Action of a New Mammalian DNA Polymerase Inhibitor, Sulfophenylsulfonyldiacetylglycerol

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We found and previously reported a new mammalian DNA polymerase inhibitor from a sea alga, Gigartina tenella, (Ohta K., et al., Chem. Pharm. Bull., 46, 684—686, 1998). It was a new sulfophenolid compound that belonged in the class of sulfophenylsulfonyldiacetylglycerol. The biochemical properties have been investigated here. The compound, temporarly designated KM043, potently inhibited the activities of mammalian DNA polymerase α (pol. α) and DNA polymerase β (pol. β) and terminal deoxynucleotidyl transferase (TdT), and moderately, human immunodeficiency virus reverse transcriptase (HIV-RT). KM043 dose-dependently inhibited their activities, and each of their IC50 values was 0.25 μM for pol. α, 0.38 μM for TdT, 3.6 μM for pol. β, or 11.2 μM for HIV-RT, and almost complete inhibition of each was achieved at 1.0 to 2.0 μM for pol. α and TdT, 7.5 μM for pol. β and about 30 μM for HIV-RT. However, the compound did not influence the activities of prokaryotic DNA polymerases such as Escherichia coli DNA polymerase I, and DNA metabolic enzymes like DNase I. Inhibition of pol. α or β by KM043 was non-competitive with both the DNA template and the substrate deoxythymidine 5'-triphosphate (dTTP). KM043 was weakly cytotoxic to cultured HeLa-S3 cells, and the IC50 value was 80 μM. KM043 could synergistically enhance the cytoidal effect of an anti-cancer chemotherapy agent, bleomycin. In the presence of 50 μM KM043, the effect ratio of (bleomycin plus KM043)/(bleomycin only) decreased from 0.76 to 0.22.

Key words DNA polymerase inhibitor; sulfophenylsulfonyldiacetylglycerol; Gigartina tenella

Eukaryotic DNA polymerases are designated as α, β, γ, δ, ε, and ζ, each responsible for different DNA syntheses (Kornberg and Baker, 1992, a report for ζ). Our recent studies on DNA polymerases have concentrated on the interest in understanding the precise role of these polymerases in vivo and to know the factors controlling their activities.1—7) In the process of the studies, the need for an inhibitor of each polymerase has arisen, and a wide screening of the enzyme inhibitors has been tried.8) Subsequently, we have found several compounds in the class of sulfophenylsulfonyldiacetylglycerol (SQDG) as an eukaryotic DNA polymerase inhibitor from fern,9,10 which structurally coincided with the compound from cyanobacterium reported as on AIDS-antiviral agent.10) It inhibited the activities of mammalian DNA polymerase α (pol. α) and β (pol. β) in a dose-dependent manner, but did not influence human immunodeficiency virus reverse transcriptase (HIV-RT) activity in vitro. As a similar agent, Sahara et al. described that other compounds in the class of SQDG and/or sulfophenylsulfonyldiacetylglycerol (SQMG) from sea urchin showed anti-tumor activity in an experiment using transplantation tumors on mice.11) We recently confirmed that the agents from sea urchin were also mammalian DNA polymerase inhibitors (manuscript in preparation). Simbulan and Yoshida showed that several glycolipids from mammalian organisms inhibited the activities of mammalian DNA polymerases, and suggested that sulfate and sialic acid moieties in the compounds were essential for the polymerase inhibition.12) These data suggest that some of the compounds in the class of SQDG and so on can possibly be potent anti-tumor and/or AIDS-antiviral agents, and may be found by screening inhibitors of mammalian DNA polymerases.

Based on the screening concept described above, we tried to isolate DNA polymerase inhibitors from sea alga because the materials are supposed to produce plenty of natural compounds which belong to the class of SQDG. As expected, we found a new compound in the SQDG class, KM043 (1-(1'-O-α-d-sulfophenylsulfonyl)-2-palmitoyl-3-[5'E],8'E,11'E,14'E,17'E-eicosapentaenyl]-sulfophenylsulfonyldiacetylglycerol), from a marine red alga, Gigartina tenella, and previously reported the chemical structure.13) This report is devoted to showing the biochemical properties of the inhibitory effects of KM043 on the activities of immunooaffinity-purified calf thymus pol. α, calf thymus terminal deoxynucleotidyl transferase (TdT), rat recombinant pol. β, and HIV-RT, as well as the in vivo effects of KM043 using Hela-S3 cultured cells.

