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An Emulsion of Sulfoquinovosylacylglycerol with Long-Chain Alkanes Increases Its Permeability to Tumor Cells

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Abstract. The α-anomer form of sulfoquinovosyl-monoacylglycerol with a saturated C18 fatty acid (α-SQMG-$C_{18:0}$) is a natural sulfolipid that is a clinically promising antitumor agent. It forms vesicles, micelles or an emulsion in water, depending on several physicochemical conditions. The type of aggregate formed appears to strongly influence the bioactivity level. Thus, we investigated the nature of the aggregates in relation to their bioactivities.The structure of the α-SQMG-$C_{18:0}$ assembly was greatly affected by the type of additive used in the preparation. Emulsification with ethanol and *n*-decane might be more effective at inhibiting tumor cell growth than the micelle or vesicle preparations. α-SQMG-$C_{18:0}$ formed an ''emulsion-like-aggregate'' in ethanol containing an *n*-decane concentration in the range of 1.03–103 mM. These ethanol/*n*-alkane/α-SQMG-$C_{18:0}$ aggregates inhibited cell growth in a dose-dependent manner, under optimum conditions (i.e., ethanol containing 103 mM of *n*-decane or *n*-dodecane dispersed in phosphate-buffered saline or culture medium). Based on these data, we discuss the relationship between the molecular action of and antitumor activity by α-SQMG-$C_{18:0}$.

Key words: Sulfoquinovosyl-acylglycerol — Sulfoquinovosyl-monoacylglycerol — *n*-Decane — Emulsion

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Introduction

The purpose of this study was to elucidate the best *in vivo* delivery method of a clinically promising, novel antitumor chemotherapeutic agent that we previously reported, the α-anomer form of sulfoquinovosyl-monoacylglycerol (α-SQMG) [1]. This agent also was found to be a potent radiosensitizer that targeted solid tumor angiogenesis without any of the unavoidable *in vivo* side effects that all other clinical anticancer medicines have [2]. While this sounds very promising, there are caveats to address, as detailed below.

Sulfoquinovosyl-acylglycerol chemical groups (SQAGs), including α-SQMG-$C_{18:0}$, are natural sulfolipids found in plants, sea algae and sea urchin. They are reported to exhibit a wide range of biological activities, including anti-human immunodeficiency virus (HIV) activityv [3], HIV-reverse transcriptase inhibition [4, 5], P-selectin receptor inhibition [6], mammalian DNA polymerase inhibition [4, 5] and angiogenesis inhibition [3]. However, they have not become clinical agents yet due to two serious problems: mass production ability and reproducibility of their effects. Natural SQAGs are composed of a sulfoquinovose, one or two fatty acid molecules (acyl moieties) and a glycerol that joins both. They possess a variety of long acyl moieties, making it difficult to isolate a single molecular species. Fortunately, we have successfully synthesized this class of compound and the synthetic α-SQMG-$C_{18:0}$ has also displayed potent DNA polymerase inhibition, antitumor activity *in vivo* and growth inhibition using cultured human tumor cells [1, 7, 8]. Thus, the mass production of any pure SQAG

is now possible. The latter problem, in which the *in vivo* bioactivities are not always reproducible, depends upon the chemical structure itself. The sulfolipids contain both a hydrophilic (sulfoquinovose) and a hydrophobic (acyl chain) moiety. The long chain structures are three-dimensionally flexible. On contact with water, such amphiphilic agents usually form vesicles, micelles or an emulsion, depending on the concentration of the agent, its molecular structure, the presence/absence of additives and/or the method of preparation. The nature of physicochemically formed aggregates is unclear. The bioactivities of the sulfolipid probably vary according to the nature of the aggregates. Moreover, they have to be intravenously injected for *in vivo* usage since the sulfolipids are easily digested in the stomach. Therefore, considerable care as to the type of aggregates selected for intravenous delivery is required.

From this point of view, we previously investigated assemblies of the β-anomer form of sulfoquinovosyl-diacylglycerol with two palmitic acids (β-SQDG-$C_{18:0}$) for intravenous administration *in vivo*. This agent has no antitumor activity at all but is a potent immunosuppressive agent [9–12]. We succeeded in preparing vesicles by taking advantage of their chemicostructural characteristics and then confirmed their potent immunosuppressive effects in a human lymphocyte reaction [9–12]. Our results suggested that the assembled structures and their stability under physiological conditions are important factors for the biological activity of SQAGs.

In this regard, Ohta et al. [8] demonstrated that emulsified α-SQMG-$C_{18:0}$ had potent cytotoxic activity, mainly causing early S phase and M phase arrest at low concentrations. From these results, we speculated that the combination of ethanol and a long chain alkane agent could increase the permeability of α-SQMG-$C_{18:0}$ in cultured cells due to the formation of a complex in aqueous solution. Thus, we evaluated the nature of the aggregates in relation to their bioactivities.

