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In Vitro Anti-inflammatory and Anti-proliferative Activity of Sulfolipids from the Red Alga *Porphyridium cruentum*

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A sulfoglycolipidic fraction (SF) isolated from the red microalga *Porphyridium cruentum* was analyzed for fatty acid composition and assayed for ability to inhibit, in vitro, the generation of superoxide anion in primed leucocytes and the proliferation of a panel of human cancer cell-lines. Results demonstrated that SF contained large amounts of palmitic acid (26.1%), arachidonic acid (C20: 4ω-6, 36.8%), and eicopentaenoic (C20:5ω-3, 16.6%) acids, and noticeable amounts of 16:1n-9 fatty acid (10.5%). It strongly inhibited both the production of superoxide anion generated by peritoneal leukocytes primed with phorbol myristate acetate (IC_{50}: 29.5 μg/mL), and the growth of human colon adenocarcinoma DLD-1 and to a lesser extent of human breast adenocarcinoma MCF-7, human prostate adenocarcinoma PC-3, and human malignant melanoma M4 Beu cell-lines, and therefore might have a chemopreventive or chemotherapeutic potential, or both. It was found markedly more cytotoxic than sulfoquinovosyldiacylglycerols from plant used as a standard (STD), due to a stronger ability to inhibit DNA α-polymerase (IC_{50}: 378 μg/mL, vs 1784 μg/mL for STD). After a 48-h continuous treatment, IC_{50} values for growth inhibition were in the range of 20−46 μg/mL instead of 94 to >250 μg/mL for STD, and those for inhibition of metabolic activity were in the range of 34−87 μg/mL instead of >250 μg/mL for STD. The higher anti-proliferative effect was observed on colon adenocarcinoma DLD-1 cells, and the weaker effect was observed on breast adenocarcinoma MCF-7.

KEYWORDS: ***Porphyridium cruentum*; sulfolipids; sulfoquinovosylacylglycerols (SQAG); fatty acid analysis; anti-inflammatory activity; anti-proliferative activity; solid cancer cell-lines; human carcinoma cells; human melanoma cells**

INTRODUCTION

Because of its amount and diversity of nutrients, there is growing interest in the red marine microalga *Porphyridium cruentum*. Its cells are rich in protein, excrete a sulfurized polysaccharide of industrial interest (*1*), and accumulate large amounts of lipids, especially neutral lipids and glycolipids (ca 40−45% each). Triacylglycerols, the main lipid class, are of particular interest because they contain large amounts of long chain PUFAs, especially arachidonic acid (ARA: C20:4ω 6) and eicosapentaenoic acid (EPA, C20:5ω 3), which are known to exert beneficial effects on human health (*2*, *3*). Therefore, this alga represents a source of PUFAs and might also have a use as a nutritional supplement or as a source of drug. Various works have focused on the production of long chain PUFAs. It was shown that using *P. cruentrum* mutants and varying the culture configuration, growth conditions and media composition has a great effect on their biosynthesis (*2*−*4*), however, today, the biosynthesis, biological activities, and pharmaceutical values of the other class of lipids remains still largely unknown. This study focused on sulfoquinovosylacylglycerols (SQAGs) which have been identified in noticeable amounts in *P. cruentum* cells cultured under stress conditions (*4*). SQAGs are anionic glycolipids present in the photosynthetic membranes of plants and algae, and in various bacteria, in association with monogalactosyldiacylglycerols (MGDGs) and digalactosyldiacylglycerols (DGDGs). Recently, SQAGs extracted from various marine red and blue-green algae were reported to inhibit both eukaryotic DNA α- and β-polymerases and HIV-reverse transcriptase, and therefore, to exhibit antiviral and antitumor activities (*5*−*7*). This work was designed to evaluate the growth inhibitor effect of a crude sulfoglycolipidic fraction from *P. cruentum* (SF) on immortalized L-929 murine cells and on a panel of human solid cancer cells comparatively to SQDGs from plants. Its ability to inhibit superoxide anion generation in primed neutrophils was also investigated.

