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SHORT COMMUNICATION

Studies on Inhibitors of Mammalian DNA Polymerase α and β

SULFOLIPIDS FROM A PTERIDOPHYTE, *ATHYRIUM NIPONICUM*

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ABSTRACT. Three sulfolipid compounds, **1**, **2**, and **3**, have been isolated from a higher plant, a pteridophyte, *Athyrium niponicum*, as potent inhibitors of the activities of calf DNA polymerase α and rat DNA polymerase β. The inhibition by the sulfolipids was concentration dependent, and almost complete inhibition of DNA polymerase α and DNA polymerase β was achieved at 6 and 8 μg/mL, respectively. The compounds did not influence the activities of calf thymus terminal deoxynucleotidyl transferase, prokaryotic DNA polymerases such as the Klenow fragment of DNA polymerase I, T4 DNA polymerase and *Taq* polymerase, the DNA metabolic enzyme DNase I, and even a DNA polymerase from a higher plant, cauliflower. Similarly, the compounds did not inhibit the activity of the human immunodeficiency virus type 1 reverse transcriptase. The kinetic studies of the compounds showed that DNA polymerase α was inhibited non-competitively with respect to the DNA template and substrate, whereas DNA polymerase β was inhibited competitively with both the DNA template and substrate. The binding to DNA polymerase β could be stopped with non-ionic detergent, but the binding to DNA polymerase α could not. BIOCHEM PHARMACOL 55;4:537–541, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. DNA polymerase; enzyme inhibitor; anti-HIV-1 viral agent; sulfolipids; pteridophyte; *Athyrium niponicum*

We have been studying DNA polymerases for several years [1–8]. In the process of our investigations, the need for an inhibitor of each of the DNA polymerases has arisen. Since the roles of the DNA polymerases *in vivo* are still obscure, we have established an assay method to detect DNA polymerase inhibitors and have used it to screen microbial secondary metabolites and the extracts of many higher plants for the inhibitors. In the screening, several fungi, mushrooms, and higher plants were found to produce such inhibitors, and one of the strongest inhibitors was found in a pteridophyte, *Athyrium niponicum*. The compounds were isolated and determined to be previously identified sulfolipid compounds from a cyanobacterium, which are AIDS-antiviral agents [9]. Such compounds are thought to be distributed extensively throughout the plant world from the blue-green algae to ferns. The cyanobacterial sulfolipids were isolated as agents that inhibit the cytopathic effects of HIV-1¶; the molecular action mechanism of this inhibition is as yet unknown. Interestingly, the agents could also be obtained from a higher plant, and were mammalian DNA polymerase-specific inhibitors. Our present study is the first extensive investigation of the mode of the molecular action of the anti-HIV-1 viral sulfolipids as eukaryotic DNA polymerase-specific inhibitors. The sulfolipids inhibited the activity of mammalian DNA polymerase α and β *in vitro* and, unexpectedly, did not influence the activity of HIV-RT.

MATERIALS AND METHODS

Materials

Sephadex LH-20, nucleotides, and chemically synthesized template-primers such as poly(dA), poly(rA), poly(rC), and oligo(dT)$_{12-18}$ were purchased from Pharmacia. [^{3}H]dTTP (43 Ci/mmol) was purchased from the New England Nuclear Corp. All other reagents were of analyti-

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¶ *Abbreviations:* HIV-1, human immunodeficiency virus type 1; and HIV-RT, human immunodeficiency virus type 1 reverse transcriptase.

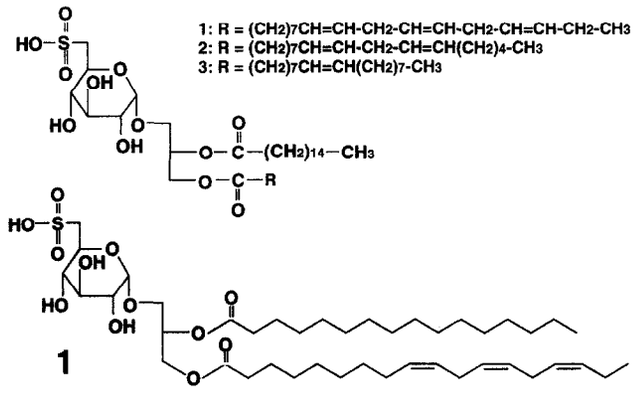

FIG. 1. Structures of sulfolipid compounds 1–3 from the pteridophyte *A. niponicum*.

cal grade and were purchased from Wako Chemical Industries.