Here, we analyze the self-assembly of a structure containing α-SQMG-$C_{18:0}$, which suppresses tumor growth *in vivo* and tumor cell or endothelial cell proliferation *in vitro*. Three types of assembly (micelle, vesicle and emulsion) were compared after formation in ethanol/*n*-alkane by cell viability assays and light scattering measurements. Our results suggest that SQMG-$C_{18:0}$ aggregates adopt a structure similar to an emulsion when formed in ethanol/*n*-alkane. The emulsified form makes α-SQMG-$C_{18:0}$ more permeable to the membrane of cells, thereby enhancing the biological activity. Therefore, we speculate that the bioactivity levels of the natural SQAG were not necessarily reproducible because of its heterogeneously assembled form.

Materials and Methods

Chemicals

α-SQMG-$C_{18:0}$ was synthesized in our laboratory as described previously [13]. Cholesterols were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's phosphate-buffered saline (PBS) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). All other reagents were of analytical grade and purchased from Wako Chemicals (Osaka, Japan).

Sample Preparation

Emulsifier solutions were prepared by dispersing powdered α-SQMG-$C_{18:0}$ into PBS (pH 7.4) containing 1, 5 or 10 wt% hexadecane. Emulsions were sonicated for 10 min at 50°C using the Branson 1510 sonicator (Branson, Shelton, CT). The final lipid concentration was 7.27 mM.

To facilitate the permeability of α-SQMG-$C_{18:0}$, powdered α-SQMG-$C_{18:0}$ was dissolved in ethanol containing a hydrocarbon (*n*-decane, -octane or -dodecane) and sonicated for 10 min at 50°C using the Branson 1510 sonicator. The final concentration of hydrocarbon was fixed at 103 mM. For investigating the activity relationship with the concentration of hydrocarbon, powdered α-SQMG-$C_{18:0}$ was dissolved in ethanol containing 0.2%, 2% and 20% (v/v) *n*-decane and sonicated. The final hydrocarbon concentrations were 10.3 mM, 103 mM and 1.03 M. This solvent was diluted with PBS and vigorously agitated for 3 min using a vortex mixer (Vortex-Genie 2; Scientific Industries, Bohemia, NY) to obtain a homogeneous mixture.

Vesicles were prepared using a conventional technique, as reported previously [9, 10]. Briefly, α-SQMG-$C_{18:0}$ and cholesterol (1:1 molar ratio) were dissolved in chloroform:methanol (70:30 ratio) in a 10 ml round-bottomed flask. The organic solvent mixture was removed using nitrogen gas, and the residual organic solvent was further removed by drying overnight in a desiccator under vacuum. The dried lipid (α-SQMG-$C_{18:0}$ and cholesterol) film was then hydrated with PBS (pH 7.4), followed by incubation at 65°C for 30 min and vigorous shaking on a vortex mixer for 5 min. The final lipid concentration was 10 mM. An Avanti Mini-extruder (Avanti Polar Lipids, Alabaster, AL) was employed, and the pore size of the polycarbonate membranes (Nucleopore Filtration Products, Clifton, NJ) was 400 and 100 nm, applied in size order. Vesicle suspensions were passed through the membranes under syringe pressure to regulate the size of the vesicles.

Powdered α-SQMG-$C_{18:0}$ was hydrated with PBS (pH 7.4), followed by incubation at 65°C for 30 min and vigorously mixed for 5 min using a vortex mixer to obtain micelles. The final lipid concentration was 10 mM.

Optical Microscopy

Images were acquired with a Carl Zeiss (Jena, Germany) Axioskop II equipped with a digital camera connected to a personal computer.

dynamic Light Scattering Measurement

The particle size and its distribution were measured with a dynamic light scattering measurement apparatus (NICOMP 380 ZSL, Particle Sizing System, Santa Barbara, CA) at a scattering angle of 90° using a wavelength of 535 nm.

