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The inhibitory action of pyrrolidine alkaloid, 1,4-dideoxy-1,4-imino-D-ribitol, on eukaryotic DNA polymerases☆

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Abstract

The pyrrolidine alkaloids mimicking the structures of pentose with nitrogen in the ring are known to be inhibitors of glycosidases. We report here that a compound belonging to this category is an inhibitor of eukaryotic DNA polymerases. Among the eight naturally occurring pyrrolidine alkaloids we tested, only one compound, 1,4-dideoxy-1,4-imino-D-ribitol (DRB), which was purified from the mulberry tree (*Morus alba*), strongly inhibited the activities of eukaryotic DNA polymerases with IC_{50} values of 21–35 μM, and had almost no effect on the activities of prokaryotic DNA polymerases, nor DNA metabolic enzymes such as human immunodeficiency virus type 1 reverse transcriptase, T7 RNA polymerase, and bovine deoxyribonuclease I. Kinetic studies showed that inhibition of both DNA polymerases α and β by DRB was competitive with respect to dNTP substrate. Whereas DNA polymerase α inhibition was noncompetitive with the template-primer, the inhibition of DNA polymerase β was found to be competitive with the template-primer. The K_i values of DNA polymerases α and β for the template-primer were smaller than those for dNTP substrate. Therefore, the affinity of DRB was suggested to be higher at the template-primer binding site than at the dNTP substrate-binding site, although DRB is an analogue of deoxyribose consisting of dNTP. Computational analyses of the eight pyrrolidine alkaloids revealed a remarkable difference in the distribution of positive and negative electrostatic charges on the surface of molecules. The relationship between the structure of DRB and the inhibition of eukaryotic DNA polymerases is discussed.

Keywords: DNA polymerase; Pyrrolidine alkaloid; 1,4-Dideoxy-1,4-imino-D-ribitol; Enzyme inhibitor

We have screened and characterized many new inhibitors of eukaryotic DNA polymerases which are directly bound to the polymerase protein and the investigated three-dimensional structural interaction between DNA polymerase β (pol β) and each of the inhibitors [1,2]. We have found many DNA polymerase inhibitors; long chain fatty acids [1,3–5], triterpenoids [6–9], steroids [2,10,11], cerebrosides [12], glycolipids [13–22], and vitamin A such as retinal [23].

This study reports that the pyrrolidine alkaloid, 1,4-dideoxy-1,4-imino-D-ribitol (DRB), was isolated as a DNA polymerase inhibitor from the mulberry tree, *Morus alba*, and exhibited characteristic inhibition spectra to DNA polymerases and DNA metabolic enzymes. Although we have found and reported on more

☆ *Abbreviations:* pol, DNA polymerase; DRB, 1,4-dideoxy-1,4-imino-D-ribitol; dTTP, 2′-deoxythymidine 5′-triphosphate.

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than 30 natural compounds which inhibit only the activities of eukaryotic DNA polymerases so far [1–24], all the compounds were water-insoluble and still not sufficient for completely investigating the processes, and subsequently, we have continued to screen for new agents. The inhibition of pol β by each of all the inhibitors tested occurred by binding to the DNA template-binding domain competitively [2–4,6,10,13,15,16].

In this paper, we describe the findings and properties of the pyrrolidine alkaloid, 1,4-dideoxy-1,4-imino-D-ribitol (DRB), as a polymerase inhibitor belonging to a new category. DRB differed in that it inhibited the polymerase activity by competing with the substrates. Since DRB inhibited the activities of mammalian DNA polymerases, α, β, δ, and ε, to the same extent, the compound must be convenient to study the catalytic subunit site of the DNA polymerases. Such types of inhibitors were extremely rare in our screening, although the reason for this is unclear. The discrimination modes of DNA polymerase inhibition by DRB, therefore, may be useful for further study of the catalytic mechanism of eukaryotic DNA polymerases. Interestingly, the tested pyrrolidine alkaloids except DRB have no such activity, therefore, we also discuss here the possible inhibition mechanisms of the compound from the viewpoint of electrostatic potential distribution on the molecular surface.

