Novel Phenalenone Derivatives from a Marine-Derived Fungus Exhibit Distinct Inhibition Spectra against Eukaryotic DNA Polymerases†

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ABSTRACT: A number of compounds used for cancer chemotherapy exert their effects by inhibiting DNA replication. New inhibitors of DNA polymerases, therefore, could be potential candidates for new anticancer drugs. This study tested the effects of two phenalenone-skeleton-based compounds, which were isolated from a marine-derived fungus *Penicillium sp.*, sculezonone-B (SCUL-B) and sculezonone-A (SCUL-A), upon DNA polymerase activity. Both compounds inhibited bovine DNA polymerases α and γ, moderately affected the activity of DNA polymerase ϵ, and had almost no effect on HIV-reverse transcriptase and an *E. coli* DNA polymerase I Klenow fragment. Most notably, whereas SCUL-A inhibited pol β ($IC_{50} = 17\ \mu M$), SCUL-B has only a weak influence upon this polymerase ($IC_{50} = 90\ \mu M$). Kinetic studies showed that inhibition of both DNA polymerases α and β by either SCUL-A or SCUL-B was competitive with respect to dTTP substrate and noncompetitive with the template-primer. Whereas pol α inhibition by SCUL-B is competitive with respect to dATP, the inhibition by SCUL-A was found to be a mixed type with dATP substrate. The K_i values of SCUL-B were calculated to be 1.8 and 6.8 μM for DNA polymerases α and γ, respectively. The K_i of DNA polymerase β for SCUL-A was 12 μM and that for DNA polymerase α, 16 μM. Therefore, deletion of the OH-group at C12 enhanced inhibition of DNA polymerase β. Since computational analyses of these two inhibitors revealed a remarkable difference in the distribution of negative electrostatic charge on the surface of molecules, we infer that different electrostatic charges might elicit different inhibition spectra from these two compounds.

In recent years, the number of known eukaryotic DNA polymerases (pol)[1] has increased to 13 (namely, α, β, γ, δ, ϵ, ζ, η, θ, τ, κ, λ, μ, and σ) plus a REV1 deoxycytidyl transferase that was found in the yeast *Saccharomyces cerevisiae* (*1*). Among these polymerases, replication of chromosomal DNA from eukaryotic cells is carried out mainly by three—pol α, δ, and ϵ (*1*, *3*), whereas pol γ is responsible for mitochondrial DNA replication (*4*), and pol β is for base excision repair (*5*−*7*). Pol ζ, η, θ, τ, κ, σ, λ, and μ and REV1 have been implicated in translesion and other processes (*1*).

Pol α, complexed with DNA primase, initiates the leading strand and Okazaki fragments of the lagging strand (*7*, *9*). After it synthesizes short DNA fragments, it is replaced by pol δ and ϵ for additional elongation (*10*). Pol α, δ, and ϵ are classified as the B family and share similar primary structures (*1*, *3*); they are equally sensitive to one inhibitor, aphidicolin (*11*).

Marine microorganisms may be rich sources of useful compounds for development of new pharmaceutical agents (*12*). We examined specific inhibitors for DNA polymerases and found that a sulfated glycosylglycerololipid, 1-*O*-(6′-sulfo-α-D-glucopyranosyl)-2,3-di-*O*-phytanyl-*sn*-glycerol (KN-208), a derivative of polar lipid isolated from an archaebacterium in an Okinawa sea sponge, strongly inhibited eukaryotic DNA polymerases (*13*). KN-208 inhibited DNA pol β most strongly among the DNA polymerases tested; it potentiated cytotoxicity of an alkylating agent, methyl methansulfonate, presumably by inhibiting base excision repair (*13*). In 1994, Shioda et al. (*14*) found that halenaquinol sulfate, a *p*-hydroquinone sulfate obtained from a marine sponge, preferentially inhibits the eukaryotic DNA polymerase α-family.

