Articles

Petasiphenol: A DNA Polymerase λ Inhibitor†

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ABSTRACT: Petasiphenol, a bio-antimutagen isolated from a Japanese vegetable, *Petasites japonicus*, selectively inhibits the activities of mammalian DNA polymerase λ (pol λ) in vitro. The compound did not influence the activities of replicative DNA polymerases such as α, δ, and ε but also showed no effect even on the pol β activity, the three-dimensional structure of which is thought to be highly similar to pol λ. The inhibitory effect of petasiphenol on intact pol λ including the BRCA1 C-terminus (BRCT) domain was dose-dependent, and 50% inhibition was observed at a concentration of 7.8 μM. The petasiphenol-induced inhibition of the pol λ activity was noncompetitive with respect to both the DNA template-primer and the dNTP substrate. Petasiphenol did not only inhibit the activity of the truncated pol λ including the pol β-like core, in which the BRCT motif was deleted in its N-terminal region. BIAcore analysis demonstrated that petasiphenol bound selectively to the N-terminal domain of pol λ but did not bind to the C-terminal region. On the basis of these results, the pol λ inhibitory mechanism of petasiphenol is discussed.

Eukaryotic cells reportedly contain three replicative DNA polymerases (pol[1] α, δ, and ε), mitochondrial DNA polymerase (pol γ), and at least nine repair types of DNA polymerase (pol β, δ, ε, ζ, η, θ, κ, λ, and μ) (*1*, *2*). We have searched for natural compounds that selectively inhibit each of these eukaryotic DNA polymerases to use as tools and molecular probes to distinguish DNA polymerases and to clarify their biological and in vivo functions (*3*−*10*). In this study, we report on a newly found compound that selectively inhibits only the pol λ activity. The natural compound is a phenolic compound, petasiphenol, produced from a higher plant, a Japanese vegetable (*Petasites japonicus*). Interestingly, the compound was originally found to be a bio-antimutagen in UV-induced mutagenic *Escherichia coli* WP2 B/r *Trp*$^-$ isolated from the same plant (*11*). To our knowledge, there have been no reports about such natural compounds specific to pol X family DNA polymerases, except for solanapyrone A as a pol β and λ inhibitor and prunasin as a pol β inhibitor, which we reported previously (*7*, *10*). The compound differed from solanapyrone A in that it inhibited only pol λ so far examined and a stronger pol λ inhibitor than solanapyrone A. No such pol λ-specific inhibitor has been reported.

Pol λ is a recently described eukaryotic DNA polymerase belonging to the pol X family (*12*) comprising enzymes involved in DNA repair processes, whose main member is pol β. Human pol λ (63.4 kDa) consists of a nuclear signal transport (residues 1−35), a BRCA1 C-terminal (BRCT) domain (residues 36−132), a proline−serine-rich region (residues 133−243), and a pol β-like core region (residues 244−575). The N-terminal part of pol λ has similarity to yeast pol IV and contains a BRCT domain (*12*). The BRCT

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[1] Abbreviations: pol, DNA-directed DNA polymerase (EC 2.7.7.7); BRCT, BRCA1 C-terminus; dRP, 5′-deoxyribose 5-phosphate; AP, apurinic/apyrimidinic; BER, base excision repair; TdT, terminal deoxynucleotidyl transferase.

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domain is present in several proteins involved in DNA repair and cell cycle checkpoint control (*13*, *14*). Recently, it has been shown that the BRCT domain is involved in protein/protein interactions (*14*). The C-terminal part of pol λ (residues 244−575) is composed of a catalytic core which is similar to pol β (8 kDa domain and 31 kDa finger, palm, and thumb polymerization domain) and has 32% amino acid identity to pol β (*13*). The truncated pol λ, in which the BRCT motif was deleted in its N-terminal region (i.e., C-terminal region containing the pol β-like core), has the DNA polymerase activity. Petasiphenol was found to directly bind to the N-terminal domain of pol λ but not to the C-terminal pol β-like core region. The compound inhibited the DNA polymerase activity of intact pol λ but did not suppress the DNA polymerase activity of the pol λ catalytic core domain. Interestingly, petasiphenol did not inhibit the activity of both the C-terminal part of pol λ in which the BRCT motif was deleted in its N-terminal region and terminal deoxynucleotidyl transferase which is an another family X polymerase species with the BRCT domain, suggesting that petasiphenol does not always recognize any of the BRCT domain structures. On the basis of these results, the inhibitory action of petasiphenol and its relation to the enzyme structure of pol λ will be discussed in this report.