Enzymes and DNA Polymerase Assays

The DNA polymerases and the DNA metabolic enzymes used were the same as those described in previous reports [10–12]. The enzyme assay methods were also described previously [10–12].

RESULTS AND DISCUSSION

Extraction and Purification of DNA Polymerase Inhibitors from the Pteridophyte A. niponicum

The pteridophyte *A. niponicum* was collected from fields in the vicinity of the city of Noda in the Chiba prefecture, Japan. The compounds were extracted with acetone from the plant leaves, and then purified through silica gel and Sephadex LH-20 column chromatography. Electron impact (EI) mass, negative fast atom bombardment high resolution (FABHR) mass, and ^{1}H-, ^{13}C-, and distortionless enhancement by polarization transfer (DEPT) NMR spectroscopic analyses suggested that the inhibitor fraction was a mixture of three structually related sulfolipids (Fig. 1). The sulfolipids were temporarily designated as sulfolipid compounds **1**, **2**, and **3**. These compounds are each composed of a 6-sulfo-D-quinovose, a C_{16}-saturated fatty acid, palmitic acid, and a C_{18}-unsaturated fatty acid such as linolenic acid, linoleic acid, or oleic acid, respectively. The ratio of compounds 1, 2, and 3 was determined to be 16.4, 15.3, and 68.3%, respectively, on gas chromatography by using alkaline hydrolysis followed by methylation. The structures of the sulfolipid compounds coincided with those of cyanobacterial sulfolipids that were isolated previously as anti-HIV-1 viral agents by Dr. K. R. Gustafson and Dr. J. H. Cardellina II of the National Cancer Institute [9]. We confirmed by HPLC, GC, and spectroscopic analyses that the pteridophytal sulfolipids **1**, **2**, and **3** coincided with the cyanobacterial sulfolipid compounds 1, 2, and 3, which were gifts from Drs. Gustafson and Cardellina II. The three compounds were extremely difficult to isolate from each other, and we used the mixture as the pteridophytal DNA polymerase inhibitor fraction in the following biochemical experiments. Whether or not the pteridophytal sulfolipids 1, 2, and 3 have anti-HIV-1 viral activity like the compounds of Gustafson is unknown, because we have no facilities to handle HIV-1 virus. At least the pteridophytal sulfolipids are structurally the same as Gustafson's compounds. Similar sulfonic acid-containing glycolipids were also discovered as an anti-cancer agent from sea urchin intestine [13]; however, the pteridophytal sulfolipids were found to have no cytotoxic activity against tumor cells *in vitro* by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay (data not shown) [14].

Inhibition by Sulfolipids of Activities of DNA Polymerases and the Other DNA Metabolic Enzymes

The mixture of the sulfolipid compounds 1, 2, and 3 was dissolved in DMSO and sonicated for 30 sec. Four microliters of the sonicated sample was mixed with 16 μL of each enzyme (final activity 0.05 units) in 50 mM Tris–HCl (pH 7.5) containing 0.1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol, and kept at 0° for 10 min. Eight microliters of each of the preincubated solutions was added to 16 μL of each of the enzyme standard reaction mixtures, and then each of the enzyme activities was measured under the conditions described in previous reports [10–12]. As shown in Fig. 2A, the sulfolipids at 6 μg/mL were found to inhibit signifcantly the activities of both calf DNA polymerase α (pol. α) and rat DNA polymerase β (pol. β), but the sulfolipids did not influence the activities of the prokaryotic DNA polymerases, i.e. the Klenow fragment of DNA polymerase I, T4 DNA polymerase and *Taq* polymerase, the DNA metabolic enzyme bovine deoxyribonuclease I (DNAse I), or DNA polymerase II (β-like) from a higher plant, cauliflower. Moreover, the sulfolipids did not inhibit the activity of HIV-RT, although the sulfolipids are reportedly AIDS-antiviral agents [9] (Fig. 2A). Calf thymus terminal deoxynucleotidyl transferase was inhibited moderately by the sulfolipids (Fig. 2A). The sulfolipids appear to be inhibitors selective to the mammalian DNA polymerases *in vitro*.

