A sulphoquinovosyl diacylglycerol is a DNA polymerase ϵ inhibitor

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Sulphoquinovosyl diacylglycerol (SQDG) was reported as a selective inhibitor of eukaryotic DNA polymerases α and β [Hanashima, Mizushina, Ohta, Yamazaki, Sugawara and Sakaguchi (2000) *Jpn. J. Cancer Res.* **91**, 1073–1083] and an immunosuppressive agent [Matsumoto, Sahara, Fujita, Shimozawa, Takenouchi, Torigoe, Hanashima, Yamazaki, Takahashi, Sugawara et al. (2002) *Transplantation* **74**, 261–267]. The purpose of this paper is to elucidate the biochemical properties of the inhibition more precisely. As expected, SQDG could inhibit the activities of mammalian DNA polymerases such as α , δ , η and κ *in vitro* in the range of 2–5 μ M, and β and λ *in vitro* in the range of 20–45 μ M. However, SQDG could inhibit only mammalian DNA polymerases ϵ (pol ϵ) activity at less than 0.04 μ M. SQDG

bound more tightly to mammalian pol ϵ than the other mammalian polymerases tested. Moreover, SQDG could inhibit the activities of all the polymerases from animals such as fish and insect, but not of the polymerases from plant and prokaryotes. SQDG should, therefore, be called a mammalian pol ϵ -specific inhibitor or animal polymerase-specific inhibitor. To our knowledge, this represents the first report about an inhibitor specific to mammalian pol ϵ .

Key words: animal DNA polymerase inhibitor, deuterostomic branch, DNA polymerase ϵ , sulphoquinovosyl diacylglycerol (SQDG), sulphoquinovosyl monoacylglycerol (SQMG).

INTRODUCTION

In recent years, it is known that eukaryotic cells contain at least three replicative DNA polymerases (EC 2.7.7.7; pols α , δ and ϵ), mitochondrial DNA polymerase (pol γ) and at least 10 repair types of DNA polymerase (pol β , δ , ϵ , ζ , η , θ , ι , κ , λ and μ) [1,2]. The purpose of the studies was to use them as tools and molecular probes to distinguish DNA polymerases and to clarify their biological and *in vivo* functions. In the process, we found two potent sulpho-glycolipid eukaryotic DNA polymerase inhibitors, sulphoquinovosyl diacylglycerol (SQDG) and sulphoquinovosyl monoacylglycerol (SQMG) [3–10], which are an immunosuppressant [11,12] and an anti-cancer chemotherapy agent [13,14], respectively. At that time, only eukaryotic pol α and β were available as DNA polymerase species for the screening, and SQDG/SQMG were found as the compounds which selectively inhibited the activities of pol α and β at about 2–4 and 25–35 μ M, respectively [8]. Now, more species of the DNA polymerase are available.

In this time, we succeeded in collecting many eukaryotic DNA polymerase species such as α , β , δ , ϵ , η , λ and κ , while pol ζ , θ and μ were not available. SQDG/SQMG could inhibit the activities of mammalian DNA polymerases such as α , β , δ , η , λ and κ . As expected from the previous data for pol α and β , both SQDG/SQMG weakly inhibited the activities of the X-family of DNA polymerases such as β and λ , and effectively inhibited pol δ , η and κ in the almost same range as pol α . However, surprisingly, SQDG inhibited not only the activities of all the polymerases from mammals tested, but also pol ϵ activity at one-hundredth of

the minimum inhibitory concentrations of the other polymerases. So far, only pol ϵ from two species, humans and *Drosophila*, are available for this experiment. SQDG did not show inhibition specificity to *Drosophila* pol ϵ , although the inhibitory effects remained in the same range as the other DNA polymerases tested. Similarly, the human pol ϵ inhibition by SQMG, which lacks one fatty acid chain from SQDG, occurred in the same range for pol α , δ , η and κ . SQDG is not a broad DNA polymerase inhibitor as reported previously [8], but a mammalian (or deuterostome) pol ϵ -specific inhibitor. To our knowledge, there has been no report about such a compound specific to mammalian pol ϵ .