MATERIALS AND METHODS

Materials. Nucleotides and chemically synthesized DNA template-primers such as poly(dA), poly(rA), and oligo(dT)$_{12-18}$ and [^{3}H]dTTP (43 Ci/mmol) were purchased from Amersham Biosciences (Buckinghamshire, U.K.). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Enzymes. DNA polymerase α (pol α) was purified from calf thymus by immunoaffinity column chromatography (*15*). Recombinant rat DNA polymerase β (pol β) was purified from *E. coli* JMpβ5 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*). The cDNA encoding full-length human pol λ (63.4 kDa), the BRCT domain of pol λ (residues 36−132), and truncated pol λ (residues 133−575) lacking the BRCT domain were generated by polymerase chain reaction (PCR) using the primers L-F1 (5′-GCAGAATTCATGGATCCCAGGGGTATCTTGAAG-3′) and L-R1 (5′-GTTCTCGAGCCAGTCCCGCTCAGCAGGTTCTCG-3′), L-F4 (5′-GCAGAATTCGTACTTGCAAAGATTCCTAGGAGG-3′) and L-R4 (5′-CCAAAGCTTGATGCTGAATCCAGCTACATCCAC-3′), and L-F3 (5′-CGGGAATTCTTCATCCCCAGTAGGTACTTGGAC-3′) and LR-1, respectively, and then constructed and purified as described previously (*10*, *19*). Pol I (α-like) and II (β-like) from a higher plant, cauliflower inflorescence, were purified according to the methods outlined by Sakaguchi et al. (*20*). The Klenow fragment of pol I and human immunodeficiency virus type 1 (HIV-1) reverse transcriptase were purchased from Worthington Biochemical Corp. (Freehold, NJ). Calf thymus terminal deoxynucleotidyl transferase, T7 RNA polymerase, and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (La Jolla, CA). Taq DNA polymerase, T4 DNA polymerase, and T4 polynucleotide kinase were purchased from Takara (Tokyo, Japan).

DNA Polymerase Assays. The reaction mixtures for pol α, β, plant, and prokaryotic DNA polymerases were described previously (*3*, *4*), and those for pol δ and ϵ were as described by Ogawa et al. (*21*). The reaction mixture for pol λ was the same as that of pol β. The substrates of the DNA polymerases used were poly(dA)/oligo(dT)$_{12-18}$ and dTTP as DNA template-primer and nucleotide substrate, respectively. The substrates of HIV-1 reverse transcriptase used were poly(rA)/oligo(dT)$_{12-18}$ and dTTP as template-primer and nucleotide substrate, respectively. The substrates of terminal deoxynucleotidyl transcriptase used were oligo(dT)$_{12-18}$ (3′-OH) and dTTP as template-primer and nucleotide substrate, respectively. Petasiphenol was dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s. Four microliters of the sonicated samples was mixed with 16 μL 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 μL) were added to 16 μL of each of the standard enzyme 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 catalyzed the incorporation of 1 nmol of deoxyribonucleotide triphosphate (i.e., dTTP) into the synthetic DNA 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*).

Other Enzyme Assays. Activities of calf DNA primase of pol α, T7 RNA polymerase, DNA topoisomerase I and II, 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. (*22*), Nakayama and Saneyoshi (*23*), Mizushina et al. (*24*), Soltis and Uhlenbeck (*25*), and Lu and Sakaguchi (*26*), respectively.

