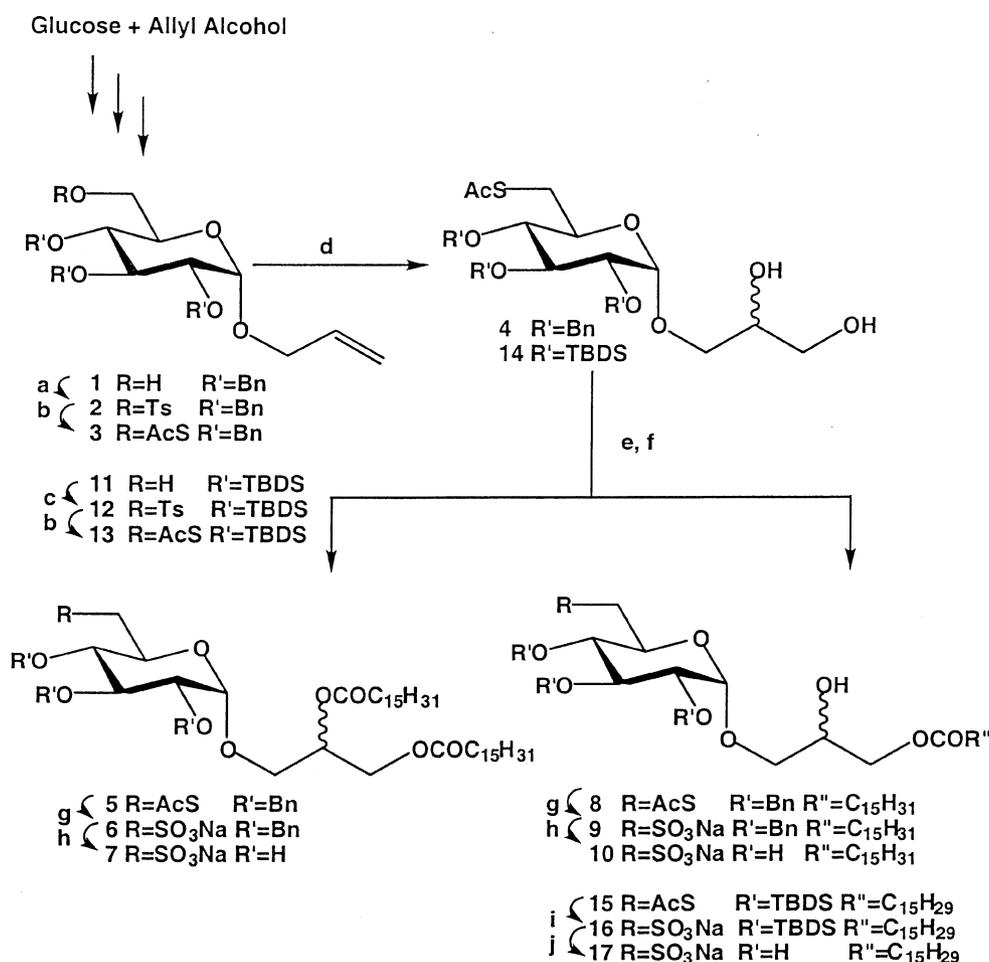


Fig. 2. The syntheses of  $\alpha$ -anomers of SQDG and SQMG. Benzyl groups (Bn: C<sub>6</sub>H<sub>5</sub>CH<sub>2</sub>-) were employed to protect the hydroxyls at C2, 3 and 4 of the glucose moiety during the synthesis of sulfolipids bearing saturated fatty acids at C1 and/or C2 on the glycerol. This figure shows an example of the reagents, reaction conditions and chemical yields for the syntheses of SQDG and SQMG. 4,6-*O*-Benzylidene-1-*O*-allyl- $\alpha$ -D-glucose was prepared from 1-*O*-allyl-D-glucose and benzaldehyde, and the crude crystalline material was recrystallized from ethanol (EtOH) to eliminate contamination with the  $\beta$ -anomer. To introduce sulfonic acid at C6 of glucose, the hydroxyl group at C6 was protected with a trityl (triphenylmethyl) group, and then the benzyl group was introduced at C2, 3 and 4. After replacement of the trityl group with tosylate (*p*-toluenesulfonate) in two steps, the thioacetate (**3**) at C6 was obtained by the reaction of potassium thioacetate (KSAc). Oxidation with osmic acid (OsO<sub>4</sub>) afforded the glycol (**4**). The fatty acids were installed into the glycerol moiety with EDCI (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide) and DMAP (4-dimethylaminopyridine). The resultant mixture of diacylglyceride (**5**) and monoacylglyceride (**8**) was separated by column chromatography to afford SQDG and SQMG, respectively. The oxidation of thioacetate by OXONE (2KHSO<sub>5</sub>, KHSO<sub>4</sub>, K<sub>2</sub>SO<sub>4</sub>) afforded