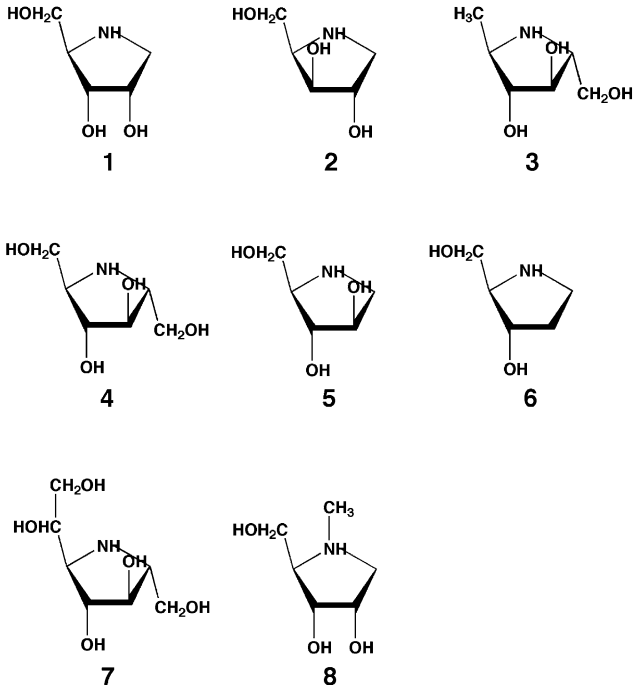

Fig. 1. Chemical structures of pyrrolidine alkaloids. Compound **1**: 1,4-dideoxy-1,4-imino-D-ribitol (DRB), compound **2**: 1,4-dideoxy-1,4-imino-D-xylitol, compound **3**: 2,5-imino-1,2,5-trideoxy-D-mannitol, compound **4**: 2,5-dideoxy-2,5-imino-D-mannitol, compound **5**: 1,4-dideoxy-1,4-imino-D-arabinitol, compound **6**: 1,4-imino-1,2,4-trideoxy-D-arabinitol, compound **7**: 2,5-dideoxy-2,5-imino-D-glycero-D-manno-heptitol, and compound **8**: 1,4-dideoxy-1,4-(methyliminiumyl)-D-ribitol.

Materials and methods

Materials. The structures of eight pyrrolidine alkaloids used in this study are shown in Fig. 1. Compounds **1** and **5** were isolated from the roots of *Morus alba* as described previously [25], compounds **2** and **3** from the bark of *Angylocalyx pynaertii* [26], compound **4** from the leaves of *Derris malaccensis* [27], compound **6** from the seeds of *Castanospermum australe* [28], and compound **7** from the bulbs of *Hyacinthus orientalis* [29]. The *N*-methyl derivative of **1** to give **8** was prepared by treatment with 37% HCHO and 80% formic acid according to the literature [27]. Deoxynucleoside triphosphates and synthetic polynucleotides such as poly(dA), poly(rA), poly(rC), and oligo(dT)$_{12-18}$, and [^{3}H]2′-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other reagents were of analytical grade and were purchased from Wako (Osaka, Japan).

Enzymes. DNA polymerase α (pol α) was purified from the calf thymus by immuno-affinity column chromatography as described previously [30]. Recombinant rat DNA polymerase β (pol β) was purified from *Escherichia coli* JMpβ5 as described by Date et al. [31]. Pol δ was purified from calf thymus [32] and pol ε was purified from HeLa cells as described previously [33]. *E. coli* Klenow fragment of pol I and human immunodeficiency virus type 1 (HIV-1) reverse transcriptase were purchased from Worthington Biochemical (Freehold, NJ, USA). Taq DNA polymerase, T4 DNA polymerase, and T4 polynucleotide kinase were purchased from Takara (Tokyo, Japan). T7 RNA polymerase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (La Jolla, CA, USA). Purified human placenta DNA topoisomerase I and II (2 U/μl) were purchased from TopoGen (Columbus, OH, USA).