MATERIALS AND METHODS

Materials

Nucleotides and chemically synthesized DNA template primers such as [³H]2'-deoxythymidine 5'-triphosphate (dTTP; 43 Ci/mmol) and poly(dA)/oligo(dT)_{12–18} were purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). All other reagents were of analytical grade and were purchased from the Wako Chemical Co. (Osaka, Japan).

Chemical synthesis of SQDG/SQMG

Structures of the chemically synthesized SQDG [1,2-di-*O*-acyl-3-*O*-(α -D-sulphoquinovosyl)-glyceride with two stearic acid molecules] and SQMG [1-mono-*O*-acyl-3-*O*-(α -D-sulphoquinovosyl)-

Abbreviations used: dTTP, 2'-deoxythymidine 5'-triphosphate; pol, DNA polymerase; SQDG, sulphoquinovosyl diacylglycerol; SQMG, sulphoquinovosyl monoacylglycerol.

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Figure 1 Chemical structures of synthesized SQDG and SQMG

(A) SQDG, 1,2-di-*O*-acyl-3-*O*-(α -D-sulphoquinovosyl)-glyceride with two stearic acids; (B) SQMG, 1-mono-*O*-acyl-3-*O*-(α -D-sulphoquinovosyl)-glyceride with a stearic acid.

glyceride with one stearic acid molecule] were synthesized according to the procedure described previously [8,9]. Chemical structures of SQDG and SQMG are shown in Figure 1. SQDG is a sulphonic analogue of D-glucose bound to a glycerol-bearing C₁₈-saturated fatty acid (stearic acid).

Enzymes

Pol α was purified from the calf thymus by immuno-affinity column chromatography as described by Tamai et al. [15]. Recombinant rat pol β was purified from *Escherichia coli* JMp β 5 as described by Date et al. [16]. Pol δ was purified from calf thymus [17], and pol ϵ was purified from HeLa cells as described previously [18]. Recombinant human pol η tagged with a 6 $\times$ His tag at its C-terminus was expressed in Sf9 insect cells using the baculovirus expression system, and was purified from the cells as described previously [19]. A truncated form of pol κ with a 6 $\times$ His tag attached at the C-terminus was overproduced using the BAC-to-BAC Baculovirus Expression System kit (Gibco-BRL) and purified as described previously [20]. Recombinant His-pol λ was overexpressed and purified according to a method described previously [21]. Fish pol α and δ were purified from the testis of cherry salmon (*Oncorhynchus masou*) [22]. Insect pol α , δ and ϵ were purified from early embryos of the fruit fly *Drosophila melanogaster* as described previously [23]. Pol I (α -like) and II (β -like) from a higher plant, cauliflower inflorescence, was purified according to the methods outlined by Sakaguchi et al. [24]. The Klenow fragment of pol I was purchased from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). *Taq* DNA polymerase and T4 DNA polymerase were purchased from Takara (Kyoto, Japan).

DNA polymerase assays

The activities of all DNA polymerases were measured by the methods described previously [25–27]. For DNA polymerases,

poly(dA)/oligo(dT)_{12–18} and dTTP, or activated calf thymus DNA and four NTPs were used as DNA template primer and nucleotide substrate, respectively. SQDG/SQMG was dissolved in DMSO at various concentrations and sonicated for 30 s. Sonicated samples (4 μ l) were mixed with 16 μ l of each enzyme (final, 0.05 unit) in 50 mM Tris/HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA, and kept at 0 °C for 10 min. These inhibitor/enzyme mixtures (8 μ l) were added to 16 μ l of each of the enzyme standard reaction mixtures, and incubation was carried out at 37 °C for 60 min, except for *Taq* DNA polymerase, which was incubated at 74 °C for 60 min. The activity without the inhibitor was considered to be 100%, and the remaining activities at each concentration of inhibitor were determined as percentages of this value. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalysed the incorporation of 1 nmol of dNTP into the synthetic DNA template primers [i.e. poly(dA)/oligo(dT)_{12–18}, the A/T ratio was 2:1; or activated calf thymus DNA] in 60 min at 37 °C under the normal reaction conditions for each enzyme [25–27].