MATERIALS AND METHODS

Biologicals and Chemicals. Normal human fibroblasts were purchased from Biopredic International (Rennes, France). M4Beu, a human melanoma cell line, was established in the laboratory of Dr. J. F. Doré

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[INSERM (National Institute for Health and Medical Research) Unit 218, Lyon, France] from a 36-year-old femal abdominal surgical waste and maintained in cell culture for almost 15 years. The cells used in this work were from the 7th to the 12th passage. Breast cancer adenocarcinoma MCF-7, prostatic adenocarcinoma PC-3, ovarian teratocarcinoma PA-1, and colon adenocarcinoma DLD-1 human cell-lines were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, U.K.). Cells of *Porphyridium cruentum* were generously provided by Dr. G. Baudimant (Aquaartis, France) as frozen cells.

Minimum essential medium (MEM) with Earle's salts and Glutamax, solution of vitamins, sodium pyruvate, nonessential amino acids, PBS, and gentamicin base were purchased from Gibco-BRL (Paisley, Scotland). Fetal calf serum was obtained from Bio West (Nuaillé, France) and Nunclon 96-well microplates were purchased from Nunc (Roskilde, Denmark). Sterile NaCl 0.15 M was from Eurobio (Les Ulis, France); Hoechst Dye 33342, orcinol spray reagent, Tris-HCl, and radioactive compounds were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France). Purified spinach SQDG (>98% pure) was from Larodan Fine Chemicals (Malmö, Sweden). DE 52 (Whatman), silicagel (Si 60, 70−230 mesh), salts, and solvents were from Merck Eurolab (Nogent-sur-marne, France). Other chemicals were from Fluka (Buchs, Switzerlands).

Extraction and Purification of SQAGs. Lipids were extracted from frozen algal biomass (50 g wet mass) by chloroform/methanol/water according to Bligh and Dyer (*8*). The lipid extract (1630 mg) was then dried under nitrogen, vortexed with 0.22 L of cold acetone (−20 °C), and stored overnight at −20 °C. The resulting pellet (793 mg) was collected by centrifugation (2500*g*, 4 °C, 20 min) and stored in C_6H_6/EtOH (1:1) at −18 °C under N_2 until further use, then fractionated by adsorption chromatography on silica gel (30 mg per mg of crude lipid). Neutral lipids, glycolipids, and phospholipids were successively eluted by $CHCl_3$ (1 mL per mg of crude lipids), Me_2CO/MeOH (9:1) (1.5 mL per mg of crude lipids), and MeOH (1 mL per mg of crude lipids) using the procedure previously described by Bergé et al. (*9*). To avoid oxidation and evaporation during storage, each fraction was evaporated and immediately stored at −18 °C in C_6H_6/EtOH (1:1) until further treatment. The glycolipidic fraction (317 mg) was then fractionated by ion-exchange chromatography according to Rouser (*10*). Briefly, 20 g of DE 52, a DEAE cellulose, were converted to their acetate form and transferred into a column (2.5 × 30 cm) which was washed with combinations of methanol and chloroform before use. After successive elution of monoglycosyl- and diglycosyl-diglycerides with mixtures of $CHCl_3$/MeOH 98:2 and 90:10, sulfoglycolipids (132 mg) were collected with a chloroform/methanol (4:1) mixture containing 2% NaOH and 50 mM CH_3COONH_4. After removing the salts with a "Folch" wash (*11*), the crude fraction was dried under nitrogen. The residue was solubilized in cold acetone (22 mL) and stored for a night at −20 °C. The soluble sulfoglycolipidic fraction (SF, 75 mg) was collected by centrifugation (2500*g*, 4 °C, 20 min), then evaporated under nitrogen and stored at −18 °C in C_6H_6/EtOH (1:1) under N_2 until further use.

TLC Analysis. At each step of purification, fractions were identified by TLC analysis on silica gel (F-254, Merck, Darmstadt, Germany) comparatively to authentic SQDGs from spinach (STD), by migration in chloroform/methanol/water (65:25:4) (*10*) then staining with orcinol reagent.

Sugar Analysis. Neutral sugars were determined by GC as their alditol acetates derivatives after hydrolysis under nitrogen in 1 M H_2SO_4 for 1 h at 100 °C (*12*).