KM043 was a potent inhibitor which selectively influenced the activities of pol. α and β, and could enhance the cytotoxicity of bleomycin.

MATERIAL AND METHODS

Enzymes Pol. α was purified from calf thymus by immuno-affinity column chromatography, as described previously.14) Pol. β was purified from a recombinant plasmid expressing rat Pol. β as described previously.15) E. coli DNA polymerase I and HIV-RT were purchased from Worthington Biochemical Corp. T4 DNA polymerase, calf thymus TdT, Taq DNA polymerase and T4 DNA polymerase were purchased from Takara. Bovine pancreas deoxyribonuclease I (DNase 1) was purchased from Stratagene Cloning System.

DNA Polymerase Assays For a routine assay in the course of purification, recombinant rat pol. β was used. The standard reaction mixture for pol. β (final volume of 24 μl) contained 50 μM Tris—HCl (pH 8.8), 20 μM of deoxythymi-
dine 5'-triphosphate (dTTP) containing $[^{32}P]$dTTP (1000 cpm/μmol), 5 μM MgCl$_2$, 15% of glycerol, 150 mM KCl, 10 μg/ml of poly(dA), 5 μg/ml of oligo(dT)$_{12-18}$, 0.05 U of rat pol. β and various amounts of the fraction to determine the inhibitory activity, and reactions were performed at 37°C for 60 min. One unit of pol. β catalyzes the incorporation of 1 nmol of dTTP into a synthetic template-primer (poly(dA)/oligo(dT)$_{12-18}$) A/T=2/1) at 37°C for 60 min.

The conditions for pol. α assay were the same as pol. β, except that KCl was omitted and the pH was 7.5.

The reaction mixture for HIV-RT-associated DNA polymerase activity contained 50 mM Tris–HCl (pH 8.0), 5 mM MgCl$_2$, 15% glycerol, 130 mM KCl, 10 μg/ml poly(rA), 5 μg/ml oligo(dT)$_{12-18}$ used as template, and 10 μM dTTP containing $[^{32}P]$dTTP.

*E. coli* DNA polymerase I assay was performed in the mixture as follows, 67 mM KPi (pH7.4), 6.7 mM MgCl$_2$, 1 mM 2-mercaptoethanol, 15% glycerol, 1 mM dithiothreitol (DTT), 50 μg/ml bovine serum albumin (BSA), 10 μg/ml poly(dA), 5 μg/ml oligo(dT)$_{12-18}$ used as template, 30 μM dTTP containing $[^{32}P]$dTTP.

The reaction mixture for calf thymus TdT contained 100 mM cacodylic acid (pH 7.2), 8 mM MgCl$_2$, 1 mM 2-mercaptoethanol, 50 μg/ml BSA, 15% glycerol, 50 μg/ml oligo (dT)$_{12-18}$, and 50 μM dTTP containing $[^{32}P]$dTTP.

T4 DNA polymerase assay was performed in the mixture as follows, 67 mM Tris–HCl (pH 8.8), 6.7 mM MgCl$_2$, 16.6 mM (NH$_4$)$_2$SO$_4$, 10 mM 2-mercaptoethanol, 6.7 mM EDTA, 50 μg/ml BSA, 15% glycerol, 10 μg/ml poly(dA), 5 μg/ml oligo(dT)$_{12-18}$, 30 μM dTTP containing $[^{32}P]$dTTP.

The reaction conditions for Taq DNA polymerase were the same as pol. β, except that they did not contain KCl.

After the incubation of each polymerase, the products which had incorporated radioactive deoxynucleoside 5'-monophosphates (dTMPs) were collected on DE81 filter paper as described and radioactivity was measured on a scintillation counter.

DNase I activity was measured as described previously.

**Investigation of Cytotoxicity on Cultured Cells by KM043**

For investigation of the primitive in vivo effect of KM043, HeLa-S3 was used.

The cells were routinely cultured using Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 250 μg/ml 2-Fungizone, 300 μg/ml l-glutamine, which was collectively used as the standard medium. The cells were routinely cultured at 37°C in the standard medium in humidified 5% CO$_2$–95% air.