In the present study, after DNA polymerase screening for inhibitors with a number of metabolites from marine

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[1] Abbreviations: pol, DNA polymerase; SCUL-A/B, sculezonone A/B; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; DMSO, dimethyl sulfoxide; HIV-RT, human immunodeficiency virus-reverse transcriptase; TCA, trichloroacetic acid; KF, Klenow fragment.

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microorganisms residing in sponges and bivalves from the Okinawa Sea, we identified two novel inhibitors for eukaryotic DNA polymerases. Sculezonone-B (SCUL-B) selectively inhibited DNA pol α and γ, but sculezonone-A (SCUL-A), lacking −OH at C12 of SCUL-B, also inhibited pol β. Here we discuss possible inhibition mechanisms of these two compounds from the viewpoint of electrostatic potential distribution on their molecular surfaces.

MATERIALS AND METHODS

Chemicals. SCUL-A and SCUL-B were purified from a broth of the fungus *Penicillium sp*., isolated from the Okinawa marine bivalve *Mytilus coruscus* as described previously (*15*). Deoxynucleoside triphosphates and the synthetic polynucleotides, poly(dA), poly(dT), poly(rA), oligo(dA)$_{12-18}$, and oligo(dT)$_{12-18}$, were purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). [*methyl*-^{3}H]dTTP (62 Ci/mmol) and [2,8-^{3}H]dATP (34.8 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA). All analytical reagents were supplied commercially by Wako, Ltd. (Osaka, Japan).

Enzymes. Pol α was purified from calf thymus (*16*). *E. coli* pol I KF was purchased from New England Biolabs (Boston, MA). Human immunodeficiency virus-reverse transcriptase (HIV-RT) was from Seikagaku Kogyo Co. (Tokyo, Japan). HeLa pol ϵ was provided by Stuart Linn (University of California, Berkeley) (*17*). Pol β was purified from *E. coli* carrying a rat pol β−cDNA recombinant plasmid (a gift from Akio Matsukage, Faculty of Science, Japan Women's University, Tokyo). Pol γ was purified from the mitochondrial fraction of bovine liver (*18*). One unit of DNA polymerase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxynucleoside triphosphates into DNA in 60 min at 37 °C under the reaction conditions described below.

DNA Polymerase Assays. Pol α was assayed in the reaction mixture (25 μL) containing the following: 40 mM potassium phosphate (pH 7.2); 8 mM $MgCl_2$; 4 mM 2-mercaptoethanol; 40 μM each of dATP, dGTP, and dCTP; 20 μM dTTP (500 cpm/pmol); and 100 μg/mL activated calf thymus DNA. Conditions used for pol α assay were also applied to *E. coli* pol I KF. Pol β was assayed in the reaction mixture (25 μL) containing the following: 0.1 M Tris-HCl (pH 8.8); 100 mM NaCl; 0.5 mM $MnCl_2$; either 100 μg/mL activated DNA with four dNTPs plus [^{3}H]dTTP or synthetic polydeoxynucleotides, such as 50 μg/mL poly(dA)-oligo(dT)$_{12-18}$ (A/T = 5) and poly(dT)-oligo(dA)$_{12-18}$ (T/A = 5), with ^{3}H-labeled dNTP complementary to the template. Pol γ was assayed in the reaction mixture (25 μL) containing the following: 50 μg/mL poly(rA)-oligo(dT)$_{12-18}$ (A/T = 5); Tris-HCl (pH 7.5); 10 mM DTT; 100 mM KCl; 20 μM [^{3}H]dTTP (1000 cpm/pmol); and 0.5 mM $MnCl_2$. Pol ϵ was assayed in a reaction mixture (25 μL) containing the following: 20 μg/mL poly(dA)-oligo(dT)$_{12-18}$ (A/T = 20); bisTris-HCl (pH 6.7); 5 mM $MgCl_2$; 1 mM DTT; and 20 μM dTTP (500 cpm/pmol). HIV-RT was assayed in a reaction mixture (25 μL) containing the following: 50 mM Tris-HCl, pH 7.5; 5 mM DTT; 20 μg/mL poly(rA)-oligo(dT)$_{12-18}$ (A/T = 10); 20 μM dTTP (500 cpm/pmol); 100 mM KCl; and 8 mM $MgCl_2$. Incubation was carried out at 37 °C for 30 min, and then acid-insoluble radioactivity was measured as previously described (*19*).