DNA polymerase assays. The activities of DNA polymerases were measured by the methods described previously [3,4]. For DNA polymerases, poly(dA)/oligo(dT)$_{12-18}$ and dTTP were used as template-primer DNA and nucleotide substrate, respectively. For HIV reverse transcriptase, poly(rA)/oligo(dT)$_{12-18}$ and dTTP were used as template-primer and nucleotide substrate, respectively. Compounds were dissolved in 10% dimethyl sulfoxide (DMSO) at various concentrations and aliquots were added to the reaction mixture for each DNA polymerase assay. 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 catalyzed the incorporation of 1 nmol of deoxyribonucleotide triphosphates (i.e., dTTP) into the synthetic template-primers (i.e., poly(dA)/oligo(dT)$_{12-18}$, A/T = 2/1) in 60 min at 37 °C under the normal reaction conditions for each enzyme [3,4]. For kinetic analyses, the concentrations of template-primer or [^{3}H]dTTPs were varied. The inhibition mode was analyzed by Lineweaver–Burk plots and K_i was obtained from Dixon plots.

Other enzyme assays. The activities of calf DNA primase of pol α, T7 RNA polymerase, T4 polynucleotide kinase, and bovine deoxyribonuclease I were measured in each of the standard assays according to the manufacturer's specifications as described by Koizumi et al. [34], Nakayama and Saneyoshi [35], Soltis and Uhlenbeck [36], and Lu and Sakaguchi [37], respectively. Telomerase activity was determined using the polymerase chain reaction (PCR)-based telomeric repeat amplification protocol as previously described [38] with some modifications [39]. The activities of DNA topoisomerase I and II were carried out as described previously [40].

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).

Temperature was set at 298 K. Calculations based on simulation images were carried out using the INSIGHT II (Accelrys, San Diego, CA, USA). Electrostatic potentials on the surface of compounds were analyzed by WebLab ViewerLite (version 3.2, Accelrys, San Diego, CA, USA) software.

Results

Isolation of pyrrolidine alkaloids from mulberry trees

Screening for DNA polymerase inhibitors was performed using 50% ethanol extracts from medicinal plants and the extract from the bark of mulberry trees (*Morus alba*) showed the strongest inhibitory activity toward DNA polymerase. Alkaloids in the extract were purified by various ion-exchange column chromatography as seen in the literature [25]. The active compound was identified as 1,4-dideoxy-1,4-imino-D-ribitol (DRB) from the ^{1}H and ^{13}C NMR spectral data. DRB has been isolated from the same plant and found to be a moderate inhibitor of mammalian digestive isomaltase [27].

Effects of pyrrolidine alkaloids on the activities of DNA polymerases and the other DNA metabolic enzymes

The eight pyrrolidine alkaloids (i.e., the purified compound **1** (DRB) and the prepared compounds **2**–**8**) were tested for their eukaryotic DNA polymerase α and β (pol α and β) inhibitory activities. Only compound **1** (DRB) inhibited the DNA polymerase activity, but the other compounds (compounds **2**–**8**) did not (Table 1).

Fig. 2 shows the inhibition dose–response curves of DRB against calf pol α, rat pol β, calf pol δ, and human pol ε. The activities of all eukaryotic DNA polymerases tested were inhibited by DRB and the IC_{50} values were 21, 28, 32, and 35 μM, respectively. On the other hand, DRB gave no significant on influence the activities of prokaryotic DNA polymerases such as the Klenow fragment of *E. coli* DNA polymerase I, Taq DNA polymerase, and T4 DNA polymerase. The three-dimensional structures of eukaryotic DNA polymerases would be greatly different from those of prokaryotic DNA polymerases. DRB also did not inhibit any of the activities of other DNA-metabolic enzymes such as calf DNA primase of pol α, HIV-1 reverse transcriptase, human telomerase, T7 RNA polymerase, DNA topoisomerase I and II, T4 polynucleotide kinase, and bovine deoxyribonuclease I (Table 2). When activated DNA was used as the template-primer DNA, the inhibition modes of DRB did not change. Therefore, the remainder of this report is devoted to an analysis of the action of DRB on eukaryotic DNA polymerases.