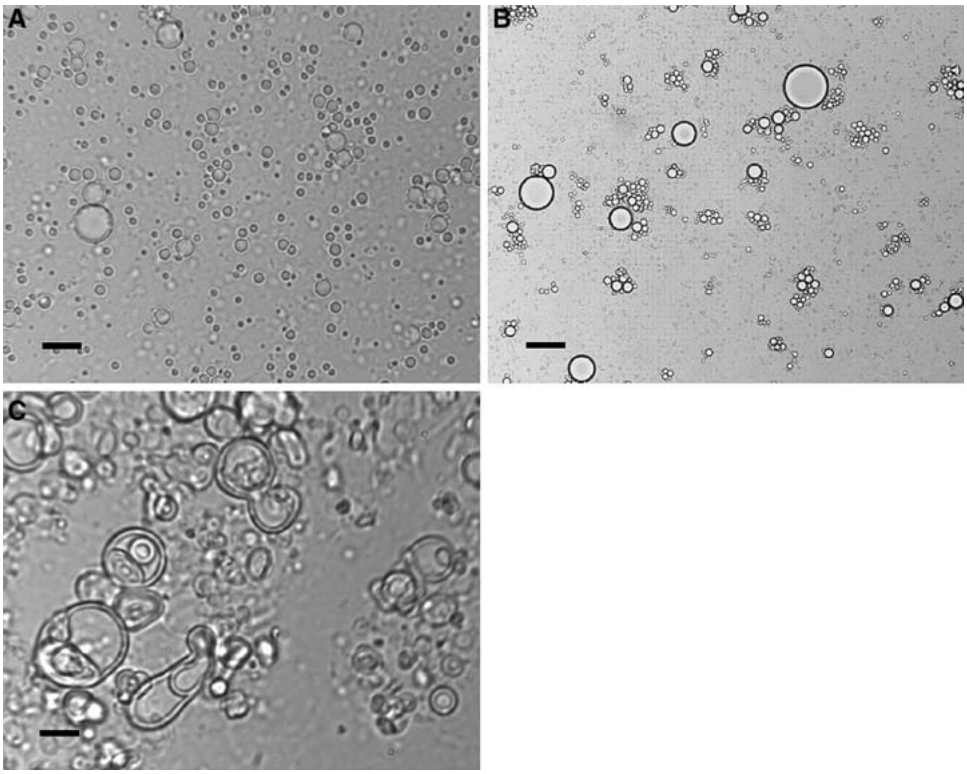

Fig. 1. Light micrographs of (*a*) α-SQMG-$C_{18:0}$ o/w (5% hexadecane/PBS) emulsion, (*b*) α-SQMG-$C_{18:0}$ oil in water (10% hexadecane/PBS) emulsion and (*c*) vesicles composed of α-SQMG-$C_{18:0}$:cholesterol (1:1). Bar = 5 μm.

Cell Lines And MTT Assay

The DLD-1 human colon adenocarcinoma cell line was purchased from Health Science Research Resources Bank, Japan Health Science Foundation (Osaka, Japan). HeLa (a human epithelial carcinoma cell line), A549 (a human adenocarcinoma cell line derived from lung cancer) and SAS (a poorly differentiated squamous cell carcinoma of the tongue) were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). These cells were cultured in RPMI-1640 medium containing streptomycin-penicillin, 10% fetal calf serum and 2 g/l $NaHCO_3$. Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (East Rutherford, NJ) and grown in RPMI-1640 medium containing 20% fetal bovine serum, heparin sodium and endothelial cell growth supplement.

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [9]. Briefly, 1×10^4 cells/well were plated in a 96-well flat-bottomed microplate and incubated for 24 h before α-SQMG-$C_{18:0}$ was applied. α-SQMG-$C_{18:0}$, prepared in several solvent systems, was diluted with medium and applied to each well. After 48 h, medium was removed from each well and fresh medium containing 1.7 mg of MTT reagent (thiazolyl blue tetrazolium bromide; Dojin Chemicals, Osaka, Japan) was added to the wells. The plate was incubated for a further 4 h. Finally, the converted dye was solubilized by 150 μl of dimethyl sulfoxide (DMSO) per well, and the A_{570} of each well was measured using an enzyme-linked immunosorbent assay plate reader.

Lactate Dehydrogenase Assay

To examine the lytic property of α-SQMG-$C_{18:0}$, the activity of lactate dehydrogenase (LDH) leaked from cells was measured according to instructions provided by Roche (Indianapolis, IN). In brief, 1×10^4 cells/well were plated in a microplate and incubated overnight. Then, SQMG dissolved in ethanol containing 2% *n*-decane was diluted with medium and applied to the cells. After a 1-h incubation, 100 μl of supernatant from each well was collected and transferred to a new microplate.

Following addition of reaction mixture containing diaphrase, oxidized nicotinamide adenine dinucleotide (NAD^+), iodotetrazolium chloride and sodium lactate, absorbance was measured at 492 nm.

TUNEL Assay

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was performed for the detection of apoptosis. DLD-1 cells were plated (2×10^4/well) in eight-well chamber slides. After overnight culture, cells were incubated with various concentrations of SQAGs for 24 h. Then, apoptosis staining was performed by the indirect TUNEL method using the Deadend™ Colorimetric TUNEL System (Promega, Madison, WI) according to the manufacturer's instructions. Finally, cells were analyzed under a microscope (Olympus IX71, Tokyo, Japan). Digital images were acquired with a charge-coupled device camera (Pixera Pro 600ES) controlled by Viewfinder version 3.0.1 software (Pixera, Los Gatos, CA).

Results and Discussion

The biological activities of several SQAG derivatives have been studied *in vivo*. These investigations revealed that α-SQMGs are highly effective at inhibiting tumor growth when used in isolation [1] or in combination with irradiation treatment [2]. Although the α-anomer form of SQMG with a saturated C18 fatty acid (α-SQMG-$C_{18:0}$) has been reported to exhibit potent inhibition of tumor growth *in vivo*, only

