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Inhibition of proliferation and induction of apoptosis in SNU-1 human gastric cancer cells by the plant sulfolipid, sulfoquinovosyldiacylglycerol

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Abstract

The sulfoglycolipid sulfoquinovosyldiacylglycerol is present in the membranes of photosynthetic organisms. This sulfolipid reportedly has pharmaceutical potential as an antiviral and antitumor agent, although no studies have examined these properties of the sulfolipids that are consumed in plant foods. This study examined the biological effects of sulfoquinovosyldiacylglycerol on the human gastric cancer cell line SNU-1. SNU-1 cells were grown in the absence and of presence of 1 μM, 100 μM or 1 mM sulfoquinovosyldiacylglycerol for up to 72 hours. Cell proliferation and viability were determined. The cells were analyzed for nuclear morphological changes by fluorescence microscopy and for DNAase-mediated DNA cleavage by flow cytometry and TUNEL detection. As indicated by cell number, the proliferation of SNU-1 cells by 72 hours of culture in the presence of 100 μM and 1 mM SQDG was inhibited 24 and 100%, respectively, as compared with the number of SNU-1 cells cultured in the absence of SQDG. Inhibition of cell proliferation by 100 μM sulfoquinovosyldiacylglycerol was in part associated with apoptotic cell death, as shown by changes in nuclear morphology and DNA fragmentation, whereas incubation of cells with 1 mM sulfoquinovosyldiacylglycerol caused necrotic cell death. Treatment of SNU-1 cells with sulfoquinovosyldiacylglycerol did not result in cell cycle arrest. The antiproliferative and apoptotic effects of sulfoquinovosyldiacylglycerol on SNU-1 gastric cancer cells revealed in this study suggest that this common dietary sulfolipid has intriguing potential as a chemopreventive or chemotherapeutic agent. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Apoptosis; Cell proliferation; SNU-1 cancer cells; Sulfolipid; Sulfoquinovosyldiacylglycerol

1. Introduction

The plant sulfolipid sulfoquinovosyldiacylglycerol (SQDG) is present in most photosynthetic organisms [1], although it is absent from some photosynthetic bacteria [2]. SQDG has also been isolated from the nonphotosynthetic bacterium *Rhizobium meliloti* [3]. SQDG is an anionic lipid with a stable sulfonic acid linkage. Owing to its distribution in plants, human intakes vary widely but typically reach milligram quantities per day in diets rich in vegetables, edible algae and photosynthetic bacterial health supplements, such as cyanobacterium [4]. Cyanobacteria, also known as blue-green algae, are prokaryotic organisms that are a rich, natural source of many macro- and micronutrients, including SQDG. Sulfolipids are 10% of algal dry weight [5]. Cyanobacteria are used as a food source and as nutritional supplements worldwide, and it has been estimated that over one million Americans currently use cyanobacterial supplements [6,7]. The most common dietary source of cyanobacteria in the United States is spirullina, a frequently used dietary supplement. Little is known about the effects of SQDG on cellular health and physiology.

Interest in the biological activity and pharmaceutical value of phytochemicals has increased in the last decade. Many phytochemicals possess selective toxicity towards

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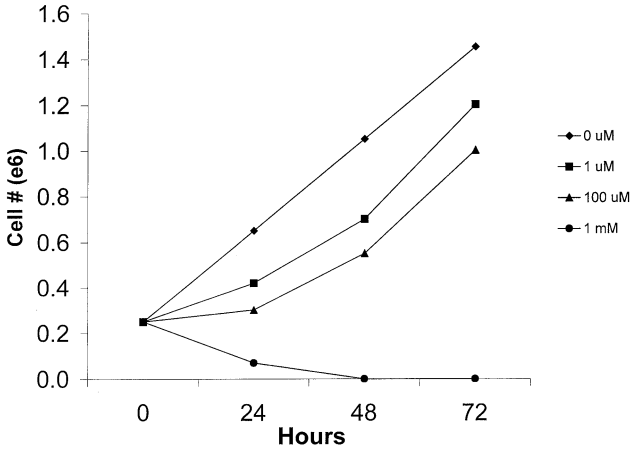

Fig. 1. Effect of SQDG on the proliferation of SNU-1 cells. SNU-1 cells were plated at 2.5×10^5 cells per well and cultured in medium with 0, 1 μM, 100 μM or 1 mM SQDG. The viable cells were counted at indicated times ($N = 5$).

tumorigenic tissues, and thus have anti-cancer properties. Recently, SQDG of cyanobacterial origin was reported to possess pharmaceutical potential, exhibiting both antiviral and antitumor activity in cultured cell lines [8–11]. SQDG has also been reported to inhibit eukaryotic DNA polymerase a and b activity [9,10]. The characteristic structural feature of SQDG is the sulfonic acid head group, referred to as sulfoquinovose [2], and the biological activity of SQDG often has been attributed to the sulfo group on the molecule. Sulfolipids, however, have not been examined for their properties as food ingredients, in particular, it is not know how they might affect cancerous cells in the gastrointestinal tract. The present study investigated the effects of SQDG on the proliferation and viability of cultured SNU-1 human gastric cancer cells.