The cytotoxicity of KM043 was investigated as follows. A high concentration of KM043 solved in dimethylsulfoxide (DMSO) was stocked. Approximately, 2×10$^5$ cells per well were inoculated in a 96-well micro plate, then KM043 stock solution was diluted with various concentrations of the standard medium, and applied to each well. After incubation for 48 h, survival rate was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

**Investigation of the Effect of KM043 on Cultured Cells Treated with a DNA-Damaging Agent**

Methyl methane sulfonate (MMS) treatment and the addition of KM043 was done as follows. Approximately, 2×10$^5$ cells per well were inoculated in a 96-well microplate and various concentrations of MMS was applied to each well. MMS was diluted with standard medium. After incubation for 16 h, each well was washed with the standard medium twice. Then, the cells were incubated with the medium containing KM043 for 48 h. After the incubation, survival rate was measured by MTT assay.

Bleomycin was added simultaneously with KM043. Bleomycin stock solution was diluted with the standard medium, and various concentrations of bleomycin were made. 2×10$^5$ cells per well were inoculated in a 96-well microplate, then the medium containing bleomycin and KM043 was applied to each well. After the incubation for 48 h, the survival rate was measured by MTT assay.

**RESULT AND DISCUSSION**

**Inhibition Effects of KM043 on Various DNA Polymerase Activities** In our continuous screening for sea algae with potent inhibitory activity against rat recombinant pol. β, KM043 was isolated from a sea alga, *Gigartina tenella* (Fig. 1). The compound was in a class of SQDG, but was structurally a new sulfolipid as described previously.

KM043 has a sulfo group in the compound which, as claimed by Simbulan and Yoshida, is essential for polymerase inhibition (Fig. 1). We preliminarily reported that KM043 could efficiently inhibit mammalian DNA polymerase activity.

As is well known, at least six classes of DNA polymerases (α, β, γ, δ, ε and ζ) are present in mammalian cells (Kornberg and Baker, 1992, and a report for ζ). 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 β and ε, appear to be related to DNA repair and/or recombination. In this study, we used calf thymus pol. α as a representative of the replicative polymerases and rat pol. β as a representative of the repair-related polymerases, and as expected, found that KM043 is a potent inhibitor of these polymerases in vitro. In Fig. 2, the inhibitory effects of KM043 to
We previously identified another potent DNA polymerase inhibitor of SQDG from fern, which is structurally similar to but different from KM043.\(^9\) The compound was bound C18:1, C18:2 or C18:3 in the secondary position of glycerol backbone, instead of C20:5 of KM043. We therefore studied the inhibitory effect of KM043 in comparison with SQDG from fern. Both KM043 and the SQDG from fern were potent inhibitors of pol. \(\alpha\) and the pol. \(\beta\) family, including TdT, but the inhibition by KM043 was slightly stronger. KM043 could completely inhibit the TdT activity at 2.0 \(\mu\)M and the HIV-RT activity at approximately 30 \(\mu\)M, whereas more than 100 \(\mu\)M of the SQDG from fern never influenced them at all. Since aphidicolin, a well-known potent inhibitor of pol. \(\alpha\), inhibited at 20 \(\mu\)M under the same condition, the KM043-effect on pol. \(\alpha\) is extremely strong. We should also emphasize that KM043 suppresses the activities of pol. \(\beta\) and TdT \emph{in vitro} to the same extent as does dideoxyTTP, a well-known potent pol. \(\beta\) inhibitor.

We previously reported that free long chain unsaturated fatty acids could also inhibit the activities of these DNA polymerases, and that the hydrocarbon chain number in those fatty acids and the number of ethylenic double bonds in the unsaturated forms were important for the inhibition.\(^9\) Since the 6-sulfo-D-quinovose in the sulfolipids itself has no inhibitory effect to the polymerases (data not shown), the inhibition by those sulfolipid compounds, as discussed previously in the fatty acid-inhibition, may depend on the binding of the fatty acid ester region to the polymerases, and the 6-sulfo-D-quinovose in the sulfolipids may strengthen the inhibitory effect. Inhibition of KM043 on the DNA polymerases, as compared with the SQDG from fern, may be strengthened by increasing the number of ethylene double bonds (\ie\ the hydrocarbon chain numbers) of the fatty acids in the structure.

\textbf{Mode of Inhibition of Pol. \(\alpha\) and \(\beta\) by KM043} To investigate the mechanisms of inhibition of pol. \(\alpha\) and \(\beta\) by KM043, next, kinetic analyses of each were performed (Figs. 3 and 4). The double reciprocal plots indicated that the inhibition of pol. \(\alpha\) by KM043 was non-competitive with the DNA template, since the \(K_m\) value obtained as 8.5 \(\mu\)g/ml was not changed in different inhibitor concentrations, while \(V_{max}\) was decreased from 65 to 28 or 15 pmol/h in the presence of 0.26 or 0.79 \(\mu\)M KM043, respectively (Fig. 3A). Similarly, the apparent \(K_m\) for the substrate was unchanged at 9.35 \(\mu\)M, although 81 and 54\% decreases in \(V_{max}\) were observed in the presence of 0.32 and 0.79 \(\mu\)M KM043, respectively (Fig. 3B).