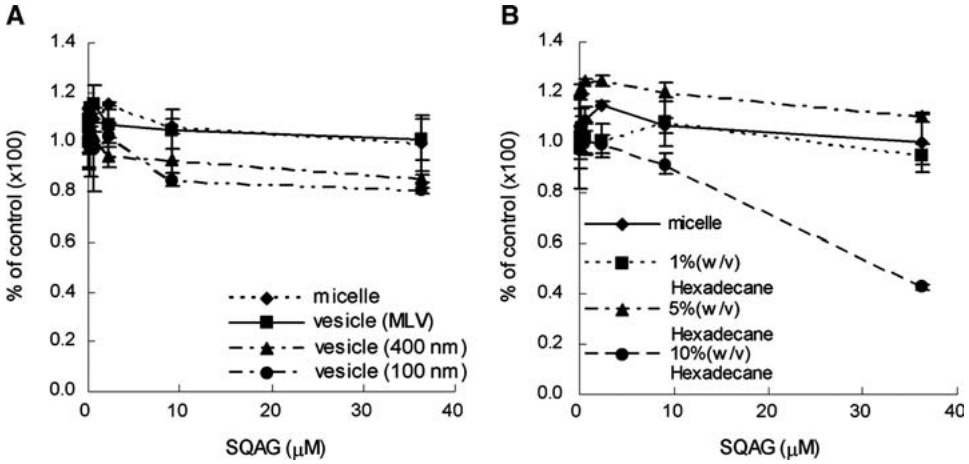

Fig. 2. The effect of SQAG on the cytotoxicity of DLD-1 cells. (*A*) Vesicles were prepared using a conventional technique. Vesicle suspensions (*squares*) were adjusted to 400 (*triangles*) or 100 (*circles*) nm. (*B*) Emulsifier solutions were prepared by dispersing powdered α-SQMG-$C_{18:0}$ into PBS (pH 7.4) containing 1 (*squares*), 5 (*triangles*) or 10 (*circles*) wt% hexadecane. Micelles were prepared as described in Materials and Methods. The data represent the average value of three experiments with the appropriate standard deviation range.

low cytotoxic activity against cultured tumor cell lines was observed [1, 2].

α-SQMG-$C_{18:0}$ contains both a hydrophilic (sulfoquinovose) and a hydrophobic (acyl chain) moiety. On contact with water, an amphiphilic agent such as α-SQMG-$C_{18:0}$ usually forms vesicles, micelles or an emulsion, depending on the concentration of the agent, its molecular structure, the presence/absence of additives and/or the method of preparation. However, the *in vivo* antitumor activity of α-SQMG-$C_{18:0}$ was not always reproducible. We often observed that the bioactivity could range from extremely strong to weak. Based upon *in vivo* experiments, we speculated that the effect of α-SQMG-$C_{18:0}$ may vary according to the nature of the aggregates. Thus, we evaluated this hypothesis.

Often, various additives must be included in the SQAG preparation mixture in order to generate either an emulsion or vesicles. Cholesterol is the common additive found in surfactant vesicles. In this study, addition of 50% (molar ratio) cholesterol to α-SQMG-$C_{18:0}$ was optimal to form vesicles (*data not shown*). The vesicles were heterogeneously assembled with a diameter of < 10 μm (Fig. 1b). For the preparation of oil in water α-SQMG-$C_{18:0}$ emulsions, hexadecane was chosen as the additive. There was a large disparity in the size of the spheres, which had a maximum diameter in the range of 5 μm (Fig. 1a). These results confirm that the resulting structures of α-SQMG-$C_{18:0}$ assemblies are greatly affected by the type of additive used in their preparation.

Each self-assembly form was evaluated by the MTT assay. In this study, micelles formed with α-SQMG-$C_{18:0}$ were prepared from a dilution of the stock solution in medium containing DMSO. DMSO is generally used as a solvent to disperse a hydrophobic agent in an aqueous medium. In Figure 2A, three different vesicle preparations of varying particle sizes were applied to the culture medium. Vesicle samples were obtained either by extrusion through a membrane (400 nm or 100 nm pore size) or without extrusion (referred to as "MLV" or multilamellar vesicles). Extrusion through a membrane with a pore size of 400 or 100 nm resulted in vesicles with an average size of 110.5 or 380.7 nm, respectively (Table 1). The average size of the MLV preparation was 1,116.2 nm (Table 1). As shown graphically in Figure 2, none of the vesicle preparations inhibited the growth of DLD-1 human colon cancer cells.

α-SQMG-$C_{18:0}$ prepared as an emulsion with 1%, 5% or 10% hexadecane (*see* Materials and Methods) had a mean particle size of 466.7, 657.2 or 715.2 nm,

Table 1. IC_{50} value and particle size of α-SQMG-$C_{18:0}$ assemblies prepared by different methods and additives*

Additive	Method	IC_{50} (μM)†	Particle size (nm)
1% Hexadecane	Sonication	-	466.7
5% Hexadecane	Sonication	-	657.2
10% Hexadecane	Sonication	32.2	715.2
None	Vortex	-	nd
Cholesterol	Vortex	-	1,116.2
Cholesterol	Extrusion (400 nm)	-	380.7
Cholesterol	Extrusion (100 nm)	-	110.5
Ethanol:decane (0.2%)	Sonication	-	nd
Ethanol:decane (2.0%)	Sonication	0.2	271.6
Ethanol:decane (20%)	Sonication	0.5	335.4
Ethanol:octane	Sonication	-	nd
Ethanol:dodecane	Sonication	20.7	220.7
Ethanol:tetradecane	Sonication	-	nd

*All samples were diluted with PBS prior to use in each experiment.
†IC_{50} values were determined by MTT assay.