Surface Plasmon Resonance. Binding analyses of the N-terminal BRCT domain fragment and C-terminal catalytic core (pol β-like) domain of pol λ and petasiphenol were performed using a Biosensor BIAcore instrument (BIACORE 3000) (BIAcore, Sweden). CM5 research grade sensor chips (BIAcore, Sweden) were used. All buffers were filtered before use. Full-length pol λ (residues 1−575, 63.4 kDa), the BRCT domain (residues 36−132, 12 kDa), and the C-terminal domain (residues 133−575, 47.4 kDa) (508, 96, and 380 μg/mL, respectively, 25 μL each; i.e., 0.2 nmol each) in coupling buffer (10 μM sodium acetate, pH 5.0) were injected over a CM5 sensor chip at 20 μL/min to capture the protein to the carboxymethyl dextran matrix of the chip by NHS/EDC coupling reaction (60 μL of mix) as described previously (*27*). Unreacted *N*-hydroxysuccinimide ester groups were inactivated using 1 M ethanolamine−HCl (pH 8.0). This reaction immobilized about 5000 response units (RU) of the protein. Binding analysis of petasiphenol was performed in running buffer including petasiphenol [5 mM potassium phosphate buffer (pH 7.0) and 10% DMSO] at a flow rate of 20 μL/min at 25 °C. Kinetic parameters were determined using the software BIA evaluation 3.2.

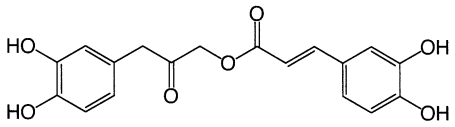

FIGURE 1: Chemical structure of petasiphenol.

Petasiphenol Docking Modeling. The generation of the BRCT domain of human pol λ was performed using the molecular modeling software Insight II/Homology (Accelrys Inc., San Diego, CA). All calculations were conducted on SGI workstations, running under the IRIX 6.5 operating system. The BRCT domain of pol λ was refined by molecular dynamics simulations using Insight II/Discover (Accelrys Inc., San Diego, CA). The binding site of petasiphenol on the BRCT domain of human pol λ was determined using the software Insight II/Binding Site Analysis (Accelrys Inc., San Diego, CA), and the molecular docking of the compound and the protein was done using a flexible docking procedure in the affinity program within the Insight II modeling software (Accelrys Inc., San Diego, CA). The calculations used a CVFF force field in the discovery program and a Monte Carlo strategy in the affinity programs (*28*). Each energy-minimized final docking position of petasiphenol was evaluated using the interactive score function in the Ludi module. The Ludi score includes contribution of the loss of translational and rotational entropy of the fragment, number and quality of hydrogen bonds, and contributions from ionic and lipophilic interactions to the binding energy.

RESULTS

Isolation of Petasiphenol. We screened for DNA polymerase inhibitors and found a natural compound from a Japanese vegetable (*P. japonicus*) collected from Akita prefecture, Japan, that inhibits mammalian DNA polymerase λ (pol λ) activity but not pol α, β, δ, and ε activity. The compound was extracted with acetone from the vegetable body (500 g dry wt). Evaporation of the solvent yielded 5 g of a green waxy material. The extract was partitioned between ethyl acetate (1 L) and water (1 L), adjusted to pH 7, and the organic layer was evaporated. The fraction was subjected to silica gel column chromatography (Wakogel C-100, 100 mesh, 5.0 × 50 cm) and then eluted with chloroform−methanol−acetate (100:10:0.5 v/v/v). The active fractions (750 mg) were purified by a second silica gel column chromatography (Wakogel C-200, 200 mesh, 2.5 × 40 cm) using benzene−methanol (10:1 v/v). The active fractions (150 mg) were subjected to a third silica gel column chromatography (Wakogel C-300, 300 mesh, 1.5 × 20 cm) and then eluted with chloroform−methanol (10:1 v/v). Finally, the active compound (20 mg) was purified by Sephadex LH-20 column chromatography (1.5 × 20 cm) eluted with chloroform−methanol (1:1 v/v). Negative FABHR (fast atom bombardment high resolution) mass and ^{1}H, ^{13}C, and DEPT (distortionless enhancement by polarization transfer) NMR spectroscopic analyses indicated that the inhibitor was found to be an agent known as petasiphenol, previously reported as a bio-antimutagen isolated from the same plant (*11*). The chemical structure of petasiphenol is shown in Figure 1.