Thus, the inhibition was also non-competitive with the substrate. From these results, KM043 may bind or interact with a domain distinct from the substrate- or template-binding sites on pol. \(\alpha\). Furthermore, the inhibition constant, \(K_i\) of KM043 obtained from Dixon plots in the data of the substrate was 0.84 \(\mu\)M and the \(K_i/K_m\) ratio was 0.09, suggesting that KM043 binds or interacts with pol. \(\alpha\) with greater affinity than the substrate, dTTP (Table 1). The inhibition of pol. \(\beta\) was also non-competitive with both the DNA template and the substrate. As shown in Fig. 4A, the \(K_m\) value was 6.06 \(\mu\)g/mL, and was unchanged in various inhibitor concentrations. On the other hand, the \(V_{max}\) value was decreased to 75 and 55\% in the presence of 6.2 and 12.4 \(\mu\)M KM043, respectively (Fig. 4A). The inhibition with the substrate was indicated in Fig. 4B. \(K_m\) was calculated as 8.5 \(\mu\)M, and \(V_{max}\) was...
Fig. 3: Kinetic Analysis of the Inhibition of DNA Pol. $\alpha$ Activity by KM043

Effects of KM043 on the $K_m$ and $V_{max}$ values of the template-primer, poly(dA):oligo(dT)$_{12-18}$, and the substrate, dTTP, were examined, and the results are shown as double-reciprocal plots. (A) The enzymatic reaction was done in the absence (•) or in the presence of 0.26 µM (△), 0.79 µM (○) KM043 with indicated concentrations of poly(dA):oligo(dT)$_{12-18}$ (2:1) as a template-primer. (B) The enzymatic reaction was done in the absence (•) or in the presence of 0.32 µM (△), 0.79 µM (○) KM043, using indicated concentrations of the substrate dTTP.

Fig. 4: Kinetic Analysis of the Inhibition of Pol. $\beta$ Activity by KM043

Effects of KM043 on the $K_m$ and $V_{max}$ values of the DNA template-primer, poly(dA):oligo(dT)$_{12-18}$, and the substrate, dTTP, were examined, and the results are shown as double-reciprocal plots. (A) The enzymatic reaction was done in the absence (•) or in the presence of 0.26 µM (△), 12.4 µM (○) KM043 with indicated concentrations of poly(dA):oligo(dT)$_{12-18}$ (2:1) as a template-primer. (B) The enzymatic reaction was done in the absence (•) or in the presence of 6.2 µM (△), 12.4 µM (○) KM043, using indicated concentrations of the substrate dTTP.

changed from 87 to 33.5 pmol/h, and 10.9 pmol/h in the presence of 6.2 and 12.4 µM KM043, respectively. In Dixon plots from Fig. 4B, the $K_i$ constant was 2.39 µM and the $K_i/K_m$ ratio was 0.28 (Table 1). KM043 also interacts with or affects a domain distinct from both the template or substrate binding sites on pol. $\beta$ as well as on pol. $\alpha$.

To determine the effect of a non-ionic detergent on the binding of KM043 to pol. $\alpha$ and $\beta$, Nonidet P-40 (NP-40) was added to the reaction mixture at a concentration of 0.05% (Fig. 5A for pol. $\alpha$, and Fig. 5B for pol. $\beta$). In the case of either pol. $\alpha$ or $\beta$, the DNA polymerase-inhibition by

Table 1. $K_m$ and $K_i$ Values for KM043

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>Calf thymus pol. $\alpha$</th>
<th>Rat recombinant pol. $\beta$</th>
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<tbody>
<tr>
<td>$K_m$ (µM)</td>
<td>9.35</td>
<td>8.50</td>
</tr>
<tr>
<td>$K_i$ (µM)</td>
<td>0.84</td>
<td>2.29</td>
</tr>
<tr>
<td>$K_i/K_m$</td>
<td>0.09</td>
<td>0.28</td>
</tr>
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$a$ $K_m$ values were obtained from kinetic analysis of the substrate dTTP (Figs. 3B and 4B). $b$ $K_i$ values were measured via Dixon plots from kinetic analysis of the substrate dTTP, which was calculated by linear regression analysis.
Fig. 5. Effect of a Detergent on the Inhibition of Pol. α (A) or Pol. β (B) Activities by KM043

Each enzyme (0.05 U) was pre-incubated with various concentrations of KM043 only (■), and KM043 + 0.05% NP-40 (●), or KM043 + 40 μg/ml BSA (▲), at 37 °C for 60 min, then assayed.