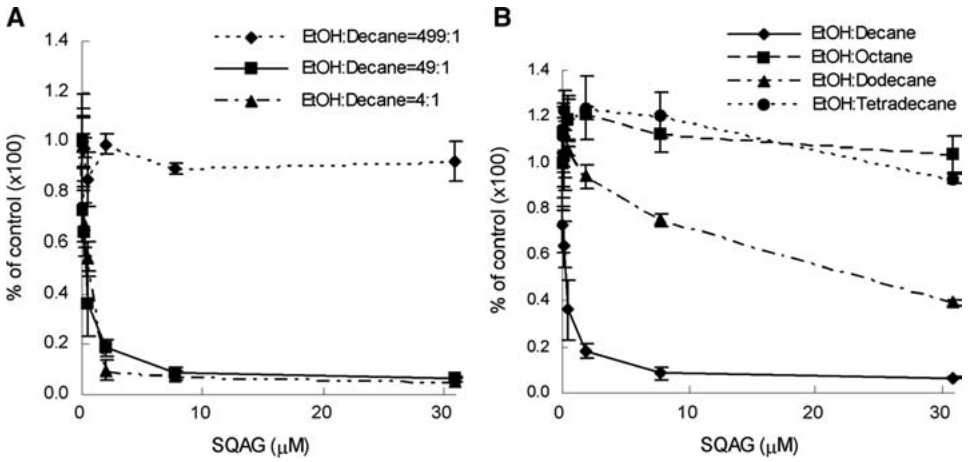

Fig. 3. The effect of SQAG on the cytotoxicity of DLD-1 cells. (*A*) Powdered α-SQMG-$C_{18:0}$ was dissolved in ethanol containing 0.2% (*diamonds*), 2% (*squares*) or 20% (*triangles*) (v/v) decane and sonicated. (*B*) Powdered αSQMG-$C_{18:0}$ was dissolved in ethanol containing oil (decane [*diamonds*], octane [*squares*], dodecane [*triangles*] or tetradecane [*circles*]). The data represent the average value of three experiments with the appropriate standard deviation range.

respectively. As shown in Figure 2B, cell viability was slightly suppressed around 30 μM α-SQMG-$C_{18:0}$ prepared in the 10% hexadecane emulsion, while 1% and 5% hexadecane/α-SQMG-$C_{18:0}$ emulsion did not inhibit cell growth. These results indicate that the emulsified form might be more effective at inhibiting tumor cell growth than the micelle or vesicle preparations.

To optimize the reaction of α-SQMG-$C_{18:0}$ with cultured cells, we investigated different methods of preparing the emulsion, such as dissolving the sulfolipid in ethanol or hydrocarbon before dispersing into water. Ji et al. [14] demonstrated that ceramide could enter cells to induce apoptosis when emulsified in the presence of ethanol and dodecane. In this report, we tested the effect of ethanol and alkane for the dispersion of α-SQMG-$C_{18:0}$ in aqueous solution, PBS(-) or culture medium.

Initially, the ratio of alkane in ethanol was examined using *n*-decane. As described in Materials and Methods, powdered α-SQMG-$C_{18:0}$ was dissolved in ethanol containing 20%, 2% or 0.2% (v/v) of *n*-decane and dispersed in medium (Fig. 3). Dynamic light scattering measurements showed that ethanol:*n*-decane (20% [v/v], 1.03 M of *n*-decane in ethanol) and ethanol:*n*-decane (2% [v/v], 103 mM of n-decane in ethanol) mixtures in PBS gave a mean particle diameter of 335.4 and 271.6 nm, respectively. However, no mean particle size could be determined for an ethanol:*n*-decane (0.2% [v/v], 10.3 mM of *n*-decane in ethanol) mixture in PBS(-). These results suggest that a 20% or 2% mixture of *n*-decane self-assembled "like an emulsion." Furthermore, addition of these aggregates to culture medium induced robust growth inhibition of DLD-1 cells (50% inhibitory concentrations [IC_{50}] for the 20% and 2% *n*-decane preparations were 500 and 200 nM, respectively; *see* Table 1). In contrast, the 0.2% *n*-decane aggregates prepared in the medium had no effect on cell growth up to 30 μM α-SQMG-$C_{18:0}$ (Fig. 3A). α-SQMG-$C_{18:0}$ formed "emulsion-like-aggregates" in ethanol containing an *n*-decane concentration in the range 1.03–103 mM. These aggregates promoted cell growth inhibition depending on the concentration of α-SQMG-$C_{18:0}$. To investigate the relationship between the number of carbon atoms in the alkane molecule and bioactivity, powdered αSQMG-$C_{18:0}$ was dissolved in ethanol containing either *n*-octane (C8), -decane (C10), –dodecane (C12) or -tetradecane (C14) and dispersed in PBS(-). The hydrocarbon concentration was fixed at 103 mM, and particle sizes were measured. The ethanol/*n*-dodecane/α-SQMG-$C_{18:0}$ mixture formed aggregates with a mean particle diameter of 220.7 nm (Table 1). However, the mean particle size could not be determined for the ethanol/*n*-octane/α-SQMG-$C_{18:0}$ mixture or the ethanol/*n*-tetradecane/α-SQMG-$C_{18:0}$ mixture. The ethanol/*n*-dodecane/α-SQMG-$C_{18:0}$ aggregates showed about 100-fold weaker inhibition of cell growth than the ethanol/*n*-decane/α-SQMG-$C_{18:0}$ aggregates (IC_{50} 20.7 μM, Table 1). Neither the ethanol/*n*-octane nor the ethanol/n-tetradecane aggregates suppressed cell growth (Fig. 3B). Thus, α-SQMG-$C_{18:0}$ must be prepared under optimum conditions (i.e., ethanol containing 10.3 mM [2% v/v] of *n*-decane or *n*-dodecane dispersed in PBS[-] or culture medium) in order to maximally suppress cell growth.