2. Materials and methods

2.1. Cell line and materials

SNU-1, a gastric carcinoma cell line, was obtained from the American Tissue Type Culture (ATTC) (Rockville, MD). Cells were cultured in RPMI 1640 medium (ATTC) that contained 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 100 units penicillin/mL (Sigma) and 100 μg streptomycin/mL (Sigma) and in an atmosphere of 5% CO_2 and 95% air at 37°C. Purified spinach SQDG (>98% pure) was purchased from Lipid Products (South Nutfield Surrey, UK).

Table 1
Effect of SQDG on the percentage viability of SNU-1 cells

SQDG concentration	Treatment time (hours)		
	24	48	72
0	98.6 ± 1.1	99.2 ± 0.8	99.2 ± 0.4
1 μM	99.8 ± 0.3	98.7 ± 0.6	99.1 ± 0.5
100 μM	98.4 ± 1.7	98.2 ± 1.0	97.9 ± 1.0
1 mM	5.4 ± 13.4*	1.0 ± 0.1*	1.0 ± 0.2*

Mean ± SD; N = 3.
* $P < 0.005$, compared with that of the control.

2.2. Cell proliferation and viability assays

Cell proliferation was measured by counting viable cells over time using Trypan Blue dye-exclusion exclusion as the basis of viability. SNU-1 cells were plated in 24-well plates at 2.5×10^5 cells per well in a complete culture medium. SQDG was dissolved in ethanol by sonication and was added to the culture media at 1 μM, 100 μM and 1 mM concentrations. The final concentration of ethanol in all cultures, including the control cell cultures, 0.5% (vol/vol,). This concentration of ethanol had no effect on the cells. The culture medium was replenished at 48-hour intervals. Cell proliferation was monitored for 72 hours. To determine the significance of differences in cell proliferation among the treated cultures, statistical analysis was performed using Student's t test.

2.3. Cellular treatment for morphologic and flow cytometric analyses

SNU-1 cells were plated at a density of 2.5×10^5 cells per well in 24-well plates. SQDG was dissolved in ethanol and added to the culture media at concentrations of 1 μM, 100 μM and 1 mM, and the cells were incubated for 24 and 48 hours. The final concentration of ethanol, which was also added to the control cells without SQDG, was 0.5%. Treatments were applied to cells in triplicate wells, and after incubation, the cells were pooled for analysis by fluorescence microscopy and flow cytometry. Cell viability was determined by Trypan Blue dye exclusion.

2.4. Characterization of nuclear morphology

For morphologic analysis, cells (5×10^5) were centrifuged at $1900 \times g$ for 5 minutes and fixed with 3.7% formaldehyde (w/v) in phosphate-buffered saline. The cells were held at room temperature for 10 minutes and then centrifuged at $1900 \times g$ for 5 minutes. The cells were resuspended in a buffer containing 1 μg bis-benzimide (Hoechst Dye H33342)/mL dimethyl sulfoxide (Sigma) and 0.1% Triton X-100 (Sigma) in phosphate-buffered saline. The dye was allowed to penetrate the cells for 10 minutes at room temperature. The cells then were centrifuged at $1900 \times g$ for 5 minutes, and the pellet was resuspended in the same buffer without Triton X-100. The cells were fixed with p-phenylenediamine (1 mg/mL) and 90% glycerol in phosphate-buffered saline and analyzed using a Zeiss uni-