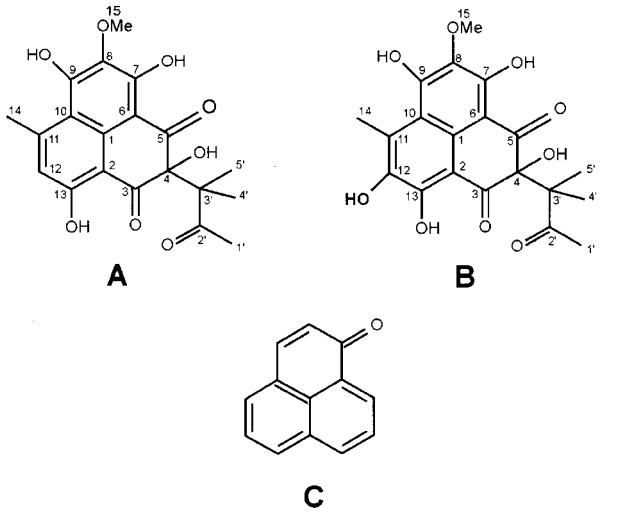

FIGURE 1: Compound structures. A: sculenozone A, $C_{20}H_{20}O_8$ (abbreviated as SCUL-A) (*15*); B: sculenozone B, $C_{20}H_{20}O_9$ (abbreviated as SCUL-B) (*15*); C: phenalen-1-dione.

Assay was performed under reaction conditions favorable to each DNA polymerase. Further, inhibition of each DNA polymerase was confirmed under other conditions using activated calf thymus DNA and/or poly(dA)-oligo(dT) to reveal template-primer-dependent differences. Inhibition was also examined in either potassium phosphate or Tris-HCl to examine possible effects of buffer systems.

Measurement of Inhibition. Compounds were dissolved in 10% dimethyl sulfoxide (DMSO) at various concentrations, and aliquots were added to the reaction mixture for each DNA polymerase assay. For kinetic analyses, concentrations of template-primer or [^{3}H]dNTPs were varied. The inhibition mode was analyzed by Lineweaver−Burk plot, and K_i was obtained from Dixon plot.

Computational Analysis. A compound model was constructed and simple-minimized. Compound models were simulated with force field parameters based on the Consistent Valence Force Field (CVFF). Group-based cutoffs, 0.95 nm for van der Waals and 0.95 nm for Coulomb interactions, were introduced. Temperature was set at 298 K. Calculations based on simulation images were carried out using the INSIGHT II package [version 98, Molecular Simulation, Inc. (MSI), San Diego, CA]. Electrostatic potentials on the surface of compounds were analyzed by WebLabViewerLite (version 3.2, MSI) software.

RESULTS

Inhibitor Screening. Screening for DNA polymerase inhibitors was performed using a library of novel compounds obtained from marine-derived fungi or bacteria residing in Okinawan marine bivalves and sponges. Among 20 compounds, we found 2 named SCUL-A (Figure 1A) and SCUL-B (Figure 1B), derivatives of phenalenone (Figure 1C), which strongly inhibited calf thymus pol α activity. As shown in Figure 1, SCUL-A has similar structure to SCUL-B except that SCUL-A lacks an OH-group at the C12 position in the phenalenone skeleton of SCUL-B (Figures 1A,B).