CPG Analysis of Fatty Acid Methyl Esters (FAMEs). An aliquot was evaporated under nitrogen and transmethylated by contact with methanol/sulfuric acid (98:2) in excess for one night at 50 °C. After cooling, 2 mL of pentane and 1 mL of water were added and vortexed. The upper organic phases containing FAME were collected and assayed by GC using a Perkin-Elmer Autosystem equipped with a FID detector. Separation was done using He as carrier gas on a fused silica column (BPX-70, 60 m long, 0.25 mm i.d., 0.25 μm film thickness) programmed from 55 °C (for 2 min) to 150 °C at 20 °C min^{-1}, then to 230 °C at 1.5 °C min^{-1}. Sample was injected with a programmable split/splitless inlet and large volume injection system (PSS) using the following temperature program: 55 °C (for 2 min) to 350 °C at 200 °C min^{-1}. FAMEs were identified by comparison of their equivalent chain length with those of authentic standards. Quantification was done using margaric acid (17:0) as internal standard. STD was reported by the manufacturer to contain 28% of C16:0, 2% of C18:0, 3% of C18.1%, 6% of C18:2, and 61% of C18:3.

Cell Proliferation and Viability Assays. *Drug Solutions.* Aliquots of SF and STD were evaporated under nitrogen, then dissolved in DMSO at 25 mg/mL. This stock solution was diluted first in DMSO, then in the culture medium to obtain a final concentration of DMSO equal to 0.5% (v/v) which was immediately used for cytotoxicity assays. The maximal concentration tested (100 μg/mL for SF, 250 μg/mL for STD) corresponded to the maximal concentration soluble in the culture medium.

Cell Culture. Stock cell cultures were done as previously described (*8*) as monolayers in 75-cm^2 culture flasks in Eagle's minimum essential medium supplemented with 10% fetal calf serum, and solutions of 5 mL of 100× vitamins, 5 mL of 100 mM sodium pyruvate, 5 mL of 100× nonessential amino acids, 5 mL of 200 mM L-glutamine, and 2 mg of gentamycin base. Cells were plated at a density of 5×10^3 cells per well in 96-well plates in 180 μL of culture medium and allowed to adhere at 37 °C in humidified atmosphere containing 5% CO_2 for 16 h. Then, 10 μL of a 20× solution of each drug concentration were added, and plates were incubated for 48 h in a humidified 37 °C incubator containing 5% CO_2 before assay.

Determination of Cellular Metabolic Activity. Metabolic activity was assayed using Resazurin (alamar blue dye) reduction test (RRT) according to O'Brien et al. (*13*) with minor modifications (*14*). Briefly, plates were rinsed with 200 μL of PBS at 37 °C, then 150 μL of a 25 μg/mL solution of resazurin in RPMI 1640 without phenol red was added to each well. Plates were incubated for 1 h at 37 °C in a humidified atmosphere with 5% of CO_2, and fluorescence was measured with an automated 96-well plate reader Fluoroskan Ascent FL (Labsystems). Excitation and emission wavelengths were, respectively, 530 and 590 nm. In the conditions of assay, fluorescence is proportional to the number of living cells in each well and IC_{50} was calculated from the curve of concentration-dependent survival percentage, defined as the fluorescence in experimental wells versus that in control wells, after subtraction of blank (medium with solvents alone) values. IC_{50} values were calculated by regression analysis of the concentration−response data and results were expressed as the mean from 3 experiments. To determine the significance of differences in cell proliferation among the treated cultures, statistical analysis was performed using Student's *t* test.

Determination of Biomass Content. Assay was performed using Hoechst dye 33342 according to Rago et al. (*15*) with minor modifications (*14*). Cells were suspended in 100 μL of a 0.01% (m/v) sodium dodecyl sulfate (SDS) solution and plates were incubated for 1 h at room temperature, then frozen at −80 °C for 1 h. After the plates thawed (approximately 15 min), 100 μL of Hoechst dye 33342 solution at 30 μg/mL in an hypersaline buffer containing 10 mM Tris−HCl pH 7.4, 1 mM EDTA, and 2 M NaCl were added to each well, then plates were incubated for 1 h at room temperature in the dark before measurement of fluorescence at 360/460 nm. Using these conditions, the fluorescence is proportional to cell biomass and IC_{50} was calculated as above.