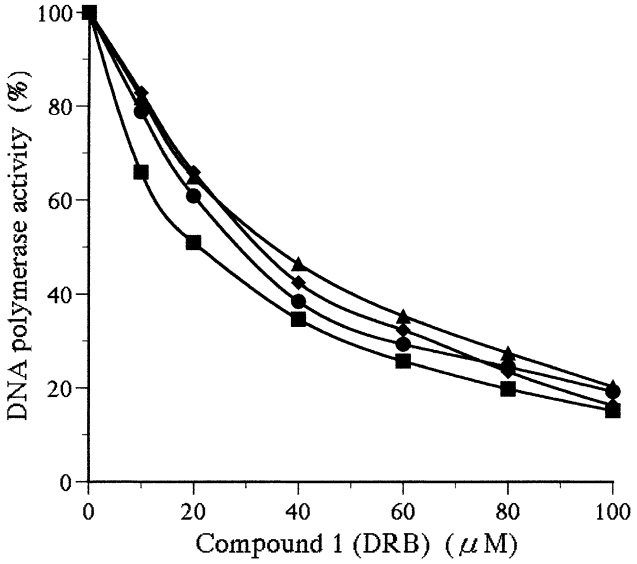

Fig. 2. Eukaryotic DNA polymerase inhibition dose–response curves of DRB. The enzymes used (0.05 U of each) were calf DNA polymerase α (closed square), rat DNA polymerase β (closed circle), calf DNA polymerase δ (closed diamond), and human DNA polymerase ε (closed triangle). The DNA polymerase activities were measured as described in the text. DNA polymerase activity in the absence of these compounds was taken as 100%.

Table 1
Concentration of pyrrolidine alkaloids (μM) giving 50% inhibition of eukaryotic DNA polymerase α and β

Compound	Eukaryotic DNA polymerase	
	α	β
1	21	28
2	>1000	>1000
3	>1000	>1000
4	>1000	>1000
5	>1000	>1000
6	>1000	>1000
7	>1000	>1000
8	>1000	>1000

The compounds were incubated with each enzyme (0.05 U). The enzymatic activity was measured as described in Materials and methods. DNA polymerase activity in the absence of the compound was taken as 100%.

Effects of reaction conditions on DNA polymerase inhibition

To determine the effects of a non-ionic detergent on the binding of DRB to the DNA polymerases, a neutral detergent, Nonidet P-40 (NP-40), was added to the reaction mixture at a concentration of 0.1%. In the absence of DRB, DNA polymerase activity was taken as 100%. The pol α or β inhibitory effect of DRB at 100 μM was not affected by the addition of NP-40 to the reaction

Table 2
IC_{50} values of DRB (compound **1**) on the activities of DNA polymerases and other DNA metabolic enzymes

Enzyme	IC_{50} value of DRB (μM)
Eukaryotic DNA polymerases	
Calf DNA polymerase α	21
Rat DNA polymerase β	28
Calf DNA polymerase δ	32
Human DNA polymerase ε	35
Prokaryotic DNA polymerase	
E. coli DNA polymerase I (Klenow fragment)	>1000
Taq DNA polymerase	>1000
T4 DNA polymerase	>1000
Other DNA metabolic enzymes	
Calf DNA primase of DNA polymerase α	>1000
HIV-1 reverse transcriptase	>1000
Human telomerase	>1000
T7 RNA polymerase	>1000
Human DNA topoisomerase I	>1000
Human DNA topoisomerase II	>1000
T4 polynucleotide kinase	>1000
Bovine deoxyribonuclease I	>1000

DRB was incubated with each enzyme (0.05 U). The enzymatic activity was measured as described in Materials and methods. Enzyme activity in the absence of the compound was taken as 100%.

