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Structure-Activity Relationship of a Novel Group of Mammalian DNA Polymerase Inhibitors, Synthetic Sulfoquinovosylacylglycerols

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We reported previously that sulfolipids in the sulfoquinovosylacylglycerol class from a fern and an alga are potent inhibitors of DNA polymerase α and β and potent anti-neoplastic agents. In developing a procedure for chemical synthesis of sulfolipids, we synthesized many derivatives and stereoisomers of sulfoquinovosylmonoacylglycerol (SQMG)/sulfoquinovosyldiacylglycerol (SQDG). Some of these molecules were stronger inhibitors than the SQMG/SQDG originally reported as natural compounds. In this study, we examined the structure-inhibitory function relationship of synthetic SQMG/SQDG and its relationship to cytotoxic activity. The inhibitory effect is probably mainly dependent on the fatty acid effect, which we reported previously, although each of the SQMG/SQDG was a much stronger inhibitor than the fatty acid alone that was present in the SQMG/SQDG. The inhibitory effect could be influenced by the chain size of fatty acids in the SQMG/SQDG. The sulfate moiety in the quinovose was also important for the inhibition. Lineweaver-Burk plots of SQMG/SQDG indicated that DNA polymerase α was non-competitively inhibited, but the SQMG/SQDG were effective as antagonists of both template-primer DNA-binding and nucleotide substrate-binding of DNA polymerase β. The SQMG had an cytotoxic effect, but the SQDG tested did not. The SQDG might not be able to penetrate into cells. Based on these results, we discuss the molecular action of SQMG/SQDG and propose drug design strategies for developing new anti-neoplastic agents.

Key words: Sulfoquinovosylmonoacylglycerol (SQMG) — Sulfoquinovosyldiacylglycerol (SQDG) — DNA polymerase — Enzyme inhibitor — Anti-neoplastic agents

At least seven classes of DNA polymerases (pol. α, β, γ, δ, ε, ζ and η) have been identified in mammalian cells.[1–3] Moreover, pol. θ may also be expressed in mammals. Recent studies have revealed the biochemical and structural properties of these polymerases, and some of their genes have been cloned. The *in vivo* functions of some of these polymerases, especially pol. β, δ, ε, ζ and η, appear to be related to DNA repair and/or recombination. On the other hand, pol. α, γ, δ and ε are mainly involved in nuclear or mitochondrial DNA replication. Our recent studies of the polymerases have focused on understanding the structure and function of these polymerases, especially pol. α and β types, and identifying the factors controlling their activities. For these studies, potent and selective inhibitors are required.[1] In screening studies, we found many inhibitors of mammalian DNA polymerases.[4–19] At present, we are engaged in analyzing the structure and function of the DNA polymerases using these inhibitors from two different viewpoints; to understand the precise role of each polymerase *in vivo*, and to develop a drug design strategy for cancer chemotherapy agents. Since DNA polymerases are essential enzymes for DNA replication and repair, and thus for cell division, inhibition of the enzymes will lead to killing of cells, especially under conditions of proliferation. The inhibitors of mammalian DNA polymerases are not only molecular tools useful for analyzing the polymerases, but should also be considered as candidates for cancer chemotherapy agents.

Several sulfolipids in the sulfoquinovosylacylglycerol class extracted from a fern and an alga were found to be DNA polymerase inhibitors.[11, 14, 15] One of them structurally coincided with a compound from a cyanobacterium reported as an AIDS anti-viral agent.[20, 21] The sulfolipids potently inhibited the activities of mammalian pol. α and pol. β and TdT, and moderately inhibited those of HIV-1 reverse transcriptase in a dose-dependent manner, but hardly influenced the activities of prokaryotic DNA polymerases such as *Escherichia coli* DNA polymerase I, and DNA metabolic enzymes such as deoxyribonuclease I. Sahara *et al.* reported that other sulfolipids from sea

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Abbreviations: pol., DNA polymerase (EC 2.7.7.7); HIV-1, human immunodeficiency virus type 1; TdT, terminal deoxynucleotidyl transferase; SQMG, sulfoquinovosylmonoacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; MIC, minimum inhibitory concentration; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; MEM, modified Eagle's medium; NP-40, Nonidet P-40; BSA, bovine serum albumin; ssDNA, single-stranded DNA.

urchin showed anti-tumor activity in studies on transplantation of human tumor into mice.[22] We recently confirmed that the agents were also mammalian DNA polymerase inhibitors (Mizushina *et al*., unpublished data). Simbulan *et al*. also showed that several glycolipids from mammals inhibited the activities of mammalian DNA polymerases, and suggested that sulfate and sialic acid moieties in the compounds were essential for the inhibition.[23] These observations indicated that some of the sulfolipids could be selective inhibitors of mammalian DNA polymerases, and might have potent anti-tumor and/or anti-viral activity. We chemically synthesized natural sulfolipids of the SQMG and SQDG classes, because our experiments required large quantities of the natural sulfolipids, and because stereoisomers of the sulfolipids and their derivatives could be useful as inhibitors. We succeeded in systematically synthesizing many kinds of SQMG/SQDG including stereoisomers of the sulfolipids and their derivatives (Hanashima *et al*., in preparation). The details of the chemical synthesis of the sulfolipids will be published elsewhere.

In this study, we examined the structure-function relationship for inhibition of DNA polymerases by synthetic derivatives and/or stereoisomers of the SQMG/SQDG, and elucidated the molecular mechanism of the inhibitory action of these synthetic sulfolipids on the DNA polymerase activity and on mammalian cell proliferation. In the SQMG/SQDG, not only the fatty acid moiety, but also the sulfate in the quinovose seem to be important for inhibition of DNA polymerase activity. The molecular mechanism of the inhibition seems to be dependent on the fatty acid chain length, as well as the sulfate moiety in the monosaccharide. This information should allow us to design novel sulfolipid inhibitors.

MATERIALS AND METHODS

Chemical synthesis of sulfolipids and their derivatives Sulfolipids and their derivatives, including stereoisomers, were synthesized according to a procedure which will be published elsewhere (Hanashima *et al*., in preparation). Structures of the synthesized compounds are summarized in Fig. 1. We chemically synthesized several types of SQMG and SQDG, and also prepared many compounds by linking a sulfate moiety (group I), monosaccharide (group II), glyceride (group III) and fatty acid(s) (group IV) (see Fig. 1A). In the symbols (e.g. C_{18}-α-SQDG), SQDG refers to sulfoquinovosyldiacylglycerol, α- refers to the stereoisomer between group II and III, and C_{18}- refers to C_{18}-saturated fatty acid (stearic acid) (see Fig. 1).