Figure 2B shows concentration–response curves of the sulfolipids to the mammalian pol. α, pol. β, and HIV-RT. The inhibition of the sulfolipids was concentration dependent, with 50% inhibition for pol. α and pol. β observed at concentrations of 1.5 and 3 μg/mL, respectively, and almost complete inhibition (more than 90–95%) was achieved at 6 and 8 μg/mL, respectively. The sulfolipids were slightly more effective on pol. α than pol. β.

We also tested the inhibitory effects of the cyanobacterial sulfolipids, which were gifts from Dr. Gustafson [9]. The cyanobacterial sulfolipids, potent anti-HIV-1 viral agents, did not inhibit the activity of HIV-RT. The failure to inhibit HIV-RT may be an important finding. A more detailed explanation of the mechanism of the anti-HIV-1 viral activity would be of interest. Each of the sulfolipids

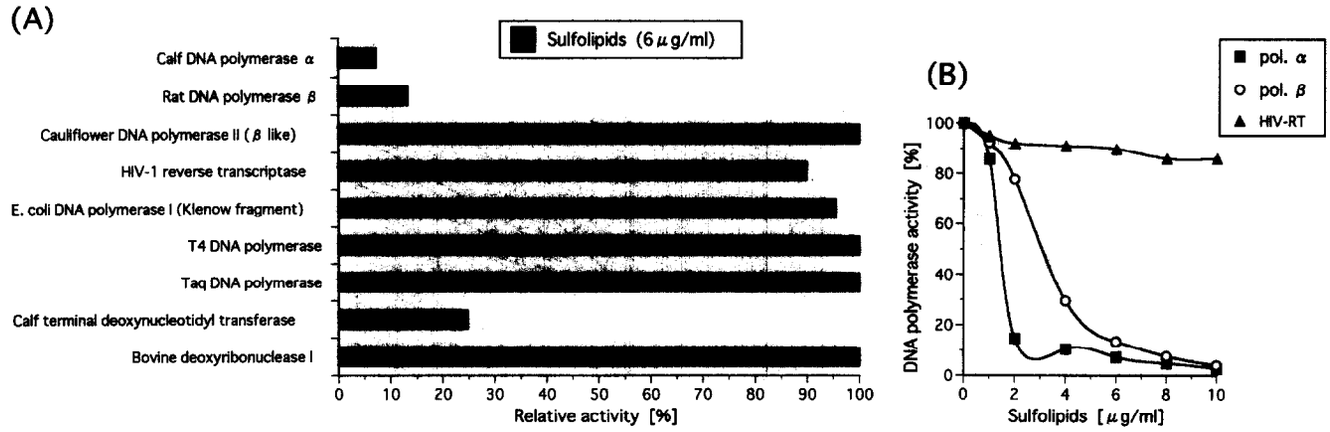

FIG. 2. (A) Effect of sulfolipids on the activities of various DNA polymerases and other enzymes. Sulfolipids (6 μg/mL) were incubated with each enzyme (0.05 units). The enzymatic activity was measured as described in previous reports [10–12]. Enzyme activity (5000 cpm) in the absence of sulfolipids was taken as 100%. (B) Inhibition of DNA polymerase α, β, and HIV-RT activities by sulfolipids. Calf thymus DNA polymerase α (■), rat DNA polymerase β (○), and HIV-RT (▲) (0.05 units each) were preincubated with the indicated concentrations (0–10 μg/mL) of purified sulfolipids and then were assayed for these enzyme activities as described in previous reports [10–12].

that we have isolated needs to be evaluated rather than a mixture of the three for this explanation.