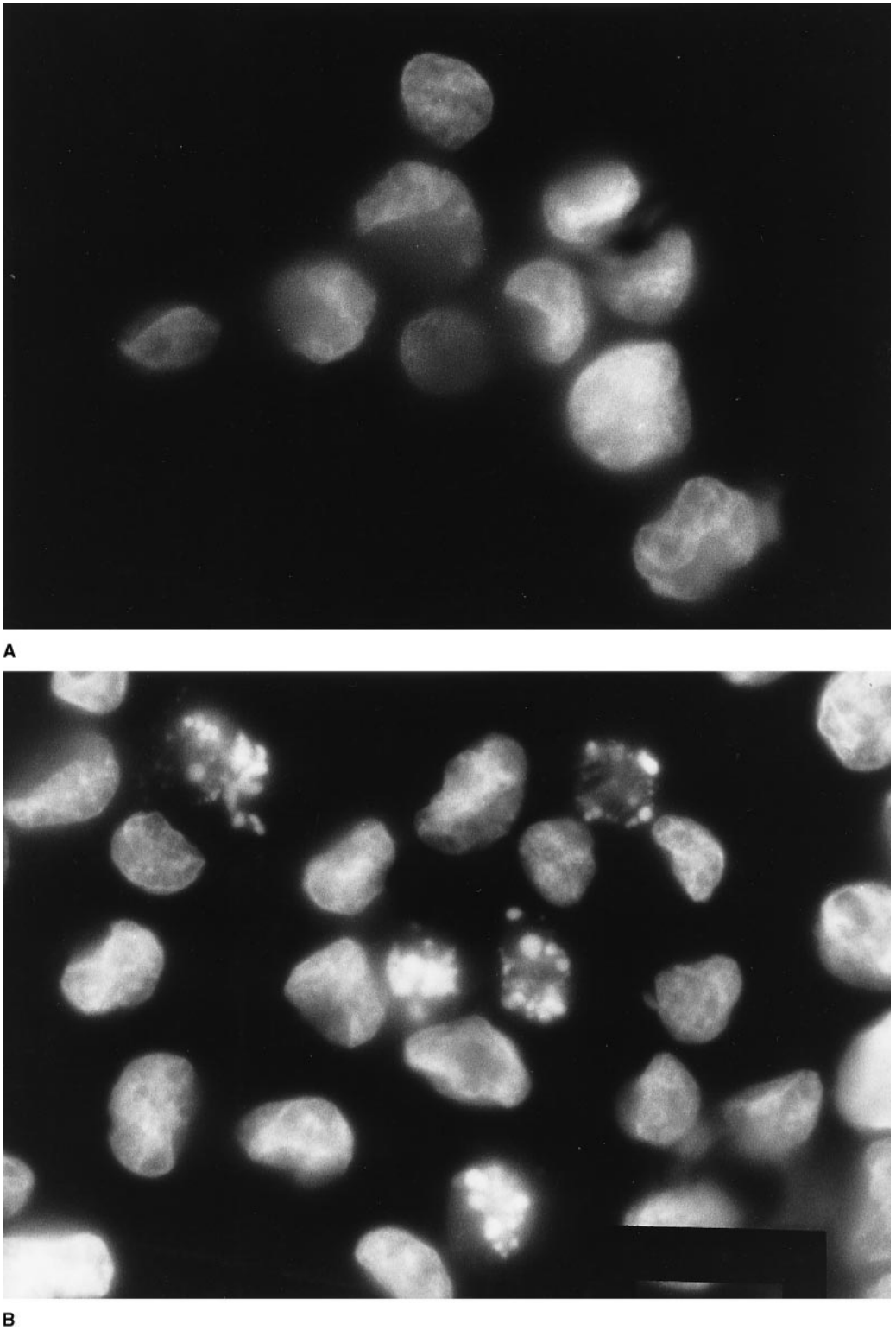

Fig. 2. Fluorescence photomicrographs of SNU-1 cells. SNU-1 cells were incubated with control medium (A) without SQDG or with (B) 100 μM SQDG for 48 hours.

versal fluorescence microscope. Microscopy was performed under conditions of normal and fluorescent illumination using phase contrast optics. The percentage of apoptotic cells was determined by counting 250 cells from random fields. DNA-complexed Hoechst dye H33342 was excited at 355 nm and emission was detected at 465 nm. This uncharged fluorochrome is a benzimidazole derivative that binds AT pairs in DNA.