Enzymes and DNA polymerase assays DNA polymerase α (pol. α) was purified from calf thymus by immuno-affinity column chromatography as described previously.[24] DNA polymerase β (pol. β) was purified from a recombinant plasmid expressing rat pol. β.[25] *E. coli* DNA polymerase I and human immunodeficiency virus type 1 (HIV-1) reverse transcriptase were purchased from Worthington Biochemical Corp. (Freehold, NJ). T4 DNA polymerase, calf thymus TdT, *Taq* DNA polymerase, T4 DNA polymerase, T7 RNA polymerase and T4 polynucleotide kinase were purchased from TaKaRa (Kyoto). Bovine pancreas deoxyribonuclease I was purchased from Stratagene Cloning Systems (La Jolla, CA).

The activities of DNA polymerases, HIV-1 reverse transcriptase and bovine deoxyribonuclease I were measured by the methods described previously.[4, 5] One unit of DNA polymerase catalyzes the incorporation of 1 nmol of dTTP into synthetic template-primer (poly(dA)/oligo$(dT)_{12-18}$, A/T=2/1) at 37°C in 60 min. The activities of RNA polymerase and polynucleotide kinase were measured in standard assays according to the manufacturer's specifications, as described by Nakayama and Saneyoshi[26] and Soltis and Uhlenbeck,[27] respectively. The activity of bovine deoxyribonuclease I was measured by the methods described previously.[28]

Investigation of cytotoxicity on cultured cells For investigation of the *in vivo* effects of SQMG/SQDG, a human stomach cancer cell line originated from a patient with cancer, NUGC-3, was used. The cells were routinely cultured using MEM supplemented with 10% fetal calf serum, 250 mg/ml fungizone, and 300 mg/ml L-glutamine as standard medium, at 37°C in a humidified atmosphere of 5% CO_2-95% air. Cytotoxicity of SQMG/SQDG was investigated as follows. High concentrations of SQMG and SQDG were dissolved in DMSO and stocked. Approximately 2×10^3 cells per well were inoculated in 96-well micro plates, then SQMG/SQDG stock solution was diluted to various concentrations with standard medium, and applied to each well. After incubation for 48 h, survival rate was determined by MTT assay.[29]

RESULTS

Inhibitory effects of synthetic SQMG/SQDG on DNA polymerase activities The sulfolipids can be separated into four moieties: monosaccharide with or without a sulfate moiety (group I & II), glyceride (group III) and fatty acid(s) (group IV) (Fig. 1). We synthesized new sulfolipids (SQMG/SQDG) (group I & II & III & IV) by assembling derivatives of each of the four parts into different kinds of sulfoquinovosylacylglycerols. The structures of the compounds are summarized in Table I. Some of the compounds, for example the SQMG/SQDG with saturated-form long chain (C_{20}- and C_{22}-) fatty acids and SQMG/SQDG with unsaturated form fatty acids, were not tested because they have not yet been synthesized. The monosaccharides such as sulfofucose and sulforhamnose were used instead of the sulfoquinovose, and the fatty

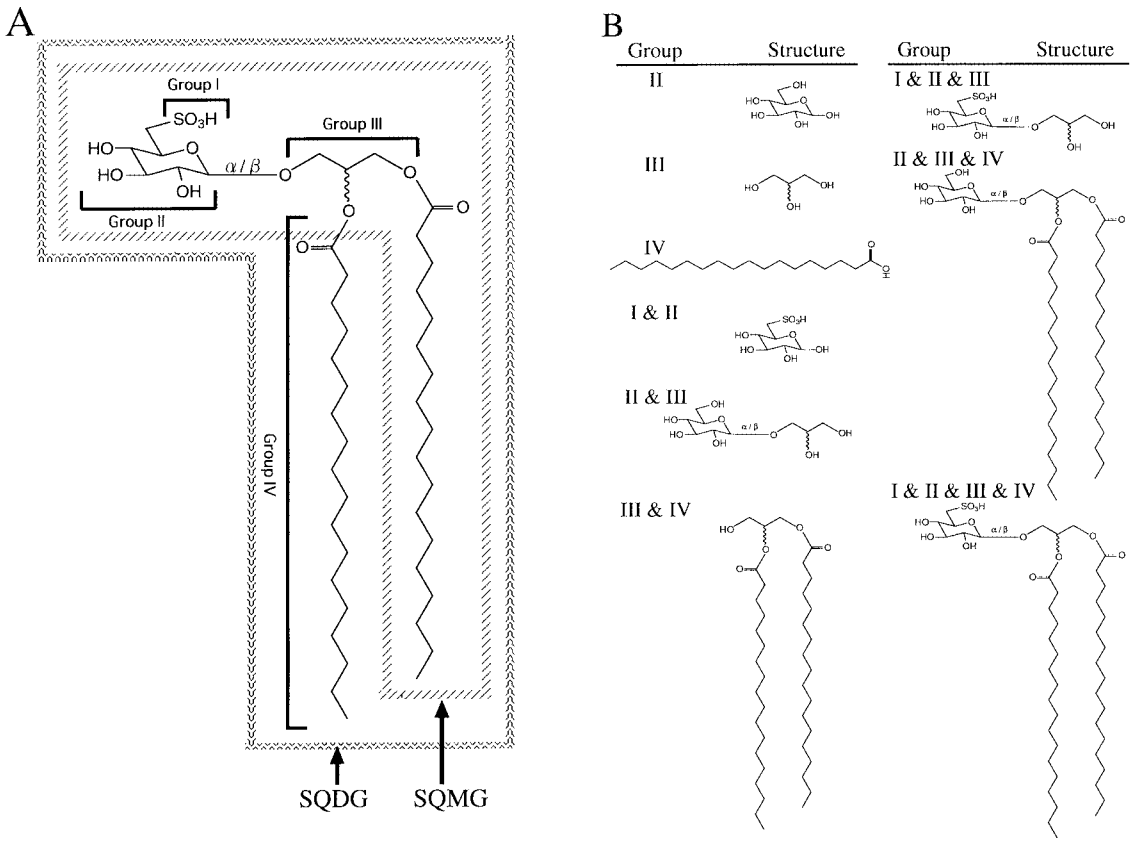

Fig. 1. Structures of derivatives and stereoisomers of sulfoquinovosylmonoacylglycerol (SQMG) and sulfoquinovosyldiacylglycerol (SQDG). (A) Chemical structure of C_{18}-SQDG. Scheme showing SQMG and SQDG separated into four moieties. Group I, 6-sulfate moiety; group II, monosaccharide; group III, glyceride; group IV, fatty acid(s). (B) Chemical combinations of parts in synthesized C_{18}-SQDG.