The cyanobacterial sulfolipids also inhibited only the activities of pol. α and pol. β at the same inhibitory concentration as those of the pteridophytal sulfolipids. The effect on pol. α was compared with that of well-known pol. α inhibitors such as aphidicolin [15]. The inhibition of the pol. β activity was much stronger than the effect of reported pol. β inhibitors: nucleotide analogs such as dideoxyTTP [16], sulfonolipids [17], a few flavonoids [18], phospholipids [19, 20], and fatty acids [10–12].

Mode of DNA Polymerase α and β Inhibition by Sulfolipids

To elucidate the mechanism of the DNA polymerase inhibition by the sulfolipids, the extent of inhibition as a function of DNA template-primer or nucleotide substrate concentration was studied (Fig. 3). In the kinetic analysis, poly (dA)/oligo $(dT)_{12\text{-}18}$ and nucleotide were used as the DNA template-primer and substrate, respectively. Double-reciprocal plots of the results showed that the inhibition of pol. α by sulfolipids was non-competitive with the DNA template, since there was no change in its apparent K_m (Michaelis constant, 34 μM), while its V_{max} (maximum velocity) decreased from 100 to 15 pmol/hr in the presence of 1, 1.5, 2, or 4 μg/mL sulfolipids (Fig. 3A). Similarly, the apparent K_m for the substrate dTTP was unchanged at a concentration of 1.3 μM, whereas an 80% decrease in the V_{max} was observed in the presence of 4 μg/mL sulfolipids (Fig. 3B). The inhibition was, therefore, also clearly non-competitive with respect to the substrate dTTP. In contrast, the sulfolipids' inhibition of pol. β activity was competitive with the DNA template and the substrate (Fig. 3, C and D). In the case of the DNA template, the apparent V_{max} was unchanged at 35 pmol/hr, whereas a 7.75-fold increase in K_m was observed in the presence of 4 μg/mL sulfolipids (Fig. 3C). The V_{max} for the substrate (dTTP) was 33 pmol/hr, and the K_m for the substrate increased with increasing sulfolipid concentration, being 5, 9, and 20 pmol/mL in the presence of 2, 3, and 6 μg/mL sulfolipids, respectively (Fig. 3D). The sulfolipids may interact with or affect both of the binding sites on pol. β, thereby decreasing its affinity for the DNA template and substrate, whereas they may bind or interact with a domain distinct from the template or substrate binding sites on pol. α. The results indicate that the sulfolipid-binding sites on pol. α and pol. β are structurally different from each other, and that the sulfolipids bind directly to the DNA template-binding or the substrate-binding site on pol. β, since they bear no structural resemblance to either the DNA template or the substrate. As reported previously [10], C_{18}-fatty acids such as linoleic acid, oleic acid, and linolenic acid can inhibit both of the polymerase activities at concentrations higher than 10 μg/mL, and the inhibition modes of the enzymes are almost same. However, the sulfolipid compounds **1**, **2**, and **3** contain the ester form of linolenic acid, linoleic acid, and oleic acid in their structures, respectively. Since the pol. β active site is sandwiched between the DNA template-binding site and the substrate-catalytic region, we speculated that the inhibition by the fatty acid occurs via its insertion into this crevice [12]. The structural resemblance between the fatty acids themselves and the fatty acid ester in the sulfolipids suggests the similarity of the action mode.