Effects of Petasiphenol on the Activities of Mammalian DNA Polymerases and Other DNA Metabolic Enzymes.

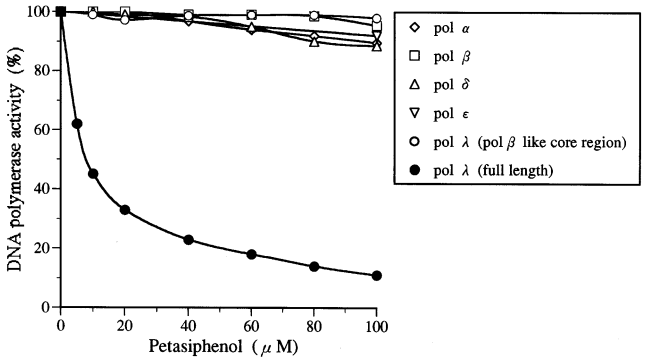

FIGURE 2: Dose−response curves of petasiphenol. The enzymes used (0.05 unit each) were calf pol α (◇), rat pol β (□), calf pol δ (△), human pol ε (▽), the pol β-like core region of human pol λ (residues 133−575) (○), and the full length of human pol λ (residues 1−575) (●). The DNA polymerase activities were measured as described in the text. The DNA polymerase activity in the absence of petasiphenol was taken as 100%.

Figure 2 shows the inhibition dose−response curves of petasiphenol against mammalian DNA polymerases. Petasiphenol was effective at inhibiting human DNA polymerase λ (pol λ) activity, and the inhibition was dose-dependent, with 50% inhibition observed at a dose of 7.8 μM (Figure 2). The compound had no influence at all on the activities of not only replicative DNA polymerases such as calf DNA polymerase α (pol α), calf DNA polymerase δ (pol δ), and human DNA polymerase ε (pol ε) but also repair-related DNA polymerase such as rat DNA polymerase β (pol β) (Figure 2). Petasiphenol had no inhibitory effect on higher plant cauliflower pol I (α-like) and II (β-like), prokaryotic DNA polymerases such as the Klenow fragment of *E. coli* pol I, Taq DNA polymerase and T4 DNA polymerase, and other DNA-metabolic enzymes such as calf terminal deoxynucleotidyl transferase (TdT), HIV-1 reverse transcriptase, T7 RNA polymerase, human DNA topoisomerase I and II, T4 polynucleotide kinase, and bovine deoxyribonuclease I (Table 1). The IC_{50} values in Table 1 did not change when the DNA template-primer was activated DNA (data not shown).

In the inhibition spectrum, interestingly, petasiphenol selectively inhibited the activity of pol λ, which has been recently identified as a new family member of pol β, in the mammalian DNA polymerases tested, and their three-dimensional structures are thought to be highly similar to each other (*12*). Since petasiphenol did not inhibit the activity of pol β (Figure 2), indicating that petasiphenol bound to the N-terminal region including the BRCT domain of pol λ directly and, subsequently, the DNA polymerase activity of the pol β-like core of pol λ. On the other hand, petasiphenol did not inhibit the activity of TdT, which is also the X family enzyme with the BRCT domain (Table 1), suggesting that the compound did not always recognize any of the BRCT domain structure. The fact that a bio-antimutagen is an inhibitor of a DNA polymerase species, pol λ, is of great interest.