Under these optimal conditions, the effects of ethanol/*n*-decane (2%)/α-SQMG-$C_{18:0}$ were examined on several kinds of culture cells. In Figure 4A (b, d, f, h), four kinds of cultured tumor cells and HUVECs were compared by the MTT assay. The inhibition effect of ethanol/*n*-decane/α-SQMG-$C_{18:0}$ on the growth of DLD, HeLa, A549 and SAS cells was clearly stronger than that of the micelle preparation (IC_{50} 0.22, 0.49, 0.65 and 0.44 μM, respectively) (Fig. 4B). By contrast, the ability of ethanol/*n*-decane/α-SQMG-$C_{18:0}$ to inhibit HUVEC growth was distinctly weaker than the four tumor cell types (Fig. 4A [j] and B). This difference might be attributed to the doubling time of each cell type since α-SQMG-$C_{18:0}$ is a DNA polymerase inhibitor. The doubling time of tumor cells in this experiment was 20–24 h, while that of HUVECs

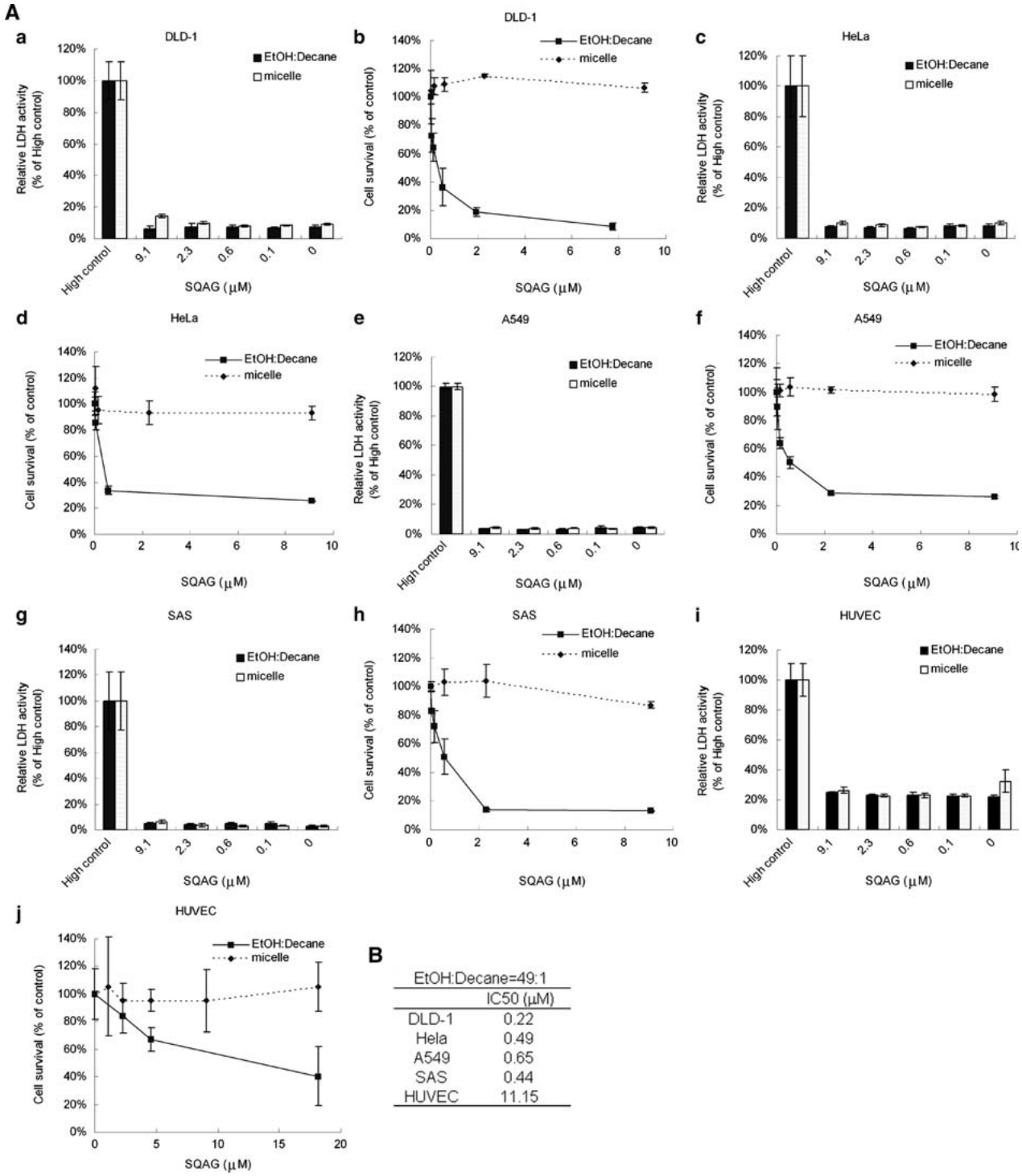

Fig. 4. Cytotoxicity and cell viability assay. (*A*) Cytotoxicity was measured using the LDH assay (*a, c, e, g, i*). High control indicates that cells were lysed completely by 2% Triton X-100. Cell viability was measured by the MTT assay (*b, d, f, h, j*). (*a, b*), DLD-1; (*c, d*), HeLa; (*e, f*), A549; (*g, h*), SAS; (*i, j*), HUVEC. (*B*) IC_{50} values were determined by the MTT assay (*b, d, f, h, j*).

was about 57 h (*data not shown*). Alternatively, the cytotoxicity of amphiphilic structure agents, such as α-SQMG-$C_{18:0}$, is often due to lysis of cells. Thus, cytosolic LDH leakage was measured simultaneously to evaluate this possibility (Fig. 4A [a, c, e, g, i]). In all cells, significant LDH leakage was not detected using the ethanol/*n*-decane solvent system. From these results, we speculate that ethanol/*n*-decane/α-SQMG-$C_{18:0}$ might efficiently penetrate cells without lysis to inhibit DNA synthesis in nuclei.