Table 3
Effects of poly(rC), bovine serum albumin (BSA), or Nonidet P-40 (NP-40) on the inhibition of DNA polymerase activities by DRB (compound **1**)

Compounds added to the reaction mixture	Calf DNA polymerase α (%)	Rat DNA polymerase β (%)
Without the compounds		
None (control)	100	100
+100 μg/ml poly(rC)	100	100
+100 μg/ml BSA	100	100
+0.1% NP-40	100	100
100 μM DRB		
100 μM DRB	15.1	19.2
100 μM DRB + 100 μg/ml poly(rC)	15.2	21.1
100 μM DRB + 100 μg/ml BSA	15.2	18.7
100 μM DRB + 0.1% NP-40	17.9	19.8

Fifty μM poly(rC) and 200 μg/ml BSA or 0.1% NP-40 was added to the reaction mixture. In the absence of DRB, DNA polymerase activity was taken as 100%.

mixture, suggesting that the binding interaction to the enzyme by DRB is hydrophilic (Table 3). We also tested whether an excess amount of a substrate analogue, poly(rC) (100 μg/ml), or a protein, BSA (100 μg/ml), could prevent the inhibitory effects of DRB, to determine whether the effects of the compound were due to their non-specific adhesion to the enzymes, or to selective binding to specific sites. Poly(rC) and BSA showed little or no influence on the effects of DRB, suggesting that the binding to the DNA polymerases occurs selectively (Table 3).

Mode of inhibition of DNA polymerase α and β by DRB

To elucidate the inhibitory mechanism, the extent of inhibition was measured as a function of the concentration of either template-primer or deoxynucleoside triphosphates in the absence or presence of DRB (Fig. 3). In the kinetic analyses, poly(dA)/oligo $(dT)_{12–18}$ and dTTP were used as the DNA template-primer and nucleotide substrate, respectively. Double reciprocal plots of the results indicated that the DRB-mediated inhibition of the pol α activity was non-competitive with the DNA template-primer and competitive with the nucleotide substrate. In the case of the DNA template-primer, 59.9% and 42.8% decreases in maximum velocity (V_{max}) were observed in the presence of 5 and 10 μM DRB, respectively, whereas the apparent Michaelis constant (K_m) was unchanged at 7.8 μM (Fig. 3A). The V_{max} for the nucleotide substrate was unchanged at 29.2 pmol/h and the K_m for the nucleotide substrate decreased from 3.6 to 2.3 μM in the presence of 10 μM DRB (Fig. 3B). On the other hand, DRB inhibited pol β competitively with both the DNA template-primer and nucleotide substrate, because the V_{max} was unchanged at a concentration of 55.6 and 62.5 pmol/h, respectively (Fig. 3C and D). These data suggested that the binding mode of DRB to both the nucleotide substrate binding site of pol α and pol β was the same. However, the mode to the DNA template-primer binding site differed from that to the nucleotide substrate binding site. DRB mimics the structure of deoxyribose consisting of nucleotide substrate and, therefore, DRB may compete with dNTP to bind to the catalytic site of the DNA polymerase.

When activated DNA and four deoxynucleoside triphosphates were used as the DNA template-primer and dNTP substrate, respectively, the inhibition of pol α and β by DRB was the same as when using poly(dA)/oligo$(dT)_{12–18}$ and dTTP (data not shown). The mode of inhibition was suggested to be ineffective for pyrimidine deoxynucleoside triphosphates (dCTP and dTTP) and purine deoxynucleoside triphosphates (dATP and dGTP).

The inhibition constant (K_i) value for pol α, obtained from Dixon plots from Fig. 3, was found to be 8.5 and 12 μM for the DNA template-primer and nucleotide substrate, respectively, and the K_i values for pol β were 2.4 and 14 μM for the DNA template-primer and nucleotide substrate, respectively (Dixon plot data are not shown). The affinity of DRB might be higher at the DNA template-primer binding site than at the nucleotide substrate-binding site of both pol α and β, although the structure of DRB is more similar to nucleotide substrate than DNA template-primer.