RESULTS AND DISCUSSION

As described previously [3–10], SQDG and SQMG are potent inhibitors of mammalian pol α and pol β *in vitro*. Anti-cancer and skin-graft tests *in vivo* of sulpho-glycolipids such as SQDG/SQMG showed positive results [11–14], and subsequently we developed methods to synthesize sulpho-glycolipids chemically [8,9]. The structures of SQDG and SQMG are shown in Figure 1.

Table 1 IC₅₀ values of SQDG and SQMG on the activities of various DNA polymerases

SQDG and SQMG were incubated with each DNA polymerase (0.05 unit). The enzymic activity was measured as described in the Materials and methods section. Enzyme activity in the absence of the compound was taken as 100%.

Enzyme	IC ₅₀ value (μ M)	
	SQDG	SQMG
Mammalian DNA polymerases		
Calf pol α	2.4	2.2
Rat pol β	37	26
Calf pol δ	3.4	4.8
Human pol ϵ	0.036	3.6
Human pol η	4.4	4.0
Human pol κ	3.8	3.2
Human pol λ	45	33
Fish DNA polymerases		
Cherry salmon pol α	5.4	2.8
Cherry salmon pol δ	6.0	8.0
Insect DNA polymerases		
<i>Drosophila</i> pol α	12	11
<i>Drosophila</i> pol δ	13	15
<i>Drosophila</i> pol ϵ	5.3	10
Plant DNA polymerases		
Cauliflower pol I (α -like)	> 500	> 500
Cauliflower pol II (β -like)	> 500	> 500
Prokaryotic DNA polymerases		
<i>E. coli</i> pol I (Klenow fragment)	> 500	> 500
<i>Taq</i> pol	> 500	> 500
T4 pol	$\geq$ 500	$\geq$ 500

Figure 2 Dose–response curves of SQDG

Effects of SQDG on calf pol α (■), rat pol β (●), calf pol δ (▲) and human pol ϵ (◆) activities. The amount of each enzyme in the assay mixture was 0.05 unit.

Effects of SQDG on the activities of DNA polymerases

As described briefly in the Introduction, we succeeded in collecting many eukaryotic DNA polymerase species such as α , β , δ , ϵ , η , κ and λ , while pol ζ , θ and μ are not available yet (Table 1). Currently eukaryotes reportedly contain at least 12 species of DNA polymerases [1,2], and we are still in an era when most DNA polymerases are very difficult to collect in their purified forms in a laboratory. Table 1 shows the inhibitory effects (IC_{50}) of SQDG/SQMG against these various DNA polymerase species. SQDG/SQMG inhibited the activities of all of DNA polymerases from mammals, fish and insects, with 50% inhibition observed at a dose of 2–20 μ M for pol α , δ , η and κ , and 20–45 μ M for pol β and λ (Table 1). Each of the inhibition curves of SQDG/SQMG against these polymerases is shown to be dose-dependent (results not shown). In the experiments in Table 1, two exceptional results were observed: SQDG and SQMG have no effect on polymerases from plants such as the cauliflower and prokaryotes such as *E. coli*, and only SQDG can inhibit human pol ϵ activity at one-hundredth of the minimum inhibitory concentrations of the other polymerases (0.036 μ M; Table 1 and Figure 2). The three-dimensional structures of eukaryotic DNA polymerases would be greatly different from those of prokaryotic DNA polymerases. The inhibition constant (K_i) values of SQDG for pol α , β , δ and ϵ were determined from Dixon plots and indicated that SQDG inhibited mammalian DNA polymerases differentially and that it was a potent inhibitor of pol ϵ (Table 2). SQDG inhibited fruit-fly pol ϵ activity at 5.3 μ M, which is just at the ordinal inhibitory range of pol α and δ (Table 1).