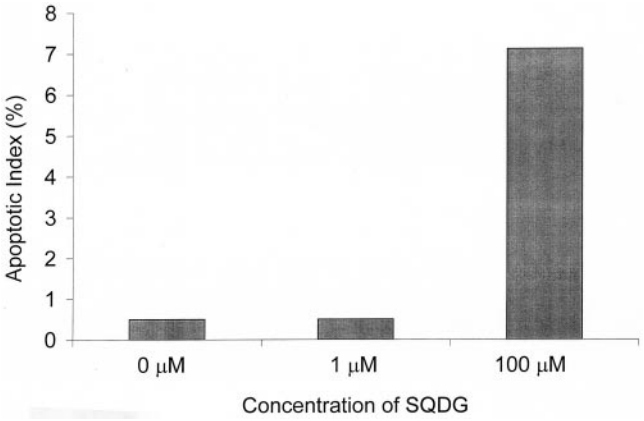

Fig. 3. SQDG-induced apoptosis of SNU-1 cells. The percentage of apoptotic events (apoptotic index) was determined by the TUNEL assay using flow cytometry. The cells were treated for 48 hours with 0, 1 μM or 100 μM SQDG.

2.5. Detection of DNA fragmentation and analysis of cell cycle

For DNA fragmentation detection, the terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using an Apo-Direct kit (Phoenix Flow Systems, San Diego, CA). Briefly, cells (approximately 1×10^6) were washed in phosphate-buffered saline, fixed in 1% paraformaldehyde for 15 minutes on ice, washed with phosphate-buffered saline and then fixed in 70% ethanol. The cells were incubated in DNA-labeling solution that contained TdT Enzyme, TdT reaction buffer and fluorescein-dUTP. After washing the cells with buffer, they were incubated with propidium iodide/RNase A solution for 30 minutes prior to flow cytometric analysis. Propidium iodide staining was used to assess the DNA cell cycle.

For cell cycle analysis, 10,000 cells were analyzed for cell cycle distribution by a Becton Dickinson FACScan flow cytometer equipped with a 488-nm argon laser and CellQUEST acquisition and analysis software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cell cycle distribution was analyzed by Mod-fit software and statistical analysis was performed using Student's *t* test. Data were analyzed using events gated for doublet discrimination on a dot plot of area versus width of propidium iodide fluorescence. Each experiment was repeated a minimum of two times.

3. Results

3.1. Effects of SQDG on the proliferation and viability of SNU-1 cells

To assess the effect of SQDG on cell proliferation, the SNU-1 cells were treated with 100 μM or 1 mM SQDG, and cell number was determined at 24, 48 and 72 hours. As shown in Fig. 1, inhibition of cell proliferation was apparent 24 hours after addition of SQDG to the culture medium. At 72 hours, cell proliferation was inhibited 24 and 100% by 100 μM and 1 mM SQDG, respectively, as compared to the control ($P < 0.05$). In a separate experiment (Table 1), the percentage of viable cells after 72 hours of treatment with 1 μM SQDG was not significantly different from the percentage of viable control cells. Less than 5% of the total cell population was Trypan Blue positive when the cells were treated with either 1 μM or 100 μM SQDG, suggesting that the primary cause of proliferation inhibition was not cytotoxicity. In contrast, cellular death was evident in the SNU-1 cells treated with 1 mM SQDG at all time points, suggesting that the primary cause of inhibition was cytotoxity.

3.2. Analysis of cellular death and cell cycle distribution

At all time points, fluorescence microscopy of Hoechst-stained cells indicated that the morphology of SNU-1 cells treated with 1 μM SQDG was not different from that of control cells. In contrast, cells treated with 100 μM SQDG exhibited marked morphological changes that were indicative of apoptosis. Morphological apoptotic changes included condensation of chromatin, membrane blebbing, fragmentation of the nucleus and formation of apoptotic bodies (Fig. 2). Apoptotic changes occurred as early as 24 hours and were maximal in cells treated for 72 hours. At all time points, cells treated with 1 mM SQDG underwent a high degree of necrosis, as indicated by the presence of cell debris that is associated with osmotic lysis. Structures indicative of apoptosis were not observed.

Flow cytometric analysis was used to determine apoptotic indices in cells stained by the TUNEL method. The proportion of SQDG-treated cells that exhibited DNAase-mediated DNA cleavage increased with time, and the apoptotic index for cells treated for 48 hours with 100 μM was approximately 8-fold greater than that of cells treated with 1 μM SQDG (Fig. 3). DNA cell cycle analysis showed an accumulation of a sub-G_0/G_1 peak in both 100 μM and 1 mM SQDG-treated cell populations (data not shown). The sub-G_0/G_1 peak represents cells with lower DNA content than that of G_0/G_1 cells and includes apoptotic and necrotic cells. There were no changes in cell cycle distribution following exposure to any concentration of SQDG tested (Table 2).