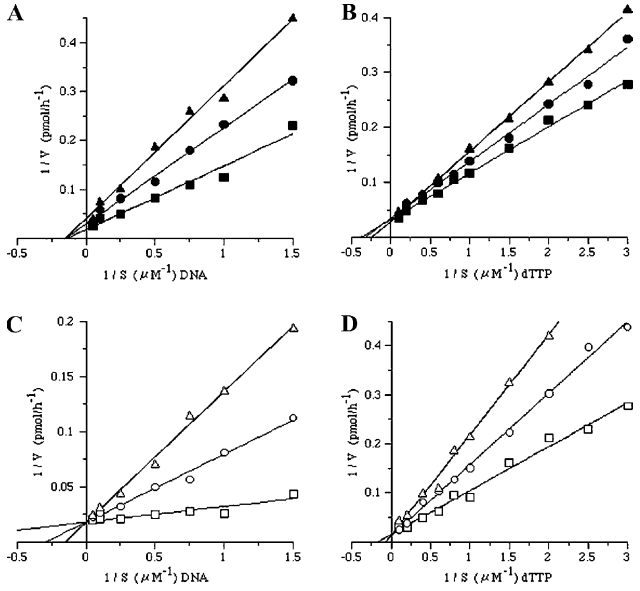

Fig. 3. Kinetic analyses of DNA polymerase α and β inhibition by DRB. (A, B) Activity of DNA polymerase α was assayed in the absence (closed square) or presence of five (closed circle) and 10 (closed triangle) μM DRB. (C, D) Activity of DNA polymerase β was assayed in the absence (open square) or presence of five (open circle) and 10 (open triangle) μM DRB. Lineweaver–Burk double-reciprocal plots obtained by varying DNA template-primer concentrations (A and C) and dTTP concentrations (B and D).

Discussion

In recent years, the number of known eukaryotic DNA polymerases (pol) has increased to 13 (α, β, γ, δ, ε, ζ, η, θ, ι, κ, λ, μ, and σ) plus a REV1 deoxycytidyl transferase found in the yeast *Saccharomyces cerevisiae* [41] and pol I-like found in rice [42]. Among these polymerases, replication of chromosomal DNA from eukaryotic cells is carried out mainly by three polymerases, pol α, δ, and ε [42–44], whereas pol γ is responsible for mitochondrial DNA replication [45], and pol β is for base excision repair [46–48]. Pol ζ, η, θ, ι, κ, λ, μ, σ, REV1 deoxycytidyl transferase and pol I-like have been implicated in translation and other processes [41,42]. However, their in vivo functions in DNA replication, repair, and recombination are mostly unknown as yet, especially in multicellular organisms. DNA polymerase inhibitors could be probes for analyzing the in vivo role of the DNA polymerases in DNA replication, repair, and recombination. Moreover, DNA polymerase inhibitors may suppress cell proliferation by inhibiting DNA replication or suppressing repair processes, making them potential candidates for new cancer drugs. Therefore, it would become more important than ever to assess the in vivo function of the DNA polymerases for inhibitor usage. From this point of view, we screened the inhibitors of eukaryotic DNA polymerases, and found more than 30 natural compounds [1–24]. They were all water-insoluble and subsequently, not sufficient for completely investigating the processes. We desperately need water-soluble DNA polymerase inhibitors.

In this study, we report on a new water-soluble inhibitor, a pyrrolidine alkaloid, 1,4-dideoxy-1,4-imino-D-ribitol (DRB), isolated from the mulberry tree, *M. alba*, which exhibits characteristic inhibition spectra to DNA polymerases and DNA metabolic enzymes. DRB was shown to be a competitive antagonist against the dNTP substrate in kinetic analyses using pol β. The inhibition by each of all the inhibitors previously found was competitive with the DNA template-primer. Since DRB inhibited the activities of mammalian DNA polymerases, α, β, δ, and ε, to the same extent, the compound must be convenient to study the catalytic subunit side of the DNA polymerases. We now speculate that the inhibitors may be suppressors that control the physiological events specific to multicellular organisms, since the action by most of the inhibitors seems to be more physiologically functional than toxic [24]. Discrimination modes of DNA polymerase inhibition by DRB, therefore, may be useful for further determination of the catalytic mechanism of eukaryotic DNA polymerases.