In order to say that SQDG is specific to the mammalian pol ϵ , we need to test whether SQDG influences pol ϵ activities from other vertebrates or other deuterostomic organisms. So far, unfortunately, many purified pol ϵ fractions are not yet available. Even for this paper, we could only take pol ϵ fractions from human and fruit fly, which could be the representatives of animal species in the deuterostomic and the protostomic branches in the Animal Kingdom, respectively. Therefore, we may say that SQDG is an inhibitor specific for deuterostome pol ϵ or vertebrate

Table 2 K_i values of SQDG/SQMG for mammalian DNA polymerases

The inhibitory mode and K_i values of SQDG for DNA polymerases were determined with respect to poly(dA)/oligo(dT)_{12–18} (DNA template primer) and dTTP (nucleotide substrate) from Dixon plots.

DNA polymerase	Poly(dA)/oligo(dT) _{12–18} K_i (μ M)	Inhibitory mode	dTTP K_i (μ M)	Inhibitory mode
SQDG				
α	0.7	Non-competitive	2.7	Non-competitive
β	3.8	Competitive	13	Competitive
δ	1.4	Non-competitive	2.8	Non-competitive
ϵ	0.028	Non-competitive	0.035	Non-competitive
SQMG				
α	0.18	Non-competitive	0.64	Non-competitive
β	5.0	Competitive	36	Competitive
δ	2.0	Non-competitive	4.2	Non-competitive
ϵ	1.8	Non-competitive	3.9	Non-competitive

pol ϵ rather than of mammalian pol ϵ . When activated DNA was used as the DNA template primer instead of synthesized DNA [i.e. poly(dA)/oligo(dT)_{12–18}], the inhibitory modes of the compounds were unchanged (results not shown).

SQDG/SQMG also did not inhibit any of the plant DNA polymerases, or any of the prokaryotic DNA polymerases, such as *E. coli* DNA polymerase I, *Taq* DNA polymerase and T4 DNA polymerase (Table 1). Specifically, SQDG/SQMG are compounds which do not influence the DNA polymerase activities from any other kingdom of species in the phylogenetic tree. At present, it is difficult to explain why the animal/plant split would be differentially affected by SQDG/SQMG. SQDG/SQMG may be able to distinguish the three-dimensional structural differences between the animal and the plant polymerases.

The effectiveness in the *in vivo* anti-cancer and skin graft tests of SQDG may, therefore, reflect the striking inhibition of human pol ϵ (Figure 2). The remainder of this article is devoted to an analysis of the mode of action of SQDG on human pol ϵ .

Mode of human pol ϵ inhibition by SQDG

Next, to elucidate the inhibition mechanism of SQDG, the extent of inhibition as a function of DNA template primer or nucleotide substrate concentrations was studied (Figure 3 and Table 3). The effect of SQDG on the pol ϵ activity was examined with various concentrations of poly(dA)/oligo(dT)_{12–18} (Figure 3A) and dTTP (Figure 3B). In the kinetic analysis, 0–30 μ M poly(dA)/oligo(dT)_{12–18} and 0–10 μ M dTTP were used as the DNA template primer and nucleotide substrate concentrations, respectively. In the case of the DNA template primer, the apparent Michaelis constant (K_m) was unchanged at 6.25 μ M, whereas the maximum velocity (V_{max}) was changed to 20.8 and 15.4 pmol/h in the presence of 25 and 50 nM SQDG, respectively (Figure 3C). The K_m for the substrate (dTTP) was unchanged at 1.82 μ M, and the V_{max} for the substrate decreased from 58.8 to 24.4 pmol/h in the presence of 50 nM SQDG (Figure 3D). SQDG inhibition was therefore non-competitive with respect to both the DNA template primer and the nucleotide substrate. The K_i value, obtained from Dixon plots, was found to be 28.4 and 35.0 nM for the DNA template primer and nucleotide substrate, respectively (Figures 3E and 3F). The relationship between the concentration of each dNTP and the inhibition activity was examined kinetically. By varying the concentration of any one of four dNTPs, the apparent V_{max} value decreased with an increase in SQDG concentration, whereas the apparent K_m remained unchanged