4. Discussion

Gastric cancer is a leading cause of cancer-related deaths, worldwide being second only to lung cancer as a cause of death [12,13]. A number of risk factors appear to be associated with increased incidence of gastric cancer, including dietary factors, *Helicobacter pylori* infection, cig-

Table 2
Effect of SQDG on the cell cycle distribution of SNU-1 cells

Time	Cycle phase	0 μM	1 μM	100 μM	1 mM
24 h	% G_0/G_1	45.7 ± 4.6	48.2 ± 4.2	50.7 ± 8.4	51.7 ± 7.0
	% G_2/M	39.3 ± 3.8	36.4 ± 4.6	39.0 ± 6.9	35.3 ± 1.1
	% S	15.0 ± 1.3	15.4 ± 0.9	10.3 ± 1.5	13.0 ± 8.0
48 h	% G_0/G_1	50.7 ± 4.8	49.8 ± 6.9	54.8 ± 9.1	57.7 ± 21.1
	% G_2/M	28.1 ± 11.7	36.6 ± 3.8	33.7 ± 7.6	31.2 ± 8.8
	% S	21.2 ± 16.0	13.6 ± 4.5	12.3 ± 3.0	11.2 ± 14.0

Mean ± SD; N = 3.

arette smoking and family history [14]. Among these, diet has been the most studied risk factor, and diets low in fresh fruits and vegetables have been associated with increased risk [15]. Although an inverse relationship between consumption of fruits and vegetables and incidence has been shown, the specific dietary nutrient(s) responsible for this chemoprotective effect is not known. Dietary components including flavanoids and carotenoids have potentially beneficial, albeit controversial, effects on gastric cancers. In the present study, the specific nutrient SQDG was examined in relation to its effects on cultured gastric cancer cells. Little is known about the effects of dietary SQDG on the physiology of humans. Although it is not known whether SQDG is beneficial or deleterious to health, it does have potential pharmaceutical value as an antiviral and antitumor agent [7–10].

In the present study, SQDG decreased cellular proliferation of the SNU-1 gastric cancer cell line. Ohta et al. [10] demonstrated that a sulfolipid, KM043, belonging to the SQDG family, was a potent inhibitor of eukaroytic DNA polymerase a and b. DNA polymerase a and b are found in the nucleus, where DNA polymerase a is involved in DNA replication and DNA polymerase b is involved in DNA repair. The inhibition of DNA polymerase activity is an activity of many cancer chemotherapeutic drugs. The inhibition of DNA polymerase activity in several cultured mammalian cell lines results in decreased proliferative rates and cell death occurring via apoptosis. In the future, it will be of interest to determine whether SQDG inhibits DNA polymerase in SNU-1 cells.

The inhibition of SNU-1 cell growth by SQDG was accompanied by a significant loss of cell number, suggesting that apoptosis was involved in the action of SQDG. Apoptotic programmed cell death contributes to the maintenance of cell number in many tissues [15–17]. Apoptosis is a highly conserved evolutionary process that removes either undesired but otherwise healthy cells (e.g., tissue restructuring during development) or removes damaged single cells. Cells from various human malignancies have a decreased ability to undergo apoptosis in response to various physiologic stimuli [18,19]. For this reason, the induction of apoptosis has become a target in cancer chemoprevention and chemotherapy [20–22]. In the present study, the occurrence and the mode of SNU-1 cell death was dependent on the concentration of SQDG. Morphological and flow cytometric data were consistent in establishing that little cell death occurred in response to 1 μM SQDG. However, at all time points tested, treatment of SNU-1 with 100 μM SQDG caused morphological changes characteristic of apoptosis, and flow cytometric analysis revealed DNA fragmentation. There was a transition from apoptosis to necrosis as the primary cause of death at some concentration between 100 μM and 1 mM SQDG. Thus, the transition from no apoptotic cellular death to necrosis occurred over a 10-fold concentration range.

There is a possibility that SQDG could cause cell cycle arrest. In many instances of apoptosis, a period of cell cycle 'stasis' precedes the onset of apoptosis. Many antiproliferative drugs, such as colcemid and aphidicolin, block cell cycle phase transitions [23]. At the concentrations and exposure times tested in the present study, SQDG did not induce changes in cell cycle distribution, thus it was not apparent at which phase of the cell cycle the SNU-1 cells entered apoptosis. These findings are consistent with the understanding that apoptosis can be initiated and can occur in any cell cycle phase [24].

This study demonstrates for the first time that human gastric cancer cells can be killed by micromolar concentrations of SQDG. Further studies are needed to determine the mechanisms of action of SQDG and whether the antiproliferative and apoptotic effects of SQDG may account for some chemopreventive or chemotherapeutic effects on gastric cancer cells.

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