Inhibition of Superoxide Generation in Peritoneal PMA-Activated Leukocytes. Assay was done as previously described (*14*) in peritoneal leukocytes elicited from male Wistar rats (*16*). Their viability was tested by trypan blue and found greater than 95%. Inhibition of superoxide generation was assessed in cells (5×10^6) preincubated at 37 °C for 10 min in Hank's balanced salt solution before stimulation with phorbol myristate acetate (PMA, 1 μM). The amount of superoxide was estimated as the reduction of ferrichrome C at 550 nm (*17*). Mammalian SOD (37.5 units/mL) was used as a standard.

DNA α-Polymerase Assay. Enzyme activity was assayed according to Ohta et al. (*18*) by incubating calf thymus DNA α-polymerase (0.05 U in 50 mM Tris−HCl, pH 7.5) with various concentrations of SF in DMSO for 60 min at 37 °C in a mixture (final volume 24 μL) containing 20 μM of deoxythymidine 5′-triphosphate (dTTP) solution containing [α-^{32}P]-dTTP (1000 cpm/pmol), 15% glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 μg/mL of poly(dA), and 10 μg/mL oligo(dT). Before

Table 1. Procedure for Isolation of SQAGs from *P. cruentum*

step	amount (g)	yield (%)
biomass (wet)	50.000	100.00
lipidic extract	01.630	003.26
acetone precipitation	00.793	001.59
adsorption chromatography (SiOH)	00.317	000.63
ion-exchange chromatography (DEAE)	00.132	000.26
acetone precipitation (SF)	00.075	000.15

Table 2. Fatty Acid Composition of the SQAG Fraction from *P. cruentum* (SF)

fatty acid	content (μM, %)	content (μg, %)
C16:0	28.8	26.1
C16:1n-9[a]	11.6	10.5
C18:0	0.7	0.7
C18:1n-9 cis	0.5	0.5
C18:1n-7	0.5	0.5
C18:2n-6 cis	4.1	4.0
C18:3n-6	0.5	0.5
C18:3n-3[a]	0.3	0.3
C20:2n-6	0.3	0.4
C20:3n-6[a]	2.7	2.9
C20:4n-6[a]	34.2	36.9
C20:5n-3[a]	15.5	16.6

[a] Indicates main differences with SQAGs from plants.

assay, the enzyme was purified as described by Tamai (*19*), then preincubated on ice for 10 min with SF before addition of the enzyme−inhibitor component. Each value was obtained as the average of triplicate experiments.

RESULTS

Extraction and Analysis of SF. The crude sulfolipid fraction (SF, 0.15% of dry biomass) was extracted according to well-established methods. Briefly, extraction of total lipids according to Bligh and Dyer (*8*), fractionation into glycolipids by column chromatography on silica gel (*9*), then into sulfoglycolipids (SF) by ion exchange chromatography (*10*), and purification of sulfoglycolipids by Folch wash (*11*) then acetone precipitation. Yields at each step are summarized in **Table 1**. Preliminary NMR and MS analysis have shown that SF contained sulfoquinovosylacylglycerols (SQAGs), i.e., glycolipids exhibiting a glycerol backbone with a polar headgroup of sulfoquinovose. The present study confirmed that the sugar linked at C3 of glycerol is a galactopyranosyl moiety. Because previous studies on SQAGs have demonstrated a relationship between their biological activities and their fatty acid composition (*6*, *7*), SF was assayed for fatty acids by GC−FID. Fatty acids were analyzed as fatty acid methyl esters (FAMEs), identified by comparing their retention times with those of authentic standards and quantified using margaric acid (C17:0) as internal standard. Results (**Table 2**) indicated that, in contrast with STD in which C18:3 fatty acids predominated, SF contained large amounts of ARA (C20:4n-6, 36.8%) and EPA (C20:5n-3, 16.6%), two long-chain PUFAs which were not present in STD, and noticeable amounts of the mono-unsaturated fatty acid 16:1n-9 (10.5%). Neither docopentaenoic acid (C22:5n-3) or docosahexaenoic acid (C22:6n-3) were detected.

Inhibition of Superoxide Generation by Activated Peritoneal Mono Nuclear Cells (PMNs). Because of the large amounts of unsaturated fatty acids, an antioxidant activity was expected. The assay was done in primed PMNs, known to release large amounts of hydrogen peroxide and superoxide anion (oxidative burst) to kill invading microorganisms and to have fundamental roles in allergy and inflammation. Results (**Figure 1**) demonstrated a strong, dose-dependent inhibition (IC_{50}: 29.5 μg/mL) of superoxide anion generated by the SOD-inhibitable reduction of cytochrome *c* and showed that 75 μg of SF exhibited an antioxidant activity equivalent to 12.5 units of bovine SOD.