Since the melting temperature of double-stranded DNA was not changed in the presence or absence of DRB (data not shown), and further at least the binding of DRB to DNA polymerases was not influenced by poly(rC) and BSA (Table 3), DRB inhibits the enzyme activities by interacting directly with the enzymes, rather than by binding to DNA. DRB competed with nucleotide substrate to bind competitively to the catalytic site of eukaryotic DNA polymerases from kinetic analyses (Fig. 3). Therefore, DRB might mimic the structure of deoxyribose consisting of nucleotide substrate. It can be thought that the substrate binding site of eukaryotic DNA polymerases discriminates DRB from the other pyrrolidine alkaloids. Although more than 30 inhibitors have so far been found in our laboratory, the inhibition of pol β by each of all the inhibitors tested was competitive with the DNA template-primer, and the inhibitors dominantly bound to the 8 kDa DNA-binding domain of pol β in competition with the DNA template [2–4,6,10,13,15,16]. DRB could differ from such types of inhibitors in that it inhibits the polymerase activity by competing with the substrates. Moreover, a number of the compounds used for cancer chemotherapy exert their effects by inhibiting DNA replication. Inhibitors of the DNA polymerases, therefore, could be potential candidates for new anti-cancer drugs. Fortunately, DRB was water-soluble. It would be intriguing to examine the biological activity including antitumor effects of DRB. Some of the inhibitors such as sulfo-glycolipids could be clinically promising immunosuppressive and anti-tumor agents as described previously [49–51], although they were not water-soluble. Therefore, DRB, which is a