KM043 was completely reversed by the addition of NP-40 to the reaction mixture, indicating that KM043 may bind to and interact with the hydrophobic region of these enzyme proteins (Fig. 5A and B). We also tested whether an excessive amount of BSA (40 μg/ml) could prevent the inhibitory effect of KM043 (Fig. 5A and B), to determine whether the effect of KM043 resulted from the non-specific adhesion of the sulfolipid to the enzyme, or bound selectively to special sites. BSA had a moderate influence on the effect of KM043 to both pol. α and β, suggesting that the binding to the polymerases occurs selectively (Fig. 5A and B). Namely, KM043 is suggested to inhibit these enzymes selectively by binding to the hydrophobic region.

These results appear to be without discrepancy to an assumption described above that the inhibition by KM043 depends on the binding of the fatty acid ester region to the polymerases, and that the 6-sulfo-β-quinojose in the KM043 strengthens the fatty acid-effect.

Effects of KM043 on Various Conditions of Mammalian Cultured Cells As described in introduction, our original interest was to study the in vivo roles of eukaryotic DNA polymerases. Another interest is to find a cytotoxic agent useful as an anticancer chemotherapy agent. Since the sulfolipids found here are broadly effective (although relatively specific for pol. α and TdT) on mammalian polymerases, the inhibitor compounds examined are not applicable to our original interest. KM043 should be tested to determine whether they are cytotoxic agents. They may be capable of preventing mammalian cell proliferation since they can inhibit the activity of the replicative polymerase.

To investigate the effect of KM043, a cultured HeLa S3 cell line was tested in vitro. KM043 weakly prevented the proliferation of the cells as shown in Fig. 6. The cells were influenced by the addition of KM043 in a dose–response fashion (10 to 150 μM) (Fig. 6). The concentrations of KM043 required for 50% growth inhibition (IC_{50}) were 80 μM (Fig. 6). The concentration difference of IC_{50} for the cells was more than 1000-fold to that of the 50% inhibition for pol. α in vitro (Fig. 6). The cytotoxicity was not strong. KM043 was, however, found to have an another interesting effect on the cells. We examined the effect of KM043 on the cells treated with a DNA-damaging agent, MMS (Fig. 7A) or bleomycin (Fig. 7B). MMS at less than 50 μM and bleomycin at less than 10 μg/ml did not greatly inhibit the cell proliferation (Fig. 7A and B). When KM043 at 50 μM, a dose that did not greatly change the survival rates of the cells, was followed by treatment with bleomycin, the survival rate of the cells was significantly decreased (Fig. 7B). The ratio of the survival rate of (bleomycin+KM043)/bleomycin was decreased from 0.76 to 0.22, indicating that KM043 could enhance the cytotoxicity by a DNA damaging agent, bleomycin. On the other hand, MMS had no such effect, even in the presence of 70 μM KM043 (Fig. 7A). As is well known, bleomycin-induced DNA damage might be repaired by using a mechanism including enzymes affected with KM043, perhaps pol. α pol. β and/or TdT. KM043 should be tested as an anticancer agent, and then, if promising, be developed as such.
Fig. 7. (A) Effect of KM043 with DNA Damaging Agent MMS on the Survival of HeLa S3 Cultured Cells

Approximately $2 \times 10^4$ cells/well were inoculated in a 96-well micro plate, then incubated with medium containing the indicated concentrations of MMS for 16 h. After the treatment of MMS, each well was washed with standard medium, and the incubation was continued with standard medium (●) or medium containing 70 μM KM043 (●) for 48 h. The survival rates indicated the average from three wells.

(B) Effect of KM043 with DNA Damaging Agent Bleomycin on the Survival of HeLa S3 Cultured Cells

Approximately $2 \times 10^4$ cells/well were inoculated in a 96-well micro plate and incubated with medium containing the indicated concentrations of bleomycin and 50 μM KM043 (●) or containing only the indicated concentration of bleomycin (●). After 48 h, the survival rates were measured.

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