acids were saturated species with 14 to 18 carbon atoms. For addition of fatty acids, SQMG/SQDG were synthesized, because these two types of natural product were reported to be effective DNA polymerase inhibitors.[11, 14, 15] The two fatty acid molecules in each SQDG were the same.

As shown in Table I, neither monosaccharides (group II), with or without a sulfate moiety (group I), nor glycerides (group III) showed any inhibitory effect, and the assembled compound, sulfoquinovosylacylglyceride (group I & II & III), also had no inhibitory effect. Each of the fatty acids from C_{14} to C_{18} (group IV), as described previously,[4, 6] inhibited the activities of pol. α moderately and pol. β weakly. When the fatty acid(s) (group IV) were joined to the sulfoquinovosylacylglyceride (group I & II & III) as sulfoquinovosylacylglycerol (group I & II & III & IV), the resultant compound showed a potent inhibitory effect on DNA polymerase activity. The sulfate moiety in the quinovose was essential for inhibition since neutral glycolipids (group II & III & IV) had no inhibitory effect. For compounds to show potent inhibitory effects, all four parts of the molecule were required. Sulfoquinovose could be replaced with other sulfo-monosaccharides such as sulfofucose and sulforhamnose, although the inhibitory effects of sulfofucosylmonoacylglycerol and the sulforhamnosylmonoacylglycerol were almost the same as that of SQMG. Sulfofucose alone or sulforhamnose alone, like sulfoquinovose, was not capable of inhibiting polymerase activity. SQMG/SQDG must have one or two molecules of fatty acid at the 2′ and/or 3′ position in the glycerol moiety to inhibit the polymerase-inhibitory activities. Similarly, the SQMG/SQDG could have stereoisomers of two configurations, α- or β-type, between the sulfoquinovose (group I & II) and the glyceride (group III). Both of the configuration types inhibited the polymerases to similar extents, suggesting that the configuration is not important for the inhibitory effect. Interestingly, the inhibitory effect was strongly influenced by changes of the sulfolipid structure, suggesting an approach for the design of new SQMG/SQDG.

Table I. Effects of Derivatives and Stereoisomers of Synthesized SQMG/SQDG on the Activities of DNA Polymerase α and β

Group	I	II	Isomer between I and II	III	IV	IC_{50} (μM)[a]		MIC (μM)[b]		Symbol
						Pol. α	Pol. β	Pol. α	Pol. β	
II	–	Quinovose		–	–	>1000	>1000			
II	–	Fucose		–	–	>1000	>1000			
II	–	Rhamnose		–	–	>1000	>1000			
III	–	–		+	–	>1000	>1000			
IV	–	–		–	Myristic acid (14:0)	95	440	180	>1000	
IV	–	–		–	Palmitic acid (16:0)	42	290	100	500	
IV	–	–		–	Stearic acid (18:0)	8	130	35	250	
I&II	+	Quinovose		–	–	>1000	>1000			
I&II	+	Fucose		–	–	>1000	>1000			
I&II	+	Rhamnose		–	–	>1000	>1000			
II&III	–	Quinovose	α	+	–	>1000	>1000			
II&III	–	Quinovose	β	+	–	>1000	>1000			
II&III	–	Fucose	α	+	–	>1000	>1000			
II&III	–	Fucose	β	+	–	>1000	>1000			
II&III	–	Rhamnose	α	+	–	>1000	>1000			
II&III	–	Rhamnose	β	+	–	>1000	>1000			
III&IV	–	–		+	Myristic acid (14:0)/H	750	>1000	>1000		
III&IV	–	–		+	Palmitic acid (16:0)/H	690	>1000	>1000		
III&IV	–	–		+	Stearic acid (18:0)/H	650	>1000	>1000		
III&IV	–	–		+	Myristic acid (14:0)/Myristic acid (14:0)	550	>1000	>1000		
III&IV	–	–		+	Palmitic acid (16:0)/Palmitic acid (16:0)	470	>1000	>1000		
III&IV	–	–		+	Stearic acid (18:0)/Stearic acid (18:0)	450	>1000	>1000		
I&II&III	+	Quinovose	α	+	–	>1000	>1000			
I&II&III	+	Quinovose	β	+	–	>1000	>1000			
I&II&III	+	Fucose	β	+	–	>1000	>1000			
I&II&III	+	Fucose	β	+	–	>1000	>1000			
I&II&III	+	Rhamnose	β	+	–	>1000	>1000			
I&II&III	+	Rhamnose	β	+	–	>1000	>1000			
II&III&IV	–	Quinovose	α	+	Stearic acid (18:0)/H	>1000	>1000			
II&III&IV	–	Quinovose	β	+	Stearic acid (18:0)/H	>1000	>1000			
II&III&IV	–	Quinovose	α	+	Stearic acid (18:0)/Stearic acid (18:0)	>1000	>1000			
II&III&IV	–	Quinovose	β	+	Stearic acid (18:0)/Stearic acid (18:0)	>1000	>1000			
II&III&IV	–	Fucose	α	+	Stearic acid (18:0)/H	>1000	>1000			
II&III&IV	–	Fucose	α	+	Stearic acid (18:0)/Stearic acid (18:0)	>1000	>1000			
II&III&IV	–	Rhamnose	α	+	Stearic acid (18:0)/H	>1000	>1000			
II&III&IV	–	Rhamnose	α	+	Stearic acid (18:0)/Stearic acid (18:0)	>1000	>1000			
I&II&III&IV	+	Quinovose	α	+	Myristic acid (14:0)/H	9.1	350	20	870	C_{14}-α-SQMG
I&II&III&IV	+	Quinovose	α	+	Palmitic acid (16:0)/H	4.0	34	9.1	43	C_{16}-α-SQMG
I&II&III&IV	+	Quinovose	α	+	Stearic acid (18:0)/H	2.1	16	4.2	21	C_{18}-α-SQMG
I&II&III&IV	+	Quinovose	α	+	Myristic acid (14:0)/Myristic acid (14:0)	1.8	23	5.3	37	C_{14}-α-SQDG
I&II&III&IV	+	Quinovose	α	+	Palmitic acid (16:0)/Palmitic acid (16:0)	1.2	25	3.4	45	C_{16}-α-SQDG
I&II&III&IV	+	Quinovose	α	+	Stearic acid (18:0)/Stearic acid (18:0)	0.4	37	1.1	60	C_{18}-α-SQDG
I&II&III&IV	+	Quinovose	β	+	Myristic acid (14:0)/H	11	370	28	900	C_{14}-β-SQMG
I&II&III&IV	+	Quinovose	β	+	Palmitic acid (16:0)/H	5.9	36	14	46	C_{16}-β-SQMG
I&II&III&IV	+	Quinovose	β	+	Stearic acid (18:0)/H	1.6	14	4.3	24	C_{18}-β-SQMG
I&II&III&IV	+	Quinovose	β	+	Myristic acid (14:0)/Myristic acid (14:0)	1.9	20	5.2	40	C_{14}-β-SQDG
I&II&III&IV	+	Quinovose	β	+	Palmitic acid (16:0)/Palmitic acid (16:0)	0.5	25	3.6	46	C_{16}-β-SQDG
I&II&III&IV	+	Quinovose	β	+	Stearic acid (18:0)/Stearic acid (18:0)	0.4	43	1.2	62	C_{18}-β-SQDG
I&II&III&IV	+	Fucose	α	+	Stearic acid (18:0)/H	3.3		6.4		
I&II&III&IV	+	Fucose	α	+	Stearic acid (18:0)/Stearic acid (18:0)	0.4		1.7		
I&II&III&IV	+	Rhamnose	α	+	Stearic acid (18:0)/H	2.4	19	5.2	27	
I&II&III&IV	+	Rhamnose	α	+	Stearic acid (18:0)/Stearic acid (18:0)	0.3	7.6	0.8	10.9	