Inhibition Specificity. Effects of SCUL-A and SCUL-B were examined on various DNA polymerases, i.e., pol α, β, γ, and ϵ, HIV-RT, and *E. coli* pol I KF. As shown in Figure 2A,B, both SCUL-A and SCUL-B inhibited pol α with IC_{50}

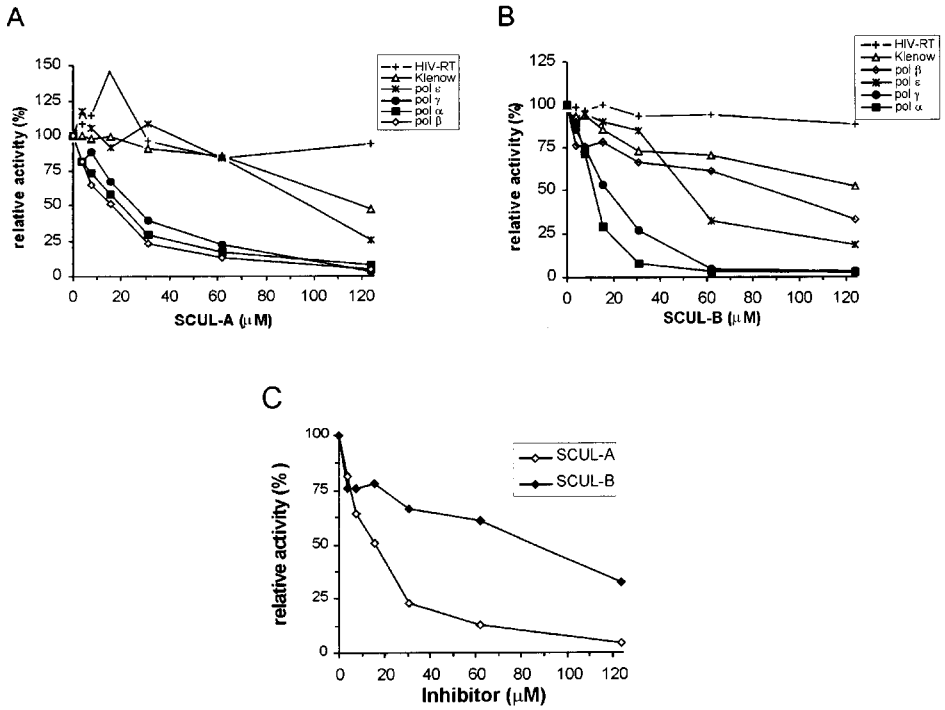

FIGURE 2: Inhibition of DNA polymerase activity by SCUL-A and SCUL-B. DNA polymerases activity was assayed in the presence of increasing concentrations of SCUL-A and SCUL-B as described under Materials and Methods. (A) SCUL-A inhibitory effects on DNA polymerase activity; (B) SCUL-B inhibitory effects on DNA polymerase activity; (C) SCUL-A and -B inhibitory effects on pol β.

Table 1: IC_{50} Values Obtained from the SCUL-A and -B Inhibition Assay against DNA Polymerases Tested

	IC_{50} (μM)	
	SCUL-A	SCUL-B
pol α	20	12
pol ϵ	105	50
pol γ	25	18.5
pol β	17	90
HIV-RT	NI[a]	NI
E. coli pol I KF	120	230

[a] No inhibition.

at approximately 20 and 12 μM, respectively (Table 1). Both compounds also inhibited pol γ (Figure 2A,B). Interestingly, pol ϵ was relatively resistant to inhibition by SCUL-A (IC_{50} > 100 μM, Table 1) and was inhibited only moderately by SCUL-B (IC_{50} = 50 μM, Table 1), although pol ϵ is structurally classified in the pol B family (*1*, *3*) (Figure 2A,B). This difference in the inhibitory effect was not due to different reaction conditions for these enzymes; pol α activity was inhibited equally under conditions used for pol ϵ assay, described under Materials and Methods (data not shown). In contrast, a remarkable difference was observed with respect to pol β inhibition. Figure 2A shows that SCUL-A inhibited pol β by an IC_{50} of 17 μM, while SCUL-B (Figure 2B) did so only weakly (IC_{50} = 90 μM). On the other hand, HIV reverse transcriptase and *E. coli* pol I KF were resistant to inhibition by both compounds (Figure 2A,B).