Effects of Reaction Conditions on DNA Polymerase λ Inhibition. To determine the effects of a nonionic detergent on the binding of petasiphenol to pol λ, Nonidet P-40 (NP-40) was added to the reaction mixture at a concentration of 0.05% and 0.1%. In the absence of the compounds, the DNA polymerase activity was taken as 100%. The pol λ inhibitory

Table 1: IC_{50} Values of Petasiphenol on the Activities of Various DNA Polymerases and Other DNA Metabolic Enzymes[a]

enzyme	IC_{50} value of petasiphenol (μM)
mammalian DNA polymerases	
calf DNA polymerase α	>500
rat DNA polymerase β	>500
calf DNA polymerase δ	>500
human DNA polymerase ϵ	>500
human DNA polymerase λ	7.6
plant DNA polymerases	
cauliflower DNA polymerase I (α-like)	>500
cauliflower DNA polymerase II (β-like)	>500
prokaryotic DNA polymerases	
E. coli DNA polymerase I (Klenow fragment)	>500
Taq DNA polymerase	>500
T4 DNA polymerase	>500
other DNA metabolic enzymes	
calf DNA primase of DNA polymerase α	>500
calf terminal deoxynucleotidyl transferase	>500
HIV-1 reverse transcriptase	>500
T7 RNA polymerase	>500
human DNA topoisomerase I	>500
human DNA topoisomerase II	>500
T4 polynucleotide kinase	>500
bovine deoxyribonuclease I	>500

[a] Petasiphenol was incubated with each enzyme (0.05 unit). The enzymatic activity was measured as described in Materials and Methods. Enzyme activity in the absence of the compound was taken as 100%.

Table 2: Effects of Poly(rC), Bovine Serum Albumin (BSA), or Nonidet P-40 (NP-40) on the Inhibition of DNA Polymerase λ Activity by Petasiphenol[a]

compounds added to the reaction mixture	human DNA polymerase λ (%)
without petasiphenol	
none (control)	100
+50 μM poly (rC)	100
+200 μg/mL BSA	100
+0.05% NP-40	100
+0.1% NP-40	100
50 μM petasiphenol	
50 μM petasiphenol	20.3
50 μM petasiphenol + 50 μM poly(rC)	21.5
50 μM petasiphenol + 200 μg/mL BSA	19.8
50 μM petasiphenol + 0.05% NP-40	44.1
50 μM petasiphenol + 0.1% NP-40	58.3
100 μM petasiphenol	
100 μM petasiphenol	9.6
100 μM petasiphenol + 50 μM poly(rC)	9.9
100 μM petasiphenol + 200 μg/mL BSA	8.6
100 μM petasiphenol + 0.05% NP-40	32.7
100 μM petasiphenol + 0.1% NP-40	38.8

[a] Poly(rC) (50 μM) and 200 μg/mL BSA or 0.1% NP-40 were added to the reaction mixture. In the absence of petasiphenol, DNA polymerase activity was taken as 100%.

effect of petasiphenol at 100 μM was slightly reversed by the addition of 0.05% NP-40 to the reaction mixture, and the pol λ inhibitory effect of 50 μM petasiphenol was moderately reversed by the addition of 0.1% NP-40 (Table 2). The pol λ inhibition−reversion by NP-40 was concentration-dependent, indicating that petasiphenol could bind to and interact with the hydrophobic region of the pol λ protein. We also tested whether an excess amount of a substrate analogue, poly(rC) (50 μM), or a protein, BSA (200 μg/mL), could prevent the inhibitory effects of petasiphenol. If petasiphenol binds to pol λ by nonspecific adhesion, the addition of the DNA and/or the protein would reduce the inhibitory activity. Poly(rC) and BSA showed no influence and/or no binding effect on the compound, suggesting that the binding to pol λ occurs selectively or binds to a specific site on pol λ (Table 2).

Mode of DNA Polymerase λ Inhibition by Petasiphenol. Next, to elucidate the mechanism of inhibition of petasiphenol on pol λ, the extent of inhibition as a function of DNA template-primer or dNTP substrate concentrations was studied (Figure 3). In kinetic analysis, poly(dA)/oligo$(dT)_{12-18}$ and dTTP were used as the DNA template-primer and dNTP substrate, respectively. Double reciprocal plots of the results showed that the petasiphenol-induced inhibition of pol λ activity was noncompetitive with both the DNA template (K_m was unchanged at 2.5 μM) and the dNTP substrate (K_m was unchanged at 1.18 μM) (Figure 3A,B). The inhibition constant (K_i) values, obtained from Dixon plots, were found to be 3.8 and 4.5 μM for the DNA template and dNTP substrate, respectively (Figure 3C,D). The inhibition by petasiphenol against the DNA template was almost as effective as that against the dNTP substrate. When activated DNA and four deoxynucleoside triphosphates were used as the DNA template-primer and dNTP substrates, respectively, the inhibition of pol λ by petasiphenol was the same inhibition mode as with the synthesized DNA template-primer (data not shown). These results suggested that petasiphenol did not directly bind to either the DNA template binding site or the dNTP substrate binding site of pol λ. Since both of the DNA template and dNTP substrate binding sites are present in the pol β-like core of pol λ (*12*), we need to study further the interaction among petasiphenol, the N-terminal BRCT domain, and the C-terminal catalytic domain of pol λ in detail.