a) 50%-inhibitory concentration.
b) Minimum inhibitory concentration.

The synthetic SQMG/SQDG tended to show stronger effects on pol. α than pol. β. The activity of pol. α was inhibited potently by the C_{14-18}-SQMG/C_{14-18}-SQDG, and the IC_{50} values (the doses that resulted in inhibition of 50 % of the activities) were in the range of 0.4 to 5.9 *μM* (Table I). On the other hand, pol. β was moderately influenced, and the range of the IC_{50} values was 14 to 43 *μM*, except for C_{14}-SQMG. The MIC (i.e., the doses that resulted in inhibition of 80% or more of the activities) were in the ranges of 1.1 to 14 *μM* for pol. α, and of 21 to 62 *μM* for pol. β, except for C_{14}-SQMG. The SQDG were stronger inhibitors of pol. α than were the SQMG, but both types of sulfolipids showed equivalent effects against pol. β. For both polymerase activities, stronger inhibitory effects were observed with longer chain size of the fatty acid in the SQMG (Table I). The same tendency was seen in the case of the SQDG for pol. α, but for pol. β the size of the fatty acids in the SQDG had little effect. For example, C_{18}-α-SQDG inhibited the pol. β activity at 37 *μM* (IC_{50}), whereas C_{14}-α- and C_{16}-α-SQDG showed inhibitory effects at 23 and 25 *μM*, respectively (Table I).

We previously reported the *in vitro* inhibition of the DNA polymerases by fatty acids alone.[4, 6] Some long-chain fatty acids could inhibit these DNA polymerase activities *in vitro*. The inhibitors were fatty acids with a hydrocarbon chain containing 18 or more carbons and with a free carboxyl end. Longer chain size of the fatty acid resulted in stronger inhibitory effects. As compared with the results for fatty acids alone (see ref. 4 and Table I), the characteristics of the fatty acids in the SQMG/SQDG were quite similar, but the inhibitory effects of the C_{14-18}-SQMG/C_{14-18}-SQDG (group I & II & III & IV), C_{14-18}-fatty acids (group IV) joined to the sulfoquinovosylacylglyceride (group I & II & III) at the carboxyl end, were much stronger than those of C_{14-18}-fatty acids alone (Table I). We investigated the inhibitory effects of C_{18}-SQMG/C_{18}-SQDG containing one or two stearic acid moieties (C_{18}) as representative examples. As shown in Fig. 2, both polymerase activities decreased dose-dependently. The IC_{50} value for pol. α of stearic acid alone was 12 *μM*, while those of C_{18}-α-SQMG and C_{18}-β-SQMG were 1.6 and 2.1 *μM*, respectively. The effectiveness of stearic acid (group I) was increased more than 6-fold by conjugation of sulfoquinovosylacylglyceride (group I & II & III). The data concerning the chain size of the fatty acid shown in Table I suggested that inhibition is mostly dependent on the fatty acid effect, and the sulfoquinovosylacylglyceride (group I & II & III) in the SQMG/SQDG may increase the inhibitory activity.

As shown in Fig. 3, the C_{18}-α-SQMG/C_{18}-α-SQDG also inhibited TdT, and had a moderate inhibitory effect on HIV-1 reverse transcriptase, but hardly affected the activities of DNA polymerases such as *E. coli* DNA polymerase I, T4 DNA polymerase and *Taq* DNA polymerase, or DNA metabolic enzymes such as T7 RNA polymerase, T4 polynucleotide and deoxyribonuclease I. All of the other SQMG/SQDG with shorter fatty acids showed similar effects (data not shown).