Kinetic Analyses. To find the inhibition mode, activity was measured as a function of the concentration of either DNA template-primer or deoxynucleoside triphosphates in the absence or presence of inhibitors. SCUL-A inhibited pol β noncompetitively with the DNA template-primer (Figure 3A) and competitively with dTTP (Figure 3B) and dATP (Figure 3C). Lineweaver−Burk double-reciprocal plots indicated that inhibition of pol α by SCUL-A was also noncompetitive with the template-primer DNA (Figure 4A) and competitive with a pyrimidine substrate, dTTP (Figure 4B); later it was found to be a mixed type with a purine substrate, dATP (Figure 4C). SCUL-B exhibited noncompetitive inhibition with poly-(dA)-oligo$(dT)_{12-18}$ as a template-primer (Figure 5A), and a competitive inhibition mode with dTTP and dATP nucleotide substrate (Figure 5B,C). Inhibition constants (K_i) obtained from Dixon plots from Figures 3D, 4D, and 5D are summarized in Table 2. K_i values of SCUL-A were 16 and 12 μM for pol α and β, respectively. Those of SCUL-B were 1.8 and 6.8 μM for pol α and γ, respectively.

DISCUSSION

DNA polymerase inhibitors may be used for two distinct applications. First, they may suppress cell proliferation by inhibiting DNA replication or suppress repair processes, making them potential candidates for new cancer drugs. Second, they could be probes for analyzing the role of each DNA polymerase in DNA replication. Recently, a number of new species of DNA polymerases have been identified (*1*), although most of their functions are unclear. Therefore, probe usage would become more important than ever to assess the in vivo function of each DNA polymerase.

This study reports two novel compounds that exhibit characteristic inhibition spectra to eukaryotic DNA polymerases. These compounds, designated as SCUL-A (Figure 1A) and SCUL-B (Figure 1B), were isolated from the *Penicillium* sp. fungus, an inhabitant in a bivalve living in the Okinawa Sea (*15*).

SCUL-B as well as SCUL-A inhibited pol α with K_i values of 1.8 and 16 μM, respectively (Table 2). With respect to the template-primer, both of them showed noncompetitive

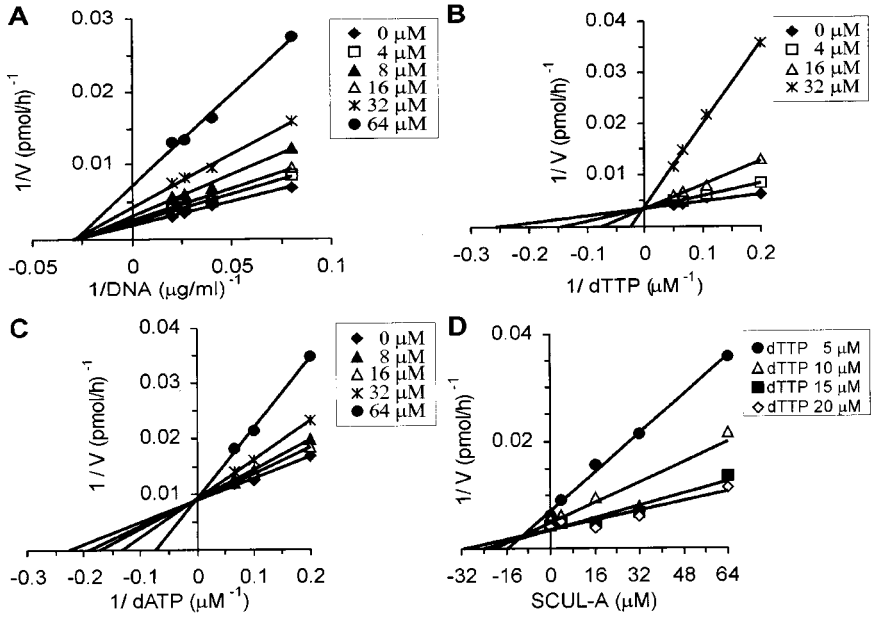

FIGURE 3: Kinetic analyses of pol β inhibition by SCUL-A. Activity of pol β was assayed in the absence (◆) or presence of 4 (□), 8 (▲), 16 (△), 32 (∗), and 64 (●) μM SCUL-A (A−D). Lineweaver−Burk double-reciprocal plots obtained by varying DNA template-primer concentration (A), dTTP concentration (B), and dATP concentration (C). Dixon plots based on data from panel B revealed the K_i value to be 12 μM (D).