Figure 3 Kinetic analysis of the inhibition of human pol ϵ by SQDG

(A and B) Relationship between the inhibition and the concentration of DNA template primer and nucleotide substrate. (C and D) Lineweaver-Burk plots. (E and F) Dixon plots. The amount of human pol ϵ in the assay mixture was 0.05 unit. (A, C) Human pol ϵ activity was measured in the absence (■) or presence of 25 (●) or 50 nM (▲) SQDG using the indicated concentrations of the DNA template primer. (B, D) Human pol ϵ activity was assayed with the indicated concentrations of the nucleotide substrate in the presence of 25 (○) or 50 nM (△) or in the absence (□) of SQDG. (E, F) The inhibition constants (K_i) were determined as 30 and 35 nM on the basis of the data in (C) and (D), respectively.

(Table 3). In the case of activated calf thymus DNA as the DNA template primer, the apparent K_m was unchanged, whereas the V_{max} decreased with increasing SQDG. From these results, it was evident that SQDG inhibited pol ϵ non-competitively with all dNTPs and the DNA template primer, and the mode of inhibition was suggested to be non-specific, i.e. not distinguishing between

pyrimidine deoxynucleoside triphosphates (dCTP and dTTP) and purine deoxynucleoside triphosphates (dATP and dGTP). The kinetic results from Lineweaver-Burk plots were observed with SQDG, and the pol α , δ and ϵ inhibitions were non-competitive, but the pol β inhibition was competitive (Table 2). SQDG would bind or interact with a domain distinct from the template or

Figure 4 Effect of detergent on the inhibition of mammalian DNA polymerase activities by SQDG

(A, B) Calf pol α ; (C, D) rat pol β ; (E, F) calf pol δ ; (G, H) human pol ϵ . (A, C, E and G) DNA polymerase activity was measured in the absence (■) or presence of 0.125 (●), 0.25 (▲) or 0.5% (◆) Nonidet P-40 using the indicated concentrations of SQDG. (B, D, F and H) DNA polymerase activity was assayed with the indicated concentrations of Nonidet P-40 in the presence of 12.5 μ M (□), 25 μ M (○), 50 μ M (△) or 100 μ M (◇) SQDG. The amount of each enzyme in the assay mixture was 0.05 unit. Enzyme activity in the absence of SQDG was taken as 100%.

substrate binding sites on pol α , δ and ϵ , whereas it directly interacts with or affects both of the binding sites on pol β . An SQDG-binding site on pol ϵ is believed to be more influential than the sites on pol α and β with regard to enzymic inhibition. As described in the next section, binding of SQDG to pol α , β and δ could easily be dissociated by Nonidet P-40, but that of pol ϵ could not (Figure 4).

Effects of reaction conditions on pol ϵ inhibition

To determine the effects of a non-ionic detergent on the binding of SQDG to pol ϵ , Nonidet P-40 was added to the reaction

mixture at concentrations of 0.125, 0.25 and 0.5%. In the absence of SQDG, each of the DNA polymerase activities was taken as 100%. The pol ϵ inhibitory effect of SQDG at 12.5 μ M was slightly reversed by the addition of 0.5% Nonidet P-40 to the reaction mixture (Figures 4G and 4H). However, the inhibitory effect of SQDG at 12.5 μ M on pol α , β and δ was completely reversed by the addition of 0.5% Nonidet P-40 (one quarter for pol ϵ ; Figures 4A–4F). The reversal of inhibition by Nonidet P-40 was concentration-dependent, suggesting that SQDG interacts with the hydrophobic region of these polymerase proteins. We also tested whether an excess amount of a substrate analogue, poly(rC) (100 μ M), or a protein, BSA (200 μ g/ml),

Table 3 Kinetic analysis with each of the four dNTPs of the inhibition of human pol ϵ by SQDG

The K_m and V_{max} values and the K_i values were determined from Lineweaver–Burk plots and Dixon plots, respectively. For each substrate, K_m , K_i and mode of inhibition remained the same for each SQDG concentration.