Modes of DNA polymerase α and β inhibition by synthetic SQMG/SQDG To investigate the mechanisms of

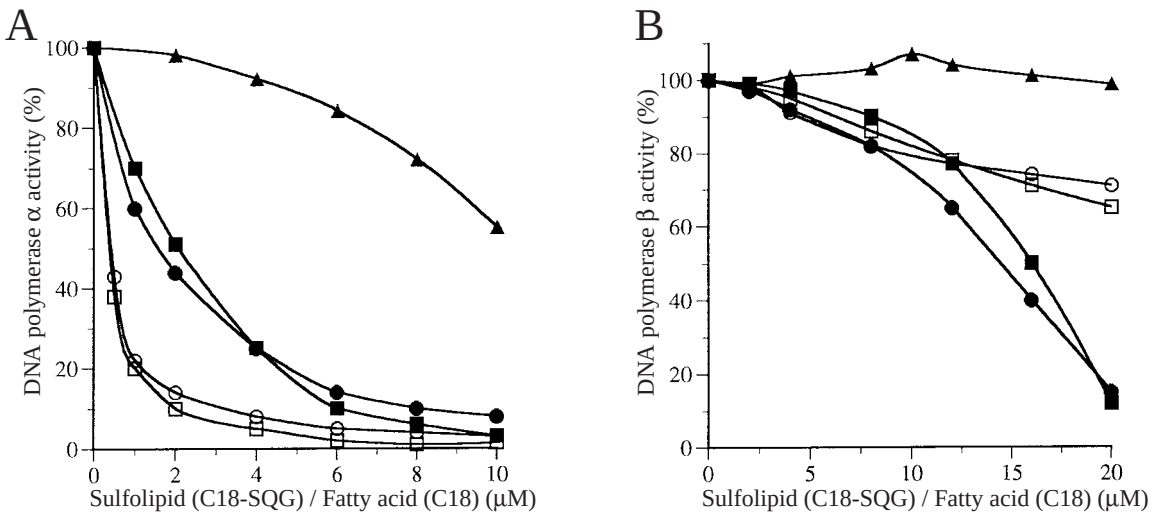

Fig. 2. Inhibition of activities of DNA polymerase α (A) and β (B) by C_{18}-α-SQMG/C_{18}-α-SQDG and stearic acid (C_{18}). Each mixture contained 0.05 units of enzyme. DNA polymerase activity (5000 cpm) in the absence of C_{18}-α-SQMG/C_{18}-α-SQDG or stearic acid was taken as 100%. The compounds tested and symbols used are as follows: C_{18}-α-SQMG, ■; C_{18}-α-SQDG, □; C_{18}-β-SQMG, ●; C_{18}-β-SQDG, ○; C_{18} (stearic acid), ▲.

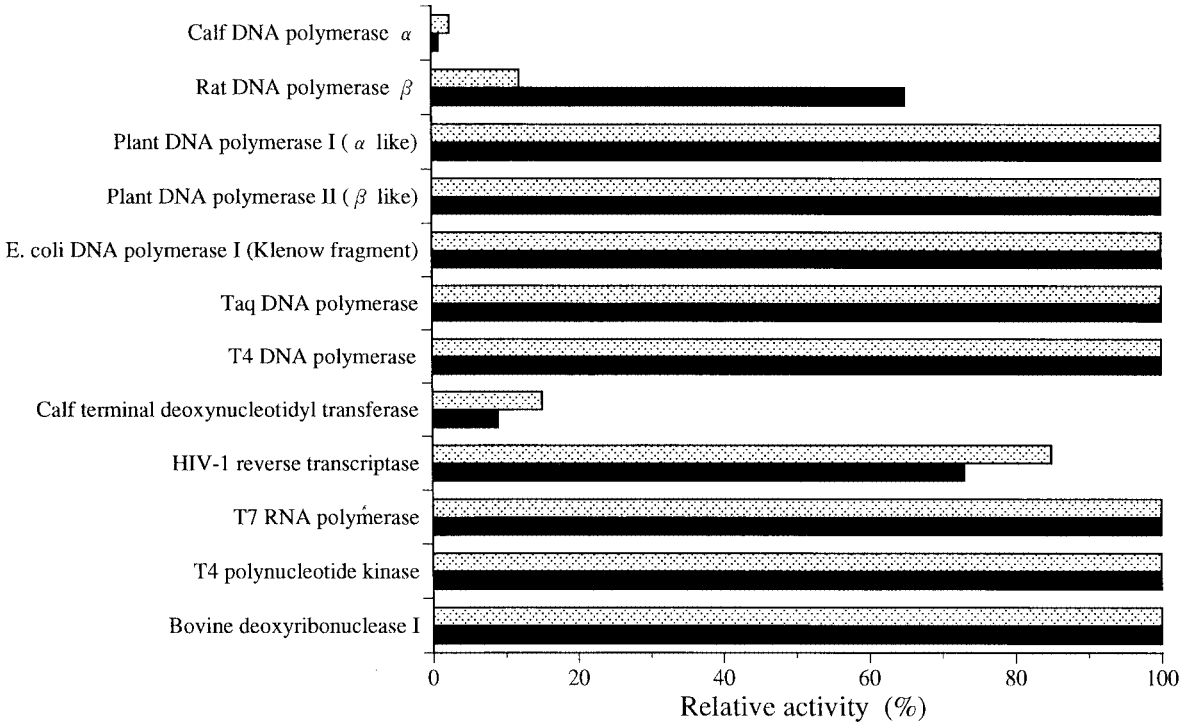

Fig. 3. Effects of C_{18}-α-SQMG/C_{18}-α-SQDG on the activities of various DNA polymerases and other DNA metabolic enzymes. C_{18}-α-SQMG/C_{18}-α-SQDG (20 *μM* each) were incubated with each enzyme (0.05 units). The enzymatic activity was measured as described in "Materials and Methods." Enzyme activity (5000 cpm) in the absence of C_{18}-α-SQMG/C_{18}-α-SQDG was taken as 100%. ▨ C_{18}-α-SQMG (20 *μM*), ■ C_{18}-α-SQDG (20 *μM*).