Binding between Petasiphenol and the N-Terminal BRCT Domain or the C-Terminal Domain of DNA Polymerase λ. To confirm the kinetic parameters precisely, the parameters for the petasiphenol binding were determined using full-length pol λ, the N-terminal BRCT domain, and the C-terminal domain containing the pol β-like core of pol λ immobilized to the sensor chip in a BIAcore. Four different concentrations of petasiphenol (2.5, 5, 10, and 20 μM) were used for the binding analysis. Both the BRCT domain and the other catalytic domain (0.2 nmol each) were conjugated to the CM5 sensor chip, and then petasiphenol was added to the conjugated proteins. Petasiphenol bound to either of full-length pol λ or the BRCT domain at approximately 240 response units (RU) and dissociated from the protein (Figure 4A). The dissociation constants (K_d) of binding of petasiphenol to full-length pol λ and the BRCT domain were determined to be 8.15 and 7.99 μM from the data in Figure 4A, indicating that the petasiphenol-binding affinity of the full-length pol λ was almost the same with that of the BRCT domain. In contrast, petasiphenol did not bind to the C-terminal catalytic domain including the pol β-like core of pol λ at all (Figure 4B). These results indicated that petasiphenol binds to the BRCT domain directly and not to the pol β-like core of pol λ.

Homology Modeling of the BRCT Domain of DNA Polymerase λ. To confirm the above assumption, we performed a homology modeling analysis of the BRCT domain of pol λ. As described in the early part of this report, petasiphenol did not inhibit the activity of TdT which contains a BRCT domain, suggesting that the compound does