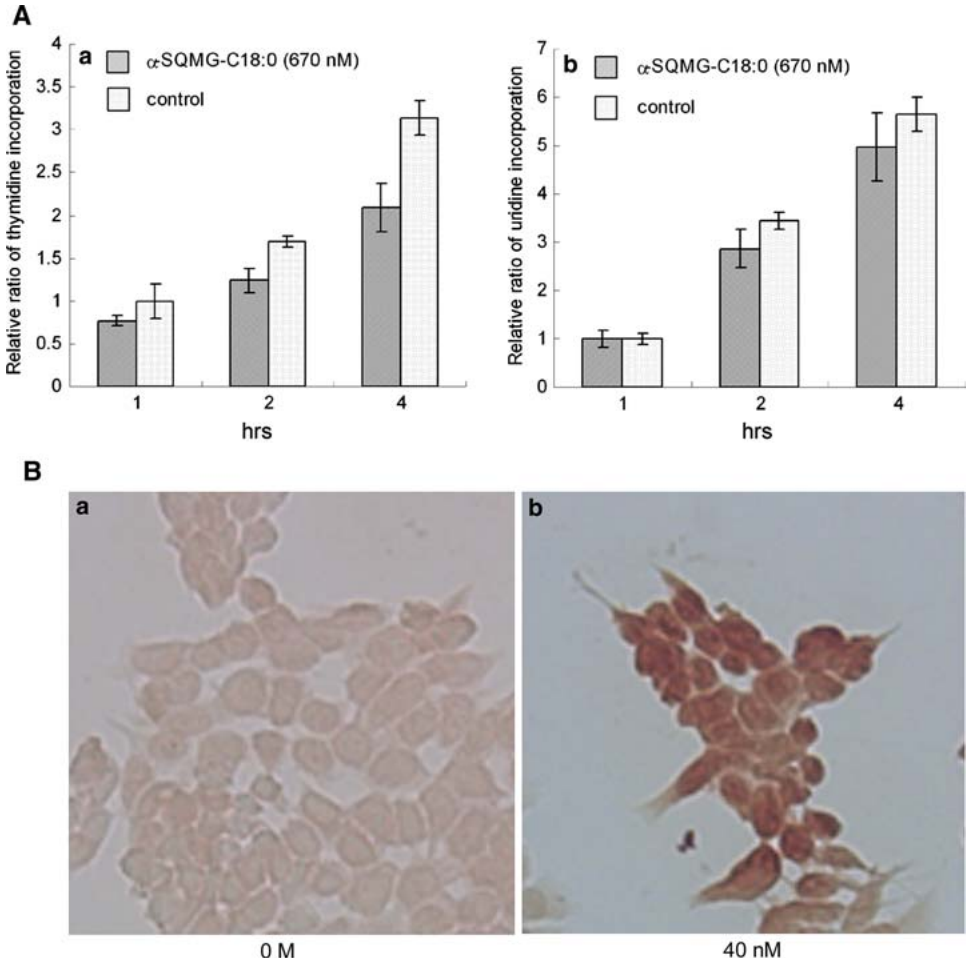

Fig. 5. The effect of ethanol/*n*-decane/α-SQMG-$C_{18:0}$ aggregates on cell metabolism. (*A*) Measurement of thymidine and uridine incorporation into DLD-1 cells incubated with α-SQMG-$C_{18:0}$. DLD-1 cells (1×10^4) were incubated with 670 nM of α-SQMG-$C_{18:0}$ in ethanol/*n*-decane (2 %), and radiolabeled (*a*) thymidine and (*b*) uridine were added at 0.5 h. These metabolites were measured simultaneously. Each value in the graph represents the average of three experiments with the appropriate standard deviation range. (*B*) Apoptosis of DLD-1 cells treated with α-SQMG-$C_{18:0}$ in ethanol/*n*-decane (2%). Apoptotic cells were determined by TUNEL as described in Materials and Methods. Representative appearance of cells treated with (*a*) control and (*b*) 40 nM SQAG in ethanol/decane. TUNEL-positive apoptotic cells exhibit a dark brown nucleus. The nucleus of nonapoptotic cells (light brown) reflects the 3,3′-diaminobenzidine background.

To evaluate the rate of DNA synthesis, the incorporation of tritium-labeled thymidine and uridine into DLD-1 cells treated with α-SQMG-$C_{18:0}$ was quantitated (Fig. 5A [a]). Ethanol/*n*-decane (2%)/α-SQMG-$C_{18:0}$ aggregates (670 nM) were administered, and the amounts of incorporated thymidine and uridine were measured at three time points. As shown in Figure 5A (a), thymidine incorporation was suppressed to 69.0% and 64.2% at 2 and 4 h, respectively. In contrast, uridine incorporation was only inhibited slightly (Fig. 5A [b]). Thus, suppression of thymidine incorporation preferentially occurred 1–2 h after administration. This suggests effective and rapid inhibition of DNA polymerase in cultured cells by α-SQMG-$C_{18:0}$. The TUNEL assay of DLD-1 cells treated with ethanol/*n*-decane (2%)/α-SQMG-$C_{18:0}$ aggregates is shown in Figure 5B. The microscopic images reveal signs of apoptosis, such as DNA strand breakage, at 40 nM α-SQMG-$C_{18:0}$ in ethanol/*n*-decane (2%) solvent.