inhibition of pol. α and pol. β by SQMG/SQDG, kinetic analyses were performed (Table II). Poly(dA)/oligo$(dT)_{12-18}$ (A/T=2/1) and dTTP were used as DNA template-primer and nucleotide substrate, respectively. Double reciprocal plots of the results showed that inhibition of pol. α by C_{18}-α-SQMG/C_{18}-α-SQDG was non-competitive with both the DNA template-primer and dTTP substrate, since there were no changes in apparent K_m, while V_{max} for both were decreased in the presence of C_{18}-α-SQMG or C_{18}-α-SQDG. On the other hand, the inhibitory effects of C_{18}-α-SQMG and C_{18}-α-SQDG on pol. β were competitive with either the template-primer or the substrate. In the case of the template-primer, the apparent V_{max} of either C_{18}-α-SQMG/C_{18}-α-SQDG was unchanged, whereas an increase in K_m was observed in the presence of C_{18}-α-SQMG or C_{18}-α-SQDG. Similar results were observed with α-SQMG/α-SQDG with the other fatty acids and β-configured SQMG/SQDG tested, which could inhibit these DNA polymerase activities; non-competitive to pol. α and competitive to pol. β with either the DNA template-primer or the substrate (data not shown). These results were consistent with those for the natural products, SQDGs from fern,[11] and long chain fatty acids[4,6] described previously. These results indicated that each of the SQMG/SQDG may bind or interact to a domain distinct from the nucleotide substrate or template-binding sites on pol. α. On the other hand, each of the SQMG/SQDG must directly interact with both the nucleotide substrate- and template-binding sites of pol. β.

The inhibition constant (K_i) values of C_{18}-α-SQMG for pol. α, obtained from Dixon plots, were found to be 0.7 and 2.7 *μM* for template-primer and nucleotide substrate respectively, corresponding to the final concentration in the reaction mixture (Table II). Similarly, the K_i values for pol. β were 3.8 and 13 *μM*, respectively. For both enzymes, the K_i values for template-primer were smaller than those for nucleotide substrate, suggesting that either C_{18}-α-SQMG or C_{18}-α-SQDG binds or interacts with the template-primer binding site on the DNA polymerases with greater affinity than their nucleotide substrate binding site. Scatchard plot analyses indicated that each of the SQMG/SQDG and the template-primer interacted one to one on pol. β. Similar results were obtained for the interaction of each of the SQMG/SQDG and the nucleotide substrate (data not shown).

When NP-40 was added to the reaction mixture at a concentration of 0.05%, only the inhibition of pol. β was reversed (Table III), indicating that each of the C_{18}-α-

Table II. Kinetic Analysis of the Inhibitory Effects of C_{18}-α-SQMG/C_{18}-α-SQDG on the Activities of DNA Polymerase α and β, as a Function of the DNA Template-primer Dose and the dTTP Substrate Concentration

Enzyme	Template-primer[c]/ Nucleotide Substrate[d]	Sulfolipid conc. (μM)	K_m[a] (μM)	V_{max}[a] (pmol/h)	K_i[b] (μM)	Inhibitory mode[a]
		C_{18}-α-SQMG				
Pol. α	Template-primer	0	2.1	200	0.7	Non-competitive
		1	2.1	160		
		2	2.1	100		
		3	2.1	67		
	Nucleotide Substrate	0	1.3	230	2.7	Non-competitive
		1	1.3	190		
		2	1.3	140		
		3	1.3	90		
Pol. β	Template-primer	0	1.7	145	3.8	Competitive
		5	3.3			
		10	6.3			
		15	7.9			
	Nucleotide Substrate	0	0.9	38	13	Competitive
		5	1.0			
		10	1.3			
		15	2.7			
		C_{18}-α-SQDG				
Pol. α	Template-primer	0	2.1	200	0.2	Non-competitive
		0.2	2.1	100		
		0.4	2.1	56		
		0.6	2.1	40		
	Nucleotide Substrate	0	1.3	230	0.6	Non-competitive
		0.2	1.3	170		
		0.4	1.3	100		
		0.6	1.3	56		
Pol. β	Template-primer	0	1.7	145	5.0	Competitive
		10	5.0			
		20	9.8			
	Nucleotide Substrate	0	0.9	38	36	Competitive
		10	1.3			
		20	1.4			
		40	3.3			

a) From Lineweaver Burk plot.
b) From Dixon plot.
c) i.e., poly(dA)/oligo$(dT)_{12-18}$.
d) i.e., dTTP.

SQMG/C_{18}-α-SQDG may bind to and interact with the hydrophobic region on pol. β protein, probably around the template-binding site.[6, 8] The sulfolipids may bind more tightly to the pol. α protein in a different manner. We also tested whether an excessive amount of protein or nucleic acid (i.e., BSA at 80 μg/ml or 40 μ*M* poly(rC), respectively) could prevent the inhibitory effect of each SQMG/SQDG, to determine whether their effects resulted from non-specific adhesion to the polymerases, or selective binding to specific sites (Table III). Neither BSA nor poly(rC) influenced the effects of the C_{18}-α-SQMG/C_{18}-α-SQDG on pol. α or pol. β, suggesting that the binding to the polymerases occurs selectively. That is, each of the SQMG/SQDG appeared to inhibit these enzymes selectively, for example by binding to the hydrophobic region.

Effects of synthetic SQMG/SQDG on cultured mammalian cells To investigate the tumoricidal effects of each of the SQMG/SQDG, the human stomach cancer cell line

NUGC-3 was tested *in vitro*. As expected, some of the C_{14-18}-SQMG/C_{14-18}-SQDG prevented proliferation of the cell line; the inhibition curves are shown in Fig. 4. In this experiment, β-SQDG/β-SQMG with saturated fatty acids, and SQDG/SQMG with unsaturated fatty acids were not tested for the reason mentioned above. The results shown in Fig. 4 indicated that the synthetic SQMG had potent growth-inhibitory effects on this cancer cell line. Unexpectedly, none of the SQDG tested showed such an inhibitory effect. The LD_{50} concentrations of these SQMG were in the range of about 18 to 35 *μM* for the α-configurations and about 20 to 38 *μM* for the β-configurations. The configuration had little effect on the cell growth inhibition. As in the case of the effects on pol. α and pol. β, stronger inhibitory effects were observed with longer chain fatty acid in the SQMG (Table I). The LD_{50} value of the most potent compound tested, C_{18}-SQMG, was approximately 20 *μM* for NUGC-3 cells, and complete inhibition occurred at about 30 *μM*. Since the IC_{50} values of the C_{18}-SQMG were about 2 *μM* for pol. α, and approximately 15 *μM* for pol. β (Table I), the LD_{50} value was 10 times the IC_{50} value for pol. α and similar to the LD_{50} value for pol. β. Comparison of the data shown in Table I and Fig. 4 indicated that both *in vivo* cell growth inhibition and *in vitro* enzyme inhibition were reduced in parallel with the chain size of the fatty acid in the SQMG. For polymerase inhibition, SQDG was more effective than SQMG (Fig. 2), but the SQDG showed no effect on cell growth, suggesting that SQDG cannot penetrate into the cells.