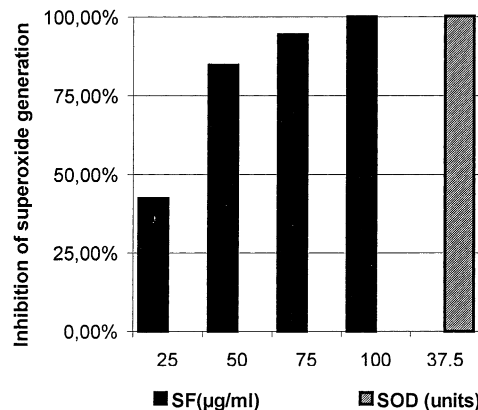

Figure 1. Inhibition of superoxide anion generated by phorbol myristate acetate in rat peritoneal leukocytes. Compared effects of SQAGs from *P. cruentrum* (SF, black) and of bovine SOD (37.5 units, gray). Values are expressed as mean for 6 tests.

SF Inhibits the Growth of Solid Cancer Cell-Lines. To assess and compare the effects of SF and STD on cell proliferation, murine immortalized cells L-929, and a panel of human solid cancer cells (colon adenocarcinoma DLD-1, breast adenocarcinoma MCF-7, prostatic adenocarcinoma PC-3 cells, and malignant melanoma cells M4-Beu) were treated with drugs at concentrations in the range of 0−100 μg/mL for SF and 0−250 μg/mL for STD (range of soluble concentrations). The metabolic activity of the culture (assayed by Resazurin reduction test, RRT) and the cell biomass (assayed with Hoechst dye 33342, a benzimidazole derivative that binds AT pairs in DNA) were examined after a 48-h continuous contact as compared to the control values ($P < 0.001$). Results (**Figure 2**) demonstrated that despite a weak inhibitory effect on breast MCF-7 cell-proliferation, both SF and STD inhibited the growth of all tested cell-lines, especially that of DLD-1 colon adenocarcinoma. They clearly showed a stronger efficacy of SF on all tested cell-lines as demonstrated by IC_{50} values for growth inhibition in the range of 20−46 μg/mL for SF instead of 94−250 μg/mL for STD. It was observed that for each cell-line, the amount of drug required to decrease the metabolic activity of the culture by 50% was markedly higher than that necessary to reduce the proliferation of cell biomass in the same proportions, indicating that many cells unable to enter in division were metabolically active, and therefore, SF and STD inhibited growth-rates by both cytotoxic and cytostatic effects and blocked the cell cycle at a step corresponding to a transient increase of cell metabolism. One can also observe that in classical conditions (i.e., in solution in DMSO), SQAGs from *P. cruentrum* appear 2 to 5 times more active than synthetic SQAGs against proliferation of colon carcinoma DLD-1 cells, suggesting a greater ability to penetrate into cancer cells. Indeed, synthetic SQAGs necessitate the use of specific solvent promoting their permeability into the cells to exhibit strong activity (*18*).

Inhibition of Mammalian α-DNA Polymerase. To determine if cell-growth inhibitory effects were related to an inhibition of DNA α-polymerase, SF and STD were examined for their ability to decrease mammalian DNA α-polymerase activity with a method previously used by Hanashima et al. (*20*) to assess IC_{50} values of synthetic SQDGs containing glucose