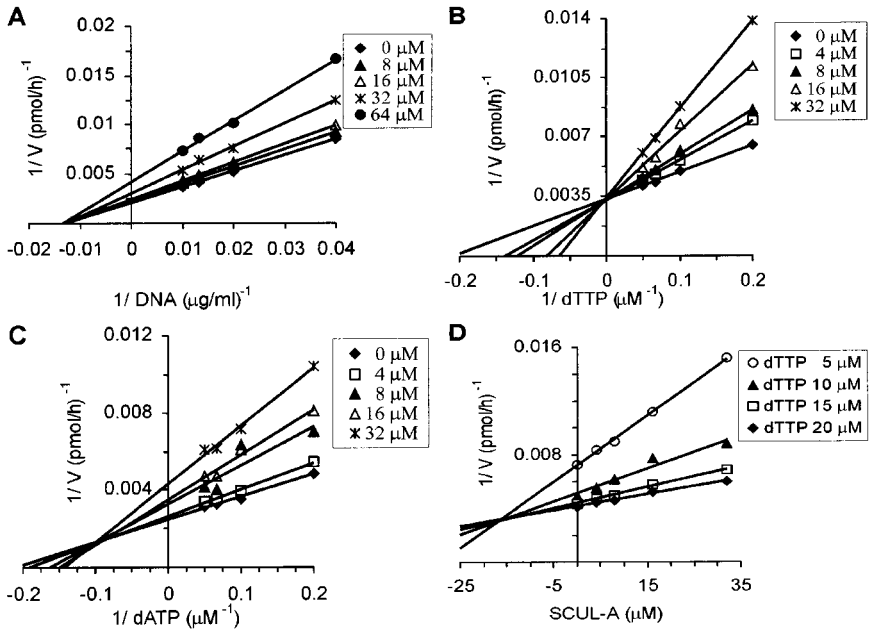

FIGURE 4: Kinetic analyses of pol α inhibition by SCUL-A. Pol α was assayed in the absence (◆) or presence of 4 (□), 8 (▲), 16 (△), 32 (∗), and 64 (●) μM SCUL-A (A−D). Lineweaver−Burk double-reciprocal plots obtained by varying the concentration of DNA template (A), dTTP substrate (B), and dATP substrate (C). (D) Dixon plot made on the basis of results from panel B, indicating a K_i value of pol α for SCUL-A at 16 μM.

inhibition (Figures 4A and 5A). The mode of inhibition by SCUL-B on pol α was competitive with substrates dTTP (Figure 5B) and dATP (Figure 5C). Inhibition of pol α by SCUL-A was, however, competitive with dTTP (Figure 4B) but was a mixed type with dATP (Figure 4C). These data suggest that the binding sites for SCUL-A and a substrate (dATP) on the pol α molecule may be in close proximity or partially overlap each other. The mutual influence between these two binding sites results in a different binding capacity and the mixed type inhibition. This mode of inhibition resembles that of aphidicolin, which inhibits B-family DNA polymerases, i.e., pol α, δ, and ϵ, in competition with pyrimidine deoxynucleoside triphosphates (dCTP) and mixed type with purine deoxynucleoside triphosphates (dGTP) (*20*, *21*). In this context, we measured inhibition of pol ϵ with these compounds. Pol ϵ was relatively resistant to both compounds, SCUL-B being only 2 times stronger than SCUL-A (Table 1). Thus, SCUL-A and SCUL-B differ from aphidicolin; using these compounds, one may be able to discriminate pol α from pol ϵ.