SQAG is known to be a potent inhibitor of mammalian DNA polymerase, particularly DNA polymerases α, β [2, 4–6] and ϵ [15]. It also inhibits mitotic centromere-associated kinesin (MCAK) [16], which regulates DNA synthesis and the cell cycle; and it can bind to angiogenetic factors such as Tie-1 and Tie-2 (Ohta et al., unpublished data). SQAG can, consequently, exhibit a wide range of biological activities, including anti-HIV activity [3], HIV-reverse transcriptase inhibition [4, 5], P-selectin receptor inhibition [6], mammalian DNA polymerase inhibition [4, 5, 7, 8] and angiogenesis inhibition [2]. In the SQAG group, synthetic α-SQMG-$C_{18:0}$ displayed potent antitumor activity *in vivo* without any of the side effects that all other anticancer medicines have [1, 2]. Therefore, an urgent question is how to safely

and effectively administer this medication to a patient.

When the administration was done using the typical hydrophobic dispersing agent DMSO, DNA polymerase α inhibition with α-SQMG-$C_{18:0}$ was observed at concentrations below ~10 μM. Inhibition of cultured cell growth required approximately 50–100 μM [1, 7] when using these micelles. This suggests that the micelle form is less permeable to the cells. As described in Figures 3A and 4, however, the α-SQMG-$C_{18:0}$ aggregates prepared with ethanol and *n*-decane could suppress the growth of several tumor cells at concentrations less than ~1 μM. As shown in Figure 3B, 10% hexadecane/α-SQMG-$C_{18:0}$ emulsion weakly inhibited cell growth, although ethanol/alkane/α-SQMG-$C_{18:0}$ emulsion potently inhibited it. We presume that ethanol promotes the fusion between emulsion and cell membrane by affecting the configuration and the fluidity of lipid molecules. The data suggest that ethanol helps importantly the absorption of emulsified α-SQMG-$C_{18:0}$ into cells and subsequently that α-SQMG-$C_{18:0}$ inhibits DNA synthesis and induces apoptosis in spite of low concentration, as depicted in Fig. 5.

Furthermore, suppression of tritium-labeled thymidine incorporation into the cells occurred within 2 h after treatment with α-SQMG-$C_{18:0}$. This suppression was not detected under the micelle condition (*data not shown*). This strongly indicates that the ethanol and *n*-alkane solvent system promotes uptake of α-SQMG-$C_{18:0}$ into the cells. Moreover, this demonstrates that by optimizing the preparation of the ethanol/decane/SQAG aggregates, the permeability of SQAG can dramatically increase, thereby allowing access to the cytosolic and/or nuclear protein targets. These findings are key to delineating the delivery mechanism of SQAG.

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