the sulfonic acid in 13% to 80% chemical yield, depending on the fatty acid (**6** and **9**). Finally the benzyl groups were removed by means of catalytic hydrogenation to afford **7** and **10**. In the case of unsaturated fatty acids, TBDMS (*t*-Bu(CH<sub>3</sub>)<sub>2</sub>Si-) groups were used for protection, because catalytic hydrogenation with H<sub>2</sub> on Pd/C caused hydrogenation of unsaturated fatty acids. (a) TsCl, pyridine, DMAP, room temperature, **2**: 91%. (b) KSAc, EtOH, reflux, **3**: 87%; KSAc, HMPA, room temperature, **13**: 80%. (c) TBDMSOTf, 2,6-lutidine, **12**: 92%. (d) OsO<sub>4</sub>, NMO, *tert*-BuOH, water, room temperature, **4**: 68%, **14**: 75%. (e) C<sub>15</sub>H<sub>31</sub>COOH, EDCI, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, room temperature, 73% (**5**: 36%, **8**: 37%). (f) C<sub>15</sub>H<sub>29</sub>COOH, EDCI, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, room temperature, **15**: 83%. (g) OXONE, KOAc, HOAc, room temperature, **6**: 76%, **9**: 83%. (h) H<sub>2</sub>, Pd/C, EtOH, room temperature, **7**: 83%, **10**: 71%. (i) OXONE, KOAc, HOAc, room temperature, **16**: 13%. (j) TFA, AcOH, THF, H<sub>2</sub>O, room temperature, **17**: 80%.

was added to cells at a dose of 50  $\mu$ g/ml, the concentrations of SQMG(14:0), (16:0) and (18:0) each corresponded to approximately 8.3  $\mu$ g/ml. In the case of

addition of M30, each sulfolipid corresponded to approximately 16.6  $\mu$ g/ml. As shown in Fig. 3A, the inhibitory activities of individual sulfolipids were weaker than those