Table III. Effects of Poly(rC), Bovine Serum Albumin (BSA) or Nonidet P-40 (NP-40) on the Inhibition of DNA Polymerase α and β Activities by C_{18}-α-SQMG/C_{18}-α-SQDG

Enzyme	Compounds added to the reaction mixture	Relative activity (%)
Pol. α	2 *μM* C_{18}-α-SQMG	
	2 *μM* C_{18}-α-SQMG	51
	2 *μM* C_{18}-α-SQMG+40 *μM* poly(rC)	48
	2 *μM* C_{18}-α-SQMG+80 *μ*g/ml BSA	53
	2 *μM* C_{18}-α-SQMG+0.05% NP-40	53
	2 *μM* C_{18}-α-SQDG	
	2 *μM* C_{18}-α-SQDG	10
	2 *μM* C_{18}-α-SQDG+40 *μM* poly(rC)	12
	2 *μM* C_{18}-α-SQDG+80 *μ*g/ml BSA	16
	2 *μM* C_{18}-α-SQDG+0.05% NP-40	15
Pol. β	20 *μM* C_{18}-α-SQMG	
	20 *μM* C_{18}-α-SQMG	12
	20 *μM* C_{18}-α-SQMG+40 *μM* poly(rC)	10
	20 *μM* C_{18}-α-SQMG+80 *μ*g/ml BSA	18
	20 *μM* C_{18}-α-SQMG+0.05% NP-40	99
	20 *μM* C_{18}-α-SQDG	
	20 *μM* C_{18}-α-SQDG	65
	20 *μM* C_{18}-α-SQDG+40 *μM* poly(rC)	59
	20 *μM* C_{18}-α-SQDG+80 *μ*g/ml BSA	63
	20 *μM* C_{18}-α-SQDG+0.05% NP-40	101

40 *μM* poly(rC), 80 *μ*g/ml BSA or NP-40 (0.05%) was added to the reaction mixture.

DISCUSSION

We have been screening for new DNA polymerase inhibitors for use in analyzing the structure and function of mammalian DNA polymerases, in order to understand

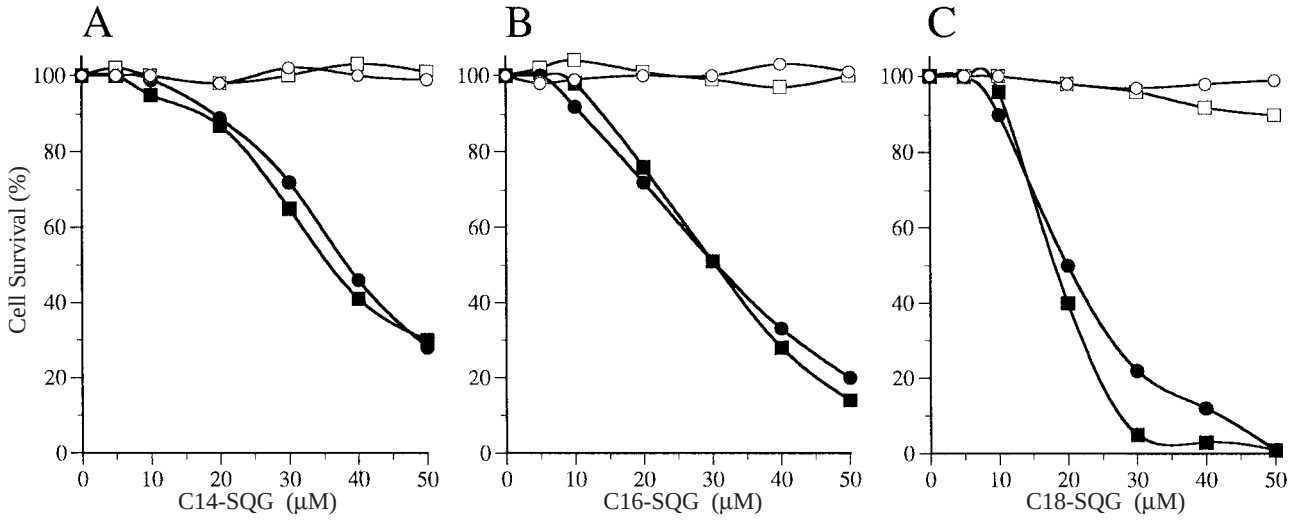

Fig. 4. Effects of SQMG/SQDG (C_{14-18}) on proliferation of NUGC-3 cancer cells. The assays were carried out under the conditions described in "Materials and Methods" with the indicated concentrations of C_{14}-SQMG/SQDG (A), C_{16}-SQMG/SQDG (B) and C_{18}-SQMG/SQDG (C). ■ α-SQMG, □ α-SQDG, ● β-SQMG, ○ β-SQDG.

their precise roles *in vivo*, and to develop drug design strategies for cancer chemotherapy agents. These inhibitors should be not only useful molecular tools for analyzing the polymerases, but also candidates as cancer chemotherapy agents. Subsequently, we synthesized many sulfoquinovosylacylglycerols, including stereoisomers and their derivatives (Hanashima *et al.*, in preparation). Some of the synthetic SQMG or SQDG compounds inhibited the activities of the DNA polymerases in the concentration ranges of 0.4 to 9.1 μM for pol. α and 14 to 43 μM for pol. β (Table I, Fig. 3). SQMG/SQDG showed markedly stronger inhibitory effects than aphidicolin on pol. α (Mizushina *et al.*, unpublished data). We should also emphasize that SQMG/SQDG suppressed the activities of pol. β *in vitro* to the same extent as dideoxyTTP, a well-known potent pol. β inhibitor.[16] Due to their strong inhibitory effects, these SQMG/SQDG could be useful as DNA polymerase-specific inhibitors in studies to determine the precise roles of each polymerase. As shown in Fig. 1A, the molecules of the SQMG/SQDG (group I & II & III & IV) consist of one/two saturated long alkyl chains (group IV) as a hydrophobic moiety and one 6-sulfated glucopyranoside ring (group I & II) as a hydrophilic site, bound to a glyceride moiety (group III) by ether linkages.