Substrate	SQDG (nM)	K_m	V_{max} (pmol/h)	K_i (nM)	Inhibitory mode
Activated DNA	0	12.5 μ g/ml	167	25.4	Non-competitive
	20		95.2		
	40		66.7		
	60		50.0		
dATP	0	3.13 μ M	118	34.6	Non-competitive
	20		74.1		
	40		54.1		
	60		42.5		
dTTP	0	3.16 μ M	125	33.1	Non-competitive
	20		80.6		
	40		59.5		
	60		47.2		
dGTP	0	3.08 μ M	128	36.3	Non-competitive
	20		78.1		
	40		56.5		
	60		44.2		
dCTP	0	3.10 μ M	118	35.5	Non-competitive
	20		75.2		
	40		55.2		
	60		43.7		
Poly(dA)/ oligo(dT) _{12–18}	0	6.25 μ M	45.5	28.4	Non-competitive
	25		20.8		
	50		15.4		
	75		11.8		
dTTP	0	1.82 μ M	58.8	35.0	Non-competitive
	25		35.7		
	50		24.4		
	75		18.3		

could prevent the inhibitory effects of 100 μ M SQDG, to see whether the binding to pol ϵ occurs selectively or by binding to a specific site on pol ϵ . These results will be discussed elsewhere.

As described in this report, we found that SQDG is a potent inhibitor specific to pol ϵ . This is the first report on the finding and characterization of a pol ϵ -specific inhibitor. Although inhibition experiments with other vertebrate or deuterostome pol ϵ still remain to be carried out, SQDG should be called a mammalian pol ϵ -specific inhibitor, as the case may be, or a vertebrate (or deuterostome) pol ϵ -specific inhibitor. Since the polymerase species specificity of SQDG was extremely high, SQDG could be useful in studies to determine the precise roles of pol ϵ .

Another interesting result was that the inhibition by SQDG/SQMG is specific to the animal DNA polymerases. We recently found and reported on two other animal DNA polymerase inhibitors [28]. Like SQDG/SQMG, elenic acid [*R*-2,4-dimethyl-22-(*p*-hydroxyphenyl)-docos-3(*E*)-enoic acid] [28] and hippospongiic acid A (Y. Mizushina, C. Murakami, H. Takikawa, N. Kasai, X. Xu, K. Mori, M. Oshige, T. Yamaguchi, M. Saneyoshi, N. Shimazaki et al., unpublished work) were found as natural compounds to influence only the activities of DNA polymerases from vertebrates. Moreover, hippospongiic acid A could equally influence the activities of all mammalian DNA polymerase species tested, including α , β , δ , ϵ , ζ , κ and λ . The chemical structures of elenic acid and hippospongiic acid A were three-dimensionally similar to the long-chain fatty acid of SQDG/SQMG, suggesting that these agents bind to a three-dimensionally similar site on each vertebrate polymerase. Since SQDG/SQMG did not dis-

tinguish the deuterostome/protostome split in the Animal Kingdom's phylogenetic tree, the structures of DNA polymerases may, irrespective of the enzyme species, be separated roughly into two groups which belong to the deuterostomic or protostomic branches. If so, purification and characterization of DNA polymerases from coelenterates such as jellyfish may give a clue to the structural classification of eukaryotic DNA polymerases. Similarly, in the phylogenetic tree, there may be a rough animal/plant split in the structures of DNA polymerases, irrespective of the enzyme species.

We thank Dr S. Yoshida of Nagoya University (Nagoya, Japan) for his valuable discussions about the inhibitor. We are grateful for the donation of human pol η and pol κ from Dr F. Hanaoka and Dr C. Masutani of Osaka University (Osaka, Japan) and Dr H. Ohmori and Mr E. Ohashi of Kyoto University (Kyoto, Japan), respectively. This work was partly supported by Grant-in-Aid for Kobe Gakuin University Joint Research (B) (to Y.M. and H.Y.). Y.M. acknowledges Grant-in-Aid 14780466 for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

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Received 6 November 2002; accepted 15 November 2002

Published as BJ Immediate Publication 15 November 2002, DOI 10.1042/BJ20021737