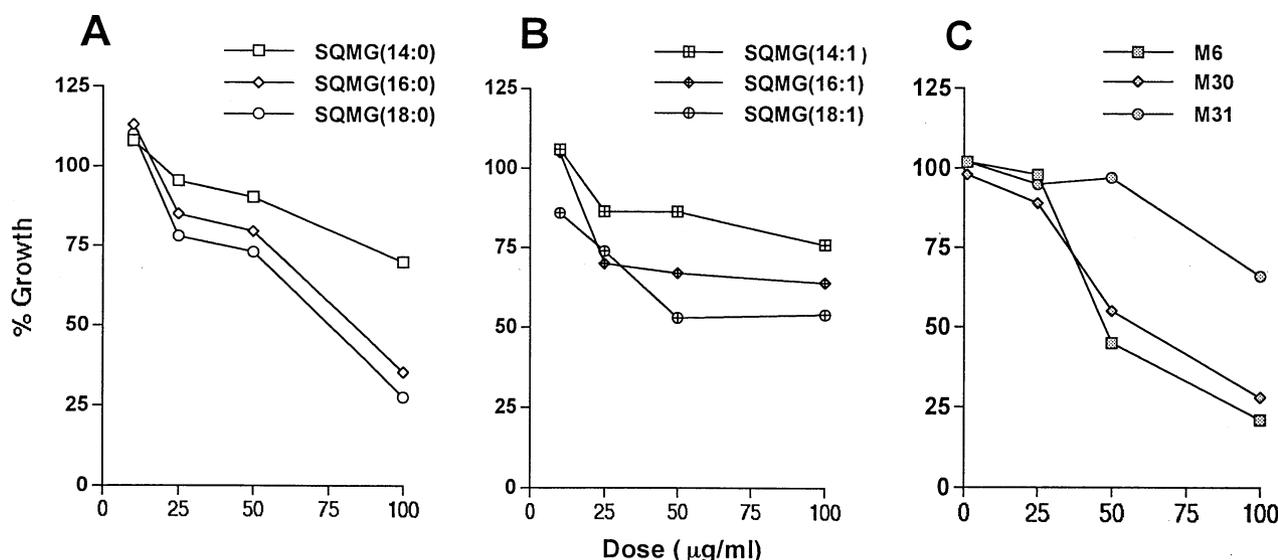


Fig. 3. *In vitro* study of growth-inhibitory activity of synthetic sulfolipids. A, B and C, the A-549 tumor growth-inhibitory activity of each sulfolipid and mixtures of sulfolipids. SQMG(14:0), (16:0), (18:0), (14:1), (16:1), (18:1), M6, M30, and M31 were added to wells of cultured A-549 cells at doses of 1, 25, 50 and 100 µg/ml, and examined 3 times by MTT assay to determine inhibitory activity (one of the 3 trials is shown).

of the mixtures at these low concentrations. Therefore, these data suggested that a synergistic effect was produced by mixtures of sulfolipid bound-saturated fatty acids *in vitro*.

***In vivo* anti-tumor effect of synthetic sulfolipids** At 43 days after implantation of A-549 cells, the mice bearing a solid tumor of 25–35 mm<sup>3</sup> were injected with synthetic sulfolipids at 3-day intervals until 64 days. As shown in Fig. 4, A, B and C, mice injected with SQMG(14:0), SQMG(16:0) and SQMG(18:0) did not show significant suppression of tumor growth at 70 days as compared to the control group. None of the mice showed any significant loss of body weight throughout the experimental period (data not shown).

On the other hand, mice treated with SQMG(14:1) at doses of 4 or 20 mg/kg showed significant growth suppression of tumors at 70 days ( $P=0.0007$  or  $P=0.000016$ , respectively) as compared to mice in the control group, and the effect was dose-dependent (Fig. 4D). These mice also had no loss of body weight throughout the treatment period (data not shown). In the SQMG(18:1) group administered 4 or 20 mg/kg, significant tumor growth suppression was also seen ( $P=0.0106$  or  $P=0.000012$ , respectively) (Fig. 4F). In this group, although tumor growth was still suppressed at a dose of 4 mg/kg throughout the period of injection, the tumor growth rate increased at 67 and 70 days after tumor implantation. However, in mice administered 20 mg/kg, tumor growth was suppressed throughout the period from the beginning

of drug injection to the end of the assay. The tumor growth was not drastically inhibited in the SQMG(16:1) group (Fig. 4E).

As shown in Fig. 4G, mice treated with M6 and M31 showed significant growth suppression of tumors at 70 days ( $P=0.01371$  and  $P=0.00732$ , respectively) as compared to mice in the control group. It seemed that the suppressive effects of M6 and M31 were weaker than that of a single application of SQMG(14:1) and (18:1) at a dose of 4 mg/kg. Since M6 included at least one of either SQMG(14:1) or (18:1) at 4 mg/kg, these data suggested that there were no synergistic effects. Although M30 possessed growth inhibitory effects *in vitro*, mice injected with M30 did not show significant suppression of tumor growth at 70 days as compared to the control group. None of the mice showed any significant loss of body weight throughout the experimental period (data not shown).

Although the cytotoxic activities of SQMG(14:1) and SQMG(18:1) were very weak *in vitro*, both agents clearly showed a suppressive effect against solid tumor *in vivo*. To understand the mechanism of the suppressive effects at the tumor sites, we assessed the pathological findings of the tumors treated with SQMG(14:1) at a dose of 20 mg/kg in comparison with SQMG(14:0) at the same dose, and a control. The tumors treated with SQMG(14:1) showed much more extensive hemorrhagic necrosis, as compared to the controls (Fig. 5, A and B). Only a small degree of hemorrhagic necrosis was observed in the tumors treated with SQMG(14:0) (Fig. 5C). Tumor-infiltrating lymphoid