In this study, we demonstrated that not only the chain size of the fatty acid, but also the sulfate moiety of the glycerolipids was important for the inhibitory activity of the synthetic SQMG/SQDG. Sulfoquinovose (group I & II), glucosylacylglyceride (group II & III), acylglycerol (group III & IV), sulfoquinovosylacylglyceride (group I & II & III) and glucosylacylglycerol, which lack the sulfate moiety (group II & III & IV), showed no inhibitory effects. The 6-sulfo-D-quinovosylacylglycerol (group I & II & III & IV) could be replaced by a 6-sulfo-D-fucosylacylglycerol or 6-sulfo-D-rhamnosylacylglycerol, so the species of the sugar appeared to have little effect on the inhibition. The molecular mode of the inhibition seems to depend on the chain length of the fatty acids. Since the 6-sulfo-D-quinovosylacylglyceride (group I & II & III) itself showed no inhibitory effect on the polymerases, the inhibitory effects of these compounds may depend on the binding of the fatty acid ester region to the polymerases, and the 6-sulfo-D-quinovosylacylglyceride in the SQMG/SQDG appeared to strengthen the inhibitory effect.

Further investigations of the structure of the binding site on both polymerases and their modes of binding are necessary. Based on the results of cross-linkage studies and NMR analyses, we previously reported the mode of binding between pol. β and long chain fatty acids, which are major part(s) of the SQMG/SQDG involved in inhibition of pol. β activity.[18] Pol. β is the smallest known DNA polymerase in animal cells, with a molecular mass of 39 kDa, and its structure is highly conserved among mammals.[1] This protein has a modular two-domain structure, with apparent flexibility within a protease-sensitive region between residues 82–86, which separates the two domains, i.e., an N-terminal domain fragment (8 kDa), which retains binding affinity for ssDNA, and a C-terminal domain fragment (31 kDa) with reduced DNA polymerase activity.[30, 31] The fatty acids bound to pol. β at the N-terminal 8 kDa domain, where they competed with the DNA template-primer molecule.[6, 18] One molecule of each of the agents at the fatty acid region competed with one molecule of template DNA, and subsequently interfered with the binding of template-primer DNA to one molecule of 8 kDa domain, indicating that the 8 kDa domain fragment bound to the fatty acids as a 1:1 complex. Therefore, the fatty acids in the SQMG/SQDG may also fit into the binding site. The solution structure of the 8 kDa domain fragment with the fatty acids has been determined in more detail by multi-dimensional NMR.[18] The binding interactions with the fatty acids were mapped to one face of the 8 kDa domain fragment by characterizing the backbone ^{1}H and ^{15}N chemical shift changes. In the 8 kDa domain fragment with the fatty acids, the structure that forms the interaction interface included helix-1, helix-2, helix-4, a turn (residues from 48 to 51) and residues adjacent to an Ω-type loop connecting helix-1 and helix-2. Since the methyl end of the fatty acids appears to bind to L11, H51 and T79 of the interface on the amino acid sheets of the 8 kDa domain fragment and the carboxyl end interacts with the K35 site, the distance between the methyl and carboxyl ends might be important for tight binding. Only the shifted cross-peaks of L11 and T79 were significantly influenced by the length of the carbon chain. Longer fatty acids could bind to the fragment more tightly. In the action of the SQMG/SQDG on pol. β, the fatty acid interaction interface on the amino acid sheets of the 8 kDa domain fragment must be mostly the same as that in the case of fatty acids in SQMG/SQDG. Since the 6-sulfo-D-quinovose in the SQMG/SQDG bound to the carboxyl end of the fatty acids, it must be located not at the sites of L11, H51 and T79, but at the K35 site. The sulfate moiety may strengthen the binding to the surface of the 8 kDa domain fragment, resulting in a further shift of the cross-peak of K35. Although the influence of SQMG/SQDG on pol. α remains to be determined in future studies, the interaction of the fatty acids and pol. β suggested that they may be useful for analyzing the structure and function of the polymerases, and in our continuing screening for SQMG/SQDG with potent inhibitory activity against polymerases. Similar experiments should be performed using pol. α, because the inhibition of cell growth by the SQMG may be due to the inhibition of both polymerases. Unfortunately, pol. α is too large for NMR analysis at present.

We were also interested in developing cancer chemotherapy agents using these inhibitors. Therefore, we investigated the effects of these compounds on a human cancer

cell line (NUGC-3) in culture. The SQMG showed potent growth inhibitory effects on the cancer cells, but none of the SQDG tested had such an effect (Fig. 4). SQMG inhibited both polymerase activity and cell growth in a dose-dependent manner (Figs. 2 and 4). SQDG were completely inactive to the cells, although their *in vitro* effects were stronger than those of SQMG, suggesting that the SQDG cannot penetrate into the cells. The LD_{50} concentrations of these C_{14-18}-SQMG were in the range of about 20 to 40 μM, which are realistic from the viewpoint of drug design. Stronger inhibitory effects were observed with longer chain size of the fatty acid in the SQMG (Fig. 4). Both *in vivo* and *in vitro* inhibitory effects were dose-dependently reduced in parallel with the chain size of the fatty acid in the SQMG. The *in vivo* inhibition must therefore depend on the strength of binding between the fatty acid ester region and the polymerase. The sulfoquinovosylacylglyceride (group I & II & III) in the SQMG appears to strengthen the binding. The binding strength depends on the tertiary structure of the SQMG, for the following reasons; the lack of growth inhibition by the fatty acid alone, the lack of a free carboxyl end in SQMG, although fatty acids in which the carboxyl end was chemically modified lost the binding effect, and the fact that each SQMG tested showed stronger inhibitory effects than the corresponding fatty acid alone. Some of the SQMG should be tested as anti-cancer agents. Drug design will be possible by investigating the tightness of the binding between the SQMG and pol β. Based on information available from the NMR structure, computer-simulation of the conformational changes in pol. β should also be useful for this purpose.

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