The most interesting feature of these compounds is their inhibitory activity against pol β (Figure 2C). SCUL-A is a phenalenone derivative similar to SCUL-B, but lacks the OH-group at C12 (Figure 1). Whereas SCUL-A strongly inhibited

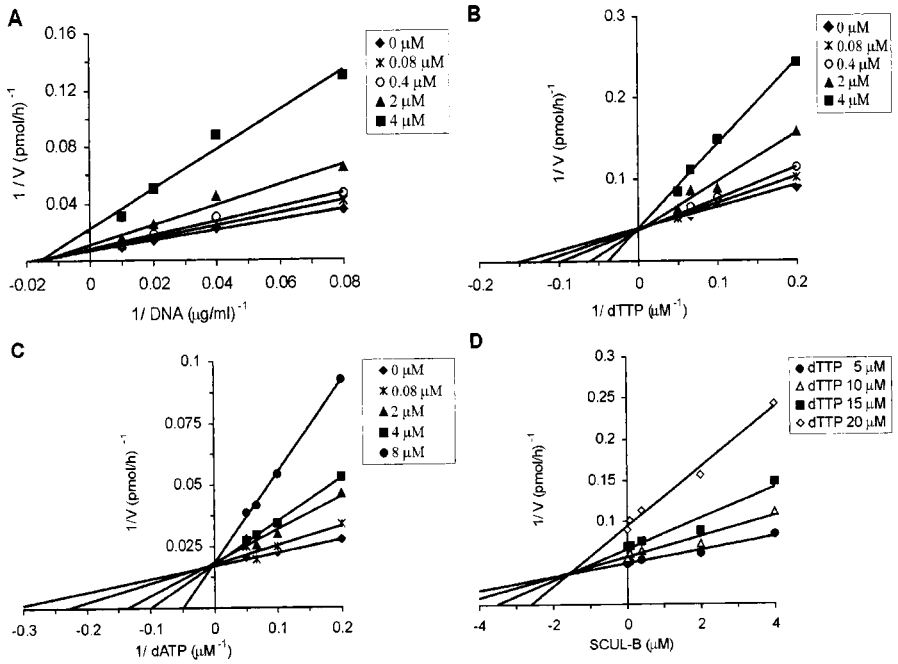

FIGURE 5: Kinetic analyses of pol α inhibition by SCUL-B. Lineweaver−Burk plots obtained by varying concentrations of template-primer DNA (A) and those obtained by varying concentrations of dTTP (B) and dATP (C). Pol α was incubated in the absence (◆) or in the presence of 0.08 (✱), 0.4 (○), 2 (▲), 4 (■), and 8 (●) μM SCUL-B. (D) Dixon plots based on the data of panel B revealed a K_i value of pol α for SCUL-B around 1.8 μM.

Table 2: K_i Values of SCUL-A and -B Inhibition on DNA Polymerase Activity

	K_i (μM)[a]	
	SCUL-A	SCUL-B
pol α	16	1.8
pol γ	ND[b]	6.8
pol β	12	ND

[a] Values represent means of three independent experiments. [b] Not determined.

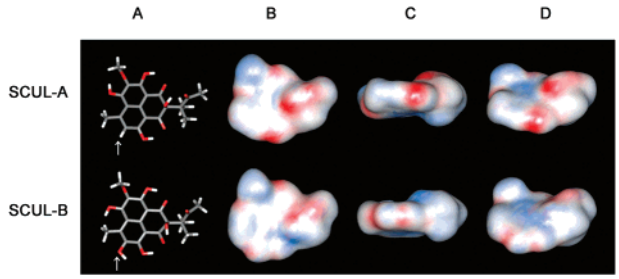

FIGURE 6: Computer graphics of SCUL-A and SCUL-B. (A) Stick models of SCUL-A and SCUL-B in the front view, built by the graphics program INSIGHT II (Molecular Simulations). White arrows indicate positions of C12-OH. (B−D) Electrostatic potentials on the molecular surface of SCUL-A and SCUL-B. (B) Front view; (C) 45° backward rotation around a horizontal axis passing through the molecule; (D) bottom view. Electrostatic potentials over molecular surfaces were analyzed using WebLabViewer Lite (version 3.2, MSI) software. Blue areas are positively charged, red are negatively charged, and white are neutral (see Materials and Methods).

pol β, SCUL-B did not (Figure 2C, Table 1). Loss of −OH from C12 of SCUL-B facilitated SCUL-A inhibitory capacity against pol β, although this did not largely affect inhibitory effects on pol α and ϵ (Figure 2A,B and Table 1).