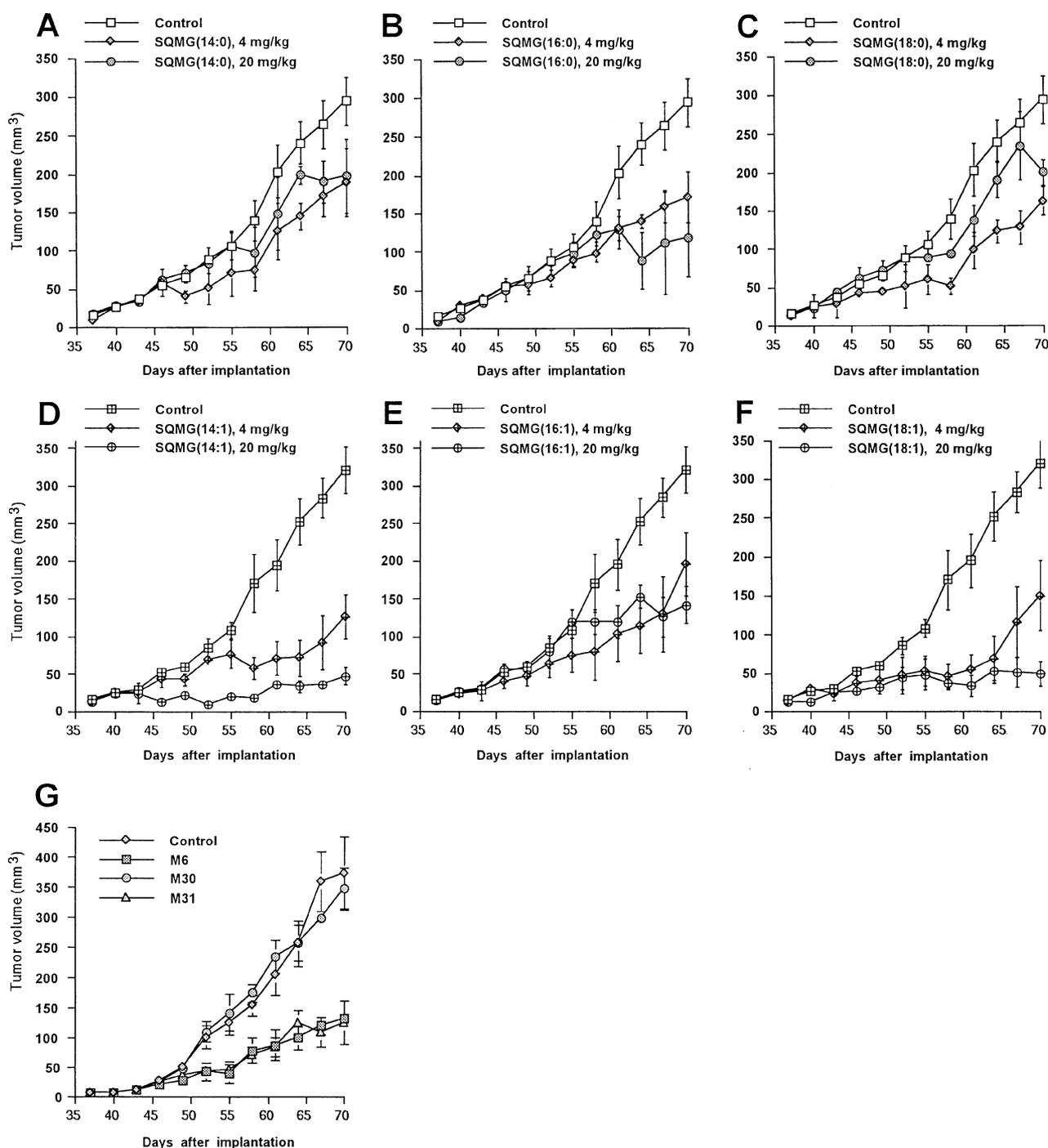


Fig. 4. *In vivo* study of anti-tumor effects of synthetic sulfolipids. To elucidate whether synthetic sulfolipids have anti-tumor effects, A-549 cells ( $5 \times 10^5$  cells/mouse) were injected s.c. into nude mice, and the mice bearing A-549 solid tumors that had reached 25–35 mm<sup>3</sup> in volume were used. In panels A, B and C, twenty-eight mice were divided into seven groups ( $n=4$ /group); mice were injected with PBS only as a control group and the other groups were injected with SQMG(14:0), SQMG(16:0) and SQMG(18:0) in PBS at a dose of 4 or 20 mg/kg. In panels D, E and F, twenty-eight mice were divided into seven groups ( $n=4$ /group); mice were injected with PBS only as a control group and the other groups were injected with SQMG(14:1), SQMG(16:1) and SQMG(18:1) in PBS at a dose of 4 or 20 mg/kg. In panel G, sixteen mice were divided into four groups ( $n=4$ /group); mice were injected with PBS only as a control group and the other groups were injected with M6, M30 and M31 in PBS at a dose of 24, 12 and 12 mg/kg, respectively. Mice were injected s.c. with PBS or synthetic sulfolipids 8 times at 3-day intervals for the period of 43–64 days after implantation. The mean ( $\pm$ SE) tumor volume from each group is shown.