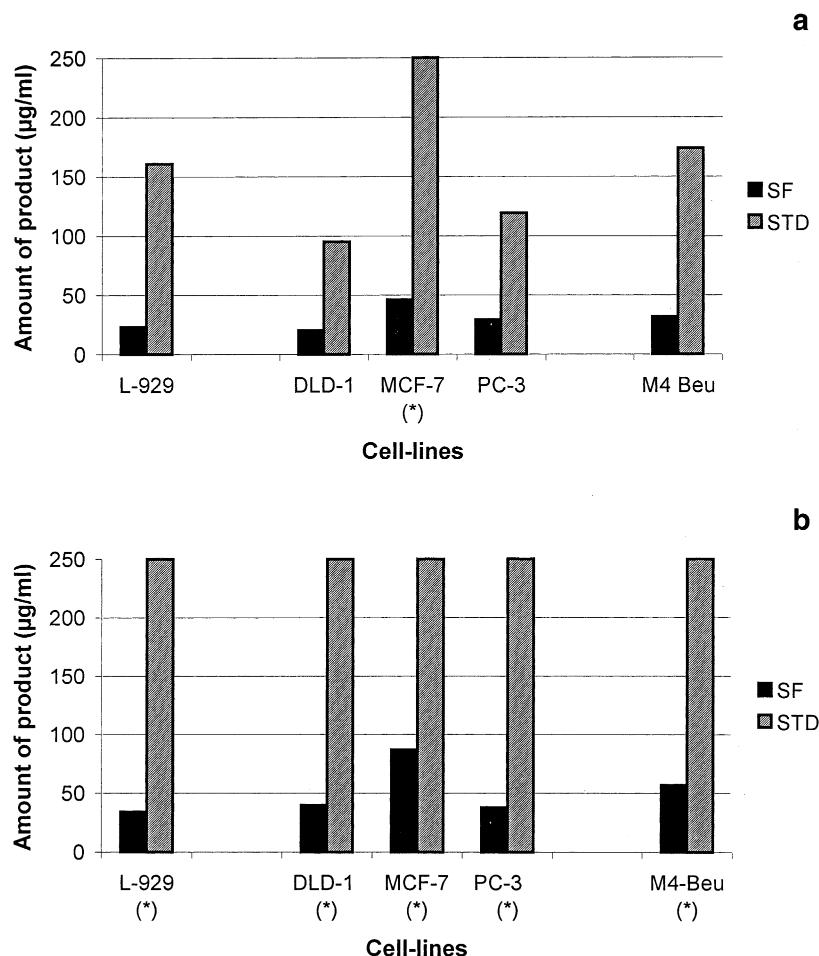

Figure 2. Anti-proliferative effects of SQAGs from *P. cruentrum* (SF, black) as compared to SQDGs from plants (STD, gray) on murine L-929 cells, and on colon carcinoma cells DLD-1, breast carcinoma cells MCF-7, prostatic carcinoma cells PC-3, and malignant melanoma M4-Beu human cell-lines. Effects were assayed by measuring (a) the biomass content (Hoechst dye 33342 assay, HO) and (b) the cellular metabolic activity (Resazurin reduction test, RRT test) of cultures submitted to a 48-h continuous contact with drugs as described in Materials and Methods. IC_{50} values (μg/mL) are expressed as mean for 3 tests. (*) IC_{50} more than 250 μg/mL.

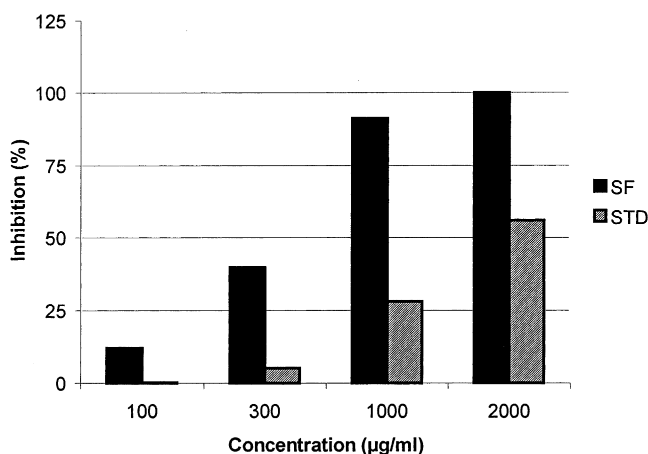

Figure 3. Compared inhibitory effects of SQAGs from *P. cuentrum* (SF, black) and SQDGs from plant (STD, gray) on mammalian DNA α-polymerase activity. Results are expressed as mean for 3 tests.

as sugar moiety. Both STD and SF inhibited the enzyme effectively with IC_{50} values equal to 378 μg/mL for SF and 1784 μg/mL for STD. On the basis of these results, and on reported data (*20*), SF appeared more effective than all tested synthetic SQAGs; however, comparison is difficult because experiments have not been conducted simultaneously. Further experiments are therefore needed to clarify this point. Nevertheless, the present results unambiguously demonstrated that SF exhibited stronger inhibition of mammalian DNA α-polymerase than SQDGs from plants. Inhibition of DNA polymerase, a nuclear enzyme involved in an early step of DNA replication, results in a decrease of proliferative rates and cell death via apoptosis due to a blockage of cell-cycle at early S-phase (*18*, *20*). This effect is in agreement with our hypothesis about a probable arrest of the cell cycle at a transient increase of cell metabolism. The higher efficacy of SF compared to STD is also in agreement with the observations of Hanashima et al. who reported that SQDGs with longer carbon chains were more effective than those with shorter carbon chains (*20*).