When a neutral detergent, 0.05% Nonidet P-40, was added to the reaction mixture, pol. β activity was no longer inhibited by sulfolipids, but pol. α activity was still inhibited (data not shown). Nonidet P-40 apparently reversed the binding of the sulfolipids to pol. β, but the binding pol. α could not be reversed, further indicating that sulfolipids

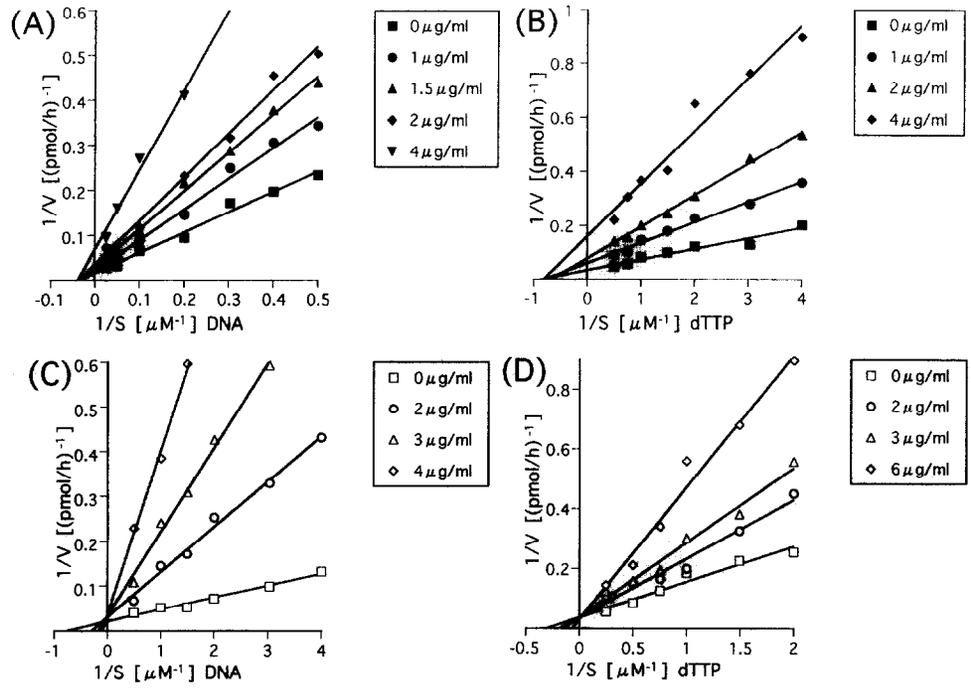

FIG. 3. Kinetic analysis of sulfolipid inhibition of DNA polymerases (0.05 units each). The effects of sulfolipids on the K_m and V_{max} values of the DNA template-primer and dTTP substrate were determined, and the results are displayed as Lineweaver–Burk plots. (A) Preincubation with DNA polymerase α in the presence of 1 μg/mL (●), 1.5 μg/mL (▲), 2 μg/mL (◆), or 4 μg/mL (▼) sulfolipids. Enzyme activity in the absence of sulfolipids is also shown (■). DNA polymerase α activity was then assayed, using the concentrations of poly(dA)/oligo(dT) (2/1) indicated as the template/primer. (B) DNA polymerase α activity was assayed with the concentrations of substrate (dTTP) indicated, after preincubation of the enzyme without (■) or with 1 μg/mL (●), 2 μg/mL (▲), and 4 μg/mL (◆) sulfolipids. (C) DNA polymerase β activity was measured in the absence (□) or presence of 2 μg/mL (○), 3 μg/mL (△), and 4 μg/mL (◇) sulfolipids at the concentrations of DNA template-primer indicated. (D) DNA polymerase β activity was assayed with the concentrations of substrate (dTTP) indicated in the presence of 2 μg/mL (○), 3 μg/mL (△), and 6 μg/mL (◇) sulfolipids or in the absence of sulfolipids (□).

interact with the hydrophobic region on pol. β. In contrast, the addition of an excess of protein (100 μg/mL BSA) or nucleic acid (50 μM poly(rC)) did not affect the binding in either case (data not shown), suggesting that the effect of the sulfolipids resulted from either the non-specific adhesion of the sulfolipids to the enzymes, or their binding selectively to special sites. The sulfolipids selectively bind to the enzyme at the hydrophobic site in the sulfolipids, i.e. the fatty acid ester region. The 6-sulfo-D-quinovose in the sulfolipids may strengthen the inhibitory effect. In this connection, we note a recent review by Joyce [21], which discusses how the configuration of nucleotide substrates influences their interaction with DNA polymerases.

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