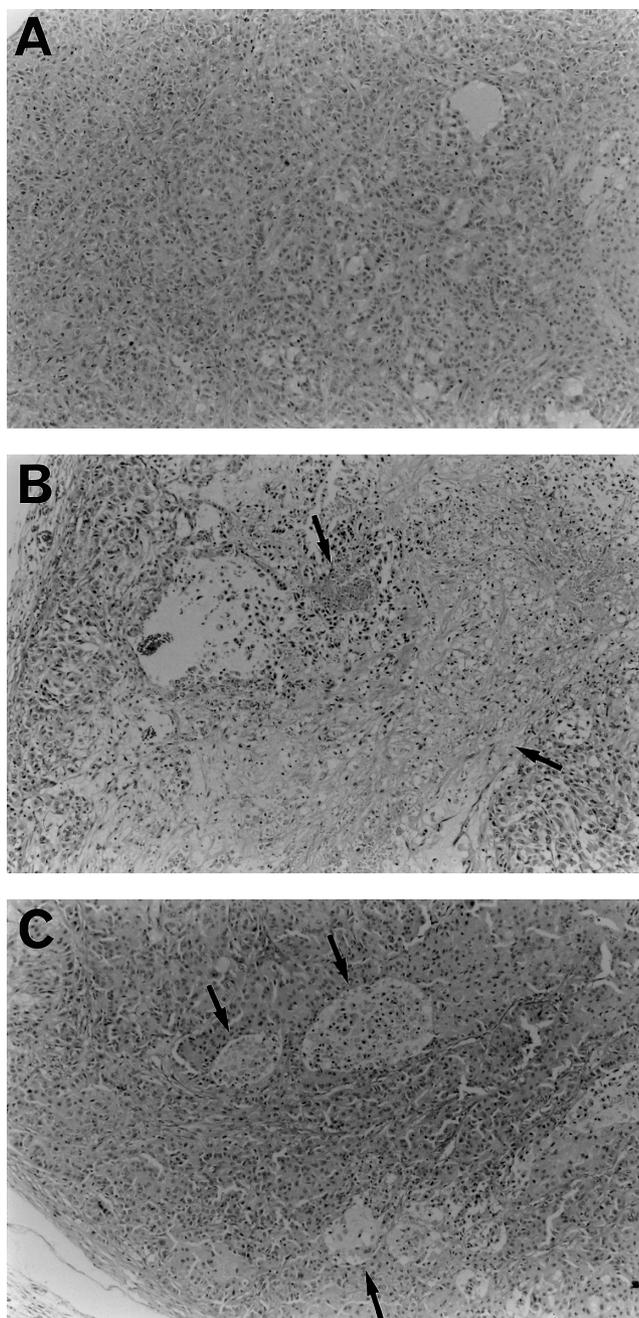


Fig. 5. Hematoxylin/eosin (HE) staining of A-549 solid tumors of mice treated with SQMG(14:1) and SQMG(14:0) at a dose of 20 mg/kg at 70 days after implantation. (A) a microscopic view (original magnification,  $\times 50$ ) of the tumor in a control mouse; (B) a microscopic view (original magnification,  $\times 50$ ) of the tumor in a mouse treated with SQMG(14:1). An area of massive hemorrhagic necrosis is indicated by arrows; (C) a microscopic view (original magnification,  $\times 50$ ) of the tumor of a mouse treated with SQMG(14:0). There are a few small areas of hemorrhagic necrosis, indicated by arrows, as compared to the controls. Tumor-infiltrating lymphoid cells were not markedly increased by treatment with these SQMGs.

cells were not markedly increased by treatment with these SQMGs. It was also noted that the main visceral organs, such as lung, heart, spleen, stomach, liver, pancreas, kidney, intestine and brain, of all the groups showed no toxic or degenerative histological appearance (data not shown).