To obtain more information about the molecular basis for differential inhibition spectra exhibited by these two compounds, computational analyses (Figure 6) were performed using molecular simulation and surface analysis software. Since these two compounds differ by only one OH-group, 3D-conformations resemble each other (Figure 6A). However, comparison of electrostatic potential surfaces for SCUL-A and -B revealed a remarkable difference in their overall disposition and rapport. The electrostatic potential at each point on a constant electronic density surface (approximating the van der Waals surface for each arrangement) is represented graphically by red color corresponding to regions where electrostatic potential is most negative and blue color corresponding to least positive regions. As shown in Figure 6B−D, SCUL-A supports an enhancement of negative electrostatic potential on certain atoms, in contrast to SCUL-B. Two strong red signals are visible around oxygen atoms bound to C4 and C13 (Figure 6B−D, upper panels) on SCUL-A, but not on SCUL-B (Figure 6B−D, lower panels). Depletion of the −OH group from C12 develops a negative charge concentration around atoms C13 and C4 that are in the proximity of C12. Intensities of neutral (white) and positively charged areas (blue) seemed to be similar in both cases. It is conceivable, therefore, that newly generated electronegative charges could be responsible for pol β inhibition by SCUL-A.

Pol β consists of two domains, 8-kDa and 31-kDa domains, connected by a protease-sensitive region. It is a basic protein showing +9 charges; the 8-kDa domain has +10 and the 31-kDa domain has −1 electric charge (*22*). The palm region of pol β is charged highly positive (*23*) and binds with DNA. The dNTP binding pocket of pol β consists of two positively charged residues from the palm region (Lys280 and Arg283), two aromatic residues located in the thumb region (Tyr271 and Phe272), plus two metal-binding aspartates (Asp190, Asp192). Biochemical as well as structural data suggest that aspartate residues, in concert

with other positively charged residues, bind the triphosphate moiety and that aromatic residues stabilize the sugar moiety (*24*−*28*). It is tempting to speculate that SCUL-A binds positively charged amino acid residues via negatively charged regions created by loss of C12-OH. The negatively charged areas around C14 and C3, developed by the depletion of C12-OH group, might increase the affinity of the compound for the substrate binding site of pol β, but not for pol α. In other words, a substrate binding site that is specific only to pol β may discriminate SCUL-B from SCUL-A. The detailed mechanism must await the analysis of the crystal structure of DNA polymerase−inhibitor complex.

Phenalenone and its derivatives have been identified as polluting substances resulted from combustion of fossil fuels. Phenalenone, itself was found to be mutagenic in *Salmonella typhimurium* TM677 and TA100 in the presence of rat liver postmitochondrial supernatant (*29*−*31*), toxic to a few species of microalgae (*32*), and carcinogenic in newborn mouse lung (*31*, *33*). Another derivative, rosselianone A, showed antifungal activity against plant diseases (*34*). Eraburenols, compounds having phenalenone skeletons modified with another moiety, inhibit cholesterol ester transfer protein (*35*, *36*). In addition to these effects, here we found that sculezonones, also two phenalenone derivatives, inhibit eukaryotic DNA polymerase activity.

Discrimination modes of SCUL-A and SCUL-B by pol β may be useful for further dissection of the catalytic mechanism of this repair DNA polymerase. Since SCUL-A and SCUL-B have a peculiar inhibition spectrum, they can be used to distinguish the activity of pol α from pol ϵ, or pol γ from pol β, in combination with other inhibitors such as aphidicolin. Also, it would be intriguing to examine biological activity including antitumor effects of SCUL-A and SCUL-B.

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