DISCUSSION

Although it is not known whether the consumption of *Porphyridium cruentum* cells is beneficial or deleterious to health, the present work shows that SF, a crude SQAG fraction rich in long-chain PUFAs, exhibits biological properties which may have beneficial effects on human health, and, therefore, might have potential pharmaceutical or nutritional value, or both. It exhibits antioxidant and anti-inflammatory effects because of its ability to inhibit the generation of superoxide anion known to be the precursor of compounds that amplify the inflammation process and allow a self-propagating response by increasing vascular permeability, adhesion of PMNs to the endothelium,

and stimulation of platelet aggregation (*21*). It also shows antiproliferative effects on various human solid cancer cell-lines, especially colon adenocarcinoma DLD-1. This panel of activity suggests a chemopreventive and/or a chemotherapeutic potential which has to be evaluated in vivo and compared with that of KM043, a SQDG fraction from another marine red alga: *Gigartina tella* (*6*), those of various plant and synthetic SQAGs, and those of reference drugs. As STD and SF differ mainly in their unsaturated fatty acid composition, the question is, what role do the unusual fatty acids such as ARA and EPA, play in SF activity? There is strong evidence that they modulate in vitro antioxidant and anti-inflammatory activities. On the basis of the presence of EPA, a ω-3 long-chain PUFA known to reduce in vivo the synthesis of pro-inflammatory eicosanoids (*22*) and pro-inflammatory cytokines IL-1, IL-6, and TN-α (*22*−*25*), to suppress lymphocyte proliferation (*23*), and to reduce both superoxide generation by neutrophils (*25*) and recruitment of phagocytes (*26*), SF might have a chemopreventive potential in man. The mechanism by which dietary lipids containing long-chain PUFAs exert their antitumor activity in the colon is not clearly understood but involves alteration of lipid composition and eicosanoid production in colonic lymphocytes (*27*) and peripheral immune cells (*28*), and changes in membrane composition that could affect cell signaling pathways (*29*). Others factors have been summarized by Diggle in her review on the relationship between PUFAs and cancer (*29*). These include lipid peroxidation allowing genetic damages, change in mitochondrial pH levels and ability to uncouple oxidative phosphorylation, and induction of transcriptional changes mainly related to PUFAs' ability to bind and activate transcriptional factors. Considering its inflammatory property, SF might contribute to decrease of the hyperimmune responses known to influence the risk for colon tumor development, however, it also contains large amounts of ω-6 fatty acids, especially ARA, known to counteract in vivo the beneficial effects of EPA by activating the leukocyte NADPH oxidase. ARA liberated in vivo by phospholipase A_2 is known to be essential for NADPH oxidase activation (*27*). Is PLA_2 able to hydrolyze ARA from SQAGs? What is the cytotoxicity of SQAGs for normal cells? Is the stronger activity of SF on DLD-1 cell membrane relative to a specific binding on membrane surface as reported for synthetic SQDGs (*30*)? Are SQAGs from *P. cruentrum* able to inhibit fatty acid synthase (FAS), recently identified as a new target for cancer therapy because of its elevated levels in cancer cells (*29*)? It is clear that experiments answering these questions and others are needed to evaluate the proliferative/cytotoxic effects of SQAGs from *P. cruentrum*, (and to a larger extent, those of SQAGs from marine origin) and therefore to assess their chemopreventive and/or chemotherapeutic potential in an in vivo situation.

In conclusion, the present results show that SF, a SQAG fraction from the red alga *P. cruentum* exhibits in vitro antioxidant, anti-inflammatory, and anti-proliferative effects, and might have a chemopreventive potential. Activity against colon carcinoma DLD-1 justifies new evaluations. New experiments are needed to isolate the most active compounds, determine their chemical structures, and identify the various factors involved in activity.

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