## DISCUSSION

It is well known that glycolipids, including sulfolipids, constitute important structural components of the cell membrane. However, accumulating evidence suggests that glycolipids act not only as structural components, but also can play certain physiological roles. For example, lysosphingolipids regulate protein kinase C activity,<sup>7-9</sup> inhibit growth of neuroblastoma cells and influence neurite outgrowth of these cells<sup>10,11</sup> and the amount of gangliosides shows a close correlation with tumorigenesis.<sup>12</sup>

On the other hand, it was reported that sulfolipids also possess distinct physiological functions.<sup>13-16</sup> It is also well-known that lysolecithin has a hemolytic effect *in vitro* that increases the permeability of the lipid bilayer, leading to easy incorporation of this molecule, as well as surfactant, into the membrane.<sup>17-19</sup> Sugiyama *et al.* reported that lysosphingolipid had strong hemolytic activity as compared to the corresponding sphingolipid.<sup>10</sup> In this study, the cytotoxic activity of synthetic sulfolipids also appeared to be weak as compared to those of lysosphingolipids,<sup>10</sup> sulfoglycolipids,<sup>4</sup> and A-5.<sup>5</sup> However, little is known about the role of fatty acids of lysosulfolipids in growth inhibition *in vitro*. In this study, it was not clear why sulfolipids containing saturated fatty acid, more particularly SQMG(16:0) and (18:0), possessed growth inhibitory activity while those containing unsaturated fatty acid did not. In addition, it was suggested that there were synergistic effects of growth inhibition *in vitro* by a mixture of sulfolipids bound to saturated fatty acids, such as in M6 and M30. This feature implies that the difference between saturated and unsaturated fatty acids is important responsible for easy incorporation into the lipid bilayer.

In this *in vivo* assessment of the anti-tumor effects of sulfolipids, those composed of unsaturated fatty acids, in particular SQMG(14:1) and (18:1), had suppressive effects on tumor growth, whereas saturated fatty acids did not. It is interesting that the results of *in vitro* and *in vivo* experiments did not correspond to observed tumor growth. In our previous report, mice given A-5 at a dose of 4 mg/kg showed significant suppression of tumor growth. However, it should be noted that SQMG(14:1) and SQMG(18:1) account for less than 1% of A-5.<sup>5</sup> If the major suppressive activity of A-5 *in vivo* is attributable to SQMG(14:1) or SQMG(18:1), 4 mg/kg of these agents should have even more dramatic anti-tumor effects, because this dose would be equivalent to the administration of a 100-fold dose of A-5. However, the current data indicate that 4 mg

of SQMG(14:1) or 4 mg of SQMG(18:1) showed a similar effect to that of A-5. The tumor growth of mice treated with these agents at a dose of 20 mg/kg was significantly suppressed. This suggested that, although SQMG(14:1) and SQMG(18:1) could possibly be responsible for the anti-tumor effect of A-5, the activity may be due to the synergistic effects produced by these two sulfolipids. To investigate this hypothesis, we performed *in vivo* assessment using mixtures of sulfolipids, M6, M30 and M31. Mice injected with M6 and M31 showed significant suppression of tumor growth as compared to the control group. However, the suppressive effects of M6 and M31 were similar to those in mice treated with A-5. Therefore, these data suggested that no synergistic effects were produced by mixtures of six or three sulfolipids, because M6 and M31 included at least 4 mg/ml each of SQMG(14:1) and (18:1).

In our previous study, the tumors in mice administered with A-5 showed, patho-histologically, much larger areas of hemorrhagic necrosis, but tumor-infiltrating lymphocytes were not markedly increased as compared with the controls. To investigate whether there were any differences in the suppressive effects at tumor sites between A-5 and synthetic sulfolipids, we assessed the pathological findings of the tumors treated with SQMG(14:1) at a dose of 20 mg/kg in comparison with SQMG(14:0) at the same dose and a control. The tumors treated with SQMG(14:1) showed much more extensive hemorrhagic necrosis, as compared to the controls, whereas only a small degree of

hemorrhagic necrosis was observed in the tumors treated with SQMG(14:0). Tumor-infiltrating lymphoid cells were not markedly increased by treatment with these SQMGs. Interestingly, these histological features were very similar to those in mice treated with A-5. Therefore, the striking anti-tumor suppressive effect seemed to occur only in conjunction with certain mechanisms that could result in hemorrhagic necrosis *in vivo*. The mechanisms by which SQMG(14:1) and (14:0) cause necrosis were not elucidated in this study, but there is a possibility that the binding of C14:1 fatty acid to SQMG may produce a strong necrotic effect which C14:0 fatty acid alone does not possess. Further study is obviously required to determine how synthetic sulfolipids induce hemorrhagic changes *in vivo*. For example, it would be of great interest to study whether these lipids can activate endothelial cells of the tumor site.

In summary, six SQMGs were synthesized, and it was demonstrated for the first time that only certain variants, such as SQMG(14:1) and SQMG(18:1), may possess anti-tumor activity against human adenocarcinoma tumor *in vivo*.

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