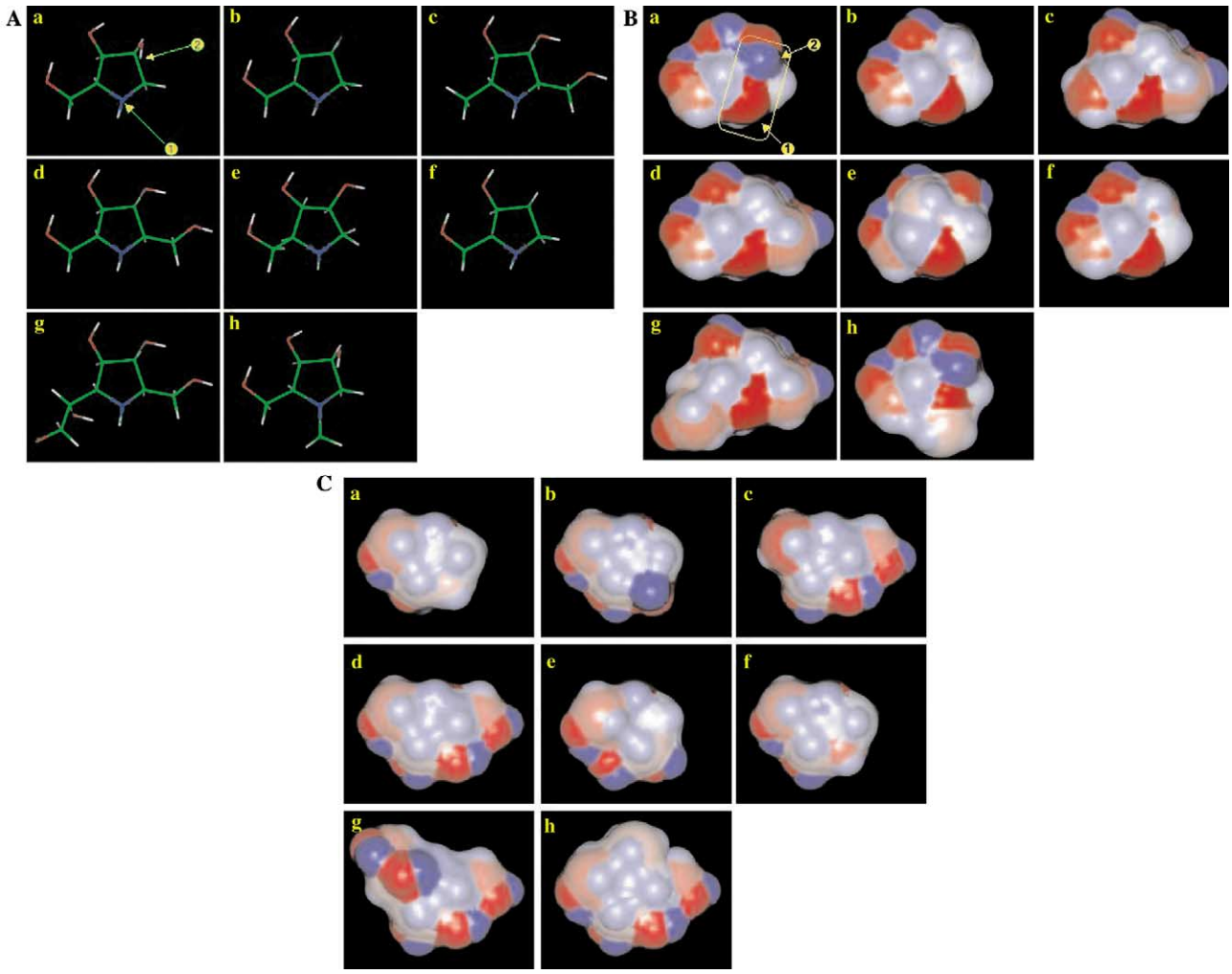

Fig. 4. Computer graphics of pyrrolidine alkaloids. (A) Stick models of compounds **1**–**8** in the front view, built by the graphics program INSIGHT II (Accelrys, San Diego, CA, USA). Panels (a–h) are compounds **1**–**8**, respectively. The carbons, nitrogens, oxygens, and hydrogens of the compounds are indicated in green, blue, red, and white, respectively. Arrows-1 and -2 indicate the position of the N atom in the ring and H atom of the –OH group from C2 in compound **1** (DRB), respectively. (B) Electrostatic potentials over molecular surfaces in the front view were analyzed using WebLab Viewer Lite (version 3.2, Accelrys, San Diego, CA, USA) software. Panels (a–h) are compounds **1**–**8**, respectively. Blue areas are positively charged, red are negatively charged, and white are neutral. Arrows-1 and -2 indicate the position of the N atom and H atom of the –OH group from C2 in compound **1** (DRB), respectively. (C) The back view of panel (B).

water-soluble compound, should be tested as an immunosuppressive or anti-tumor agent, and, if effective, could be more promising in clinical usage.

Aside from the clinical application, the substrate binding site of eukaryotic DNA polymerases suggests that DRB differs from the other pyrrolidine alkaloids tested. To obtain more information about the molecular basis for differential inhibition spectra exhibited by the eight pyrrolidine alkaloids, computational analyses were performed using molecular simulation and surface analysis software (Fig. 4A). Since these compounds can be regarded as pentoses with nitrogen in the ring, the 3D-conformations resemble each other (Fig. 4A). However, a comparison of the electrostatic potential surfaces of these compounds revealed a remarkable difference in their overall disposition and rapport (Fig. 4B). The electrostatic potential at each point on a constant electronic density surface (approximating the van der Waals surface for each arrangement) is represented graphically in red corresponding to the regions where the electrostatic potential is most negative and blue corresponding to the most positive regions. As shown in Fig. 4B(a), compound **1** (DRB) in the front view supports the enhancement of both negative and positive electrostatic potential on the N atom in the ring (the arrow-1 in Figs. 4A and B) and H atom of the –OH group from C2 (arrow-2 in Figs. 4A and B), respectively. DRB and compounds **2**–**7** have neutral electrostatic potential in the back view, but compound **8** does not (Fig. 4C). Compound **7** also has two strong red and blue signals, but the surface area of the signals of compound **7** was smaller than that of DRB (Fig. 4B(g)). The

surface area of neighboring negative and positive charges is suggested as important for DNA polymerase inhibition (i.e., the yellow area in Fig. 4B(a)).

Discrimination modes of DNA polymerase inhibition by DRB and the other pyrrolidine alkaloids may be useful for further determination of the catalytic mechanism of eukaryotic DNA polymerases. Also, it would be intriguing to examine the biological activity including the antitumor effects of DRB.

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