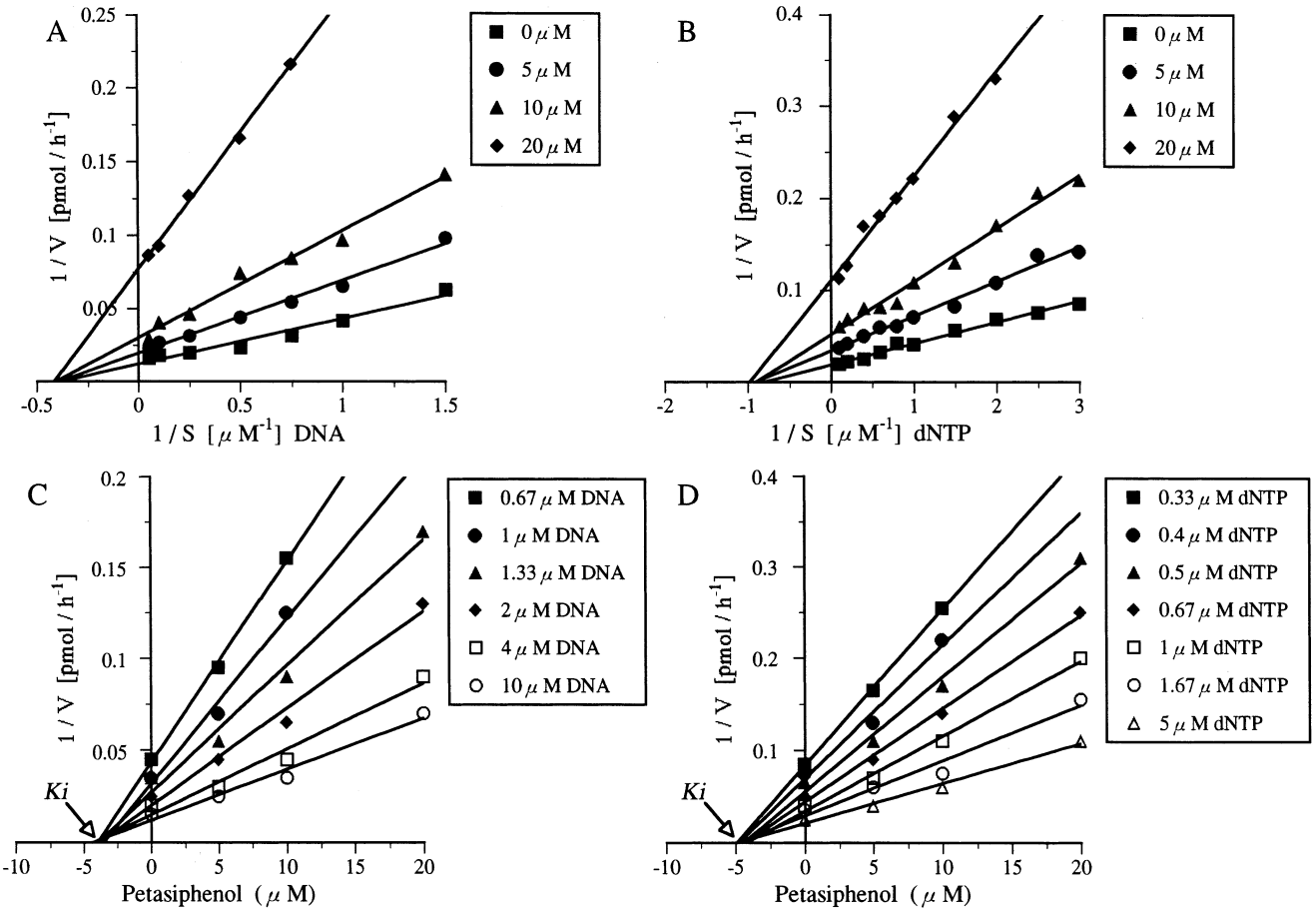

FIGURE 3: Kinetic analysis of the inhibition of pol λ by petasiphenol. (A) Pol λ activity was measured in the absence (■) or presence of 5 μM (●), 10 μM (▲), or 20 μM (◆) petasiphenol using the indicated concentrations of the DNA template-primer. (B) Pol λ activity was assayed with the indicated concentrations of the dNTP substrate in the presence of 5 μM (●), 10 μM (▲), or 20 μM (◆) petasiphenol or in the absence (□) of petasiphenol. (C, D) The inhibition constants (K_i) were determined as 3.8 and 4.5 μM from a Dixon plot made on the basis of the same data for (A) and (B), respectively. The amount of human pol λ in the assay mixture was 0.05 unit.

not recognize the BRCT domain structure of TdT. Therefore, the three-dimensional structure of the BRCT domain of pol λ with or without petasiphenol should be studied. At present, the BRCT domain structure of pol λ has not been determined by X-ray crystal or NMR analysis, but instead of that, the three-dimensional structures of the BRCT domains including human XRCC1 [Protein Data Bank (PDB) accession code: 1CDZ] (*14*), bacterial NAD^+-dependent DNA ligase (PDB accession code: 1DGS) (*29*), and human DNA ligase IIIa (PDB accession code: 1IMO) (*30*) are available. The sequence of the BRCT domain of pol λ with 97 amino acids was retrieved from the data bank in the National Center for Biotechnology Information (NCBI). The DNA sequence of the BRCT domain in human XRCC1 was used in this experiment, because it was the most similar to that of human pol λ in the three proteins. From pairwise sequence alignments, the percent identity is 13% between the BRCT domains of human pol λ and human XRCC1 (Figure 5). The multiple sequence alignment of the template was obtained from the CD search (NCBI) which compares a protein sequence against the conserved domain database with the RPS-BLAST program. The calculated three-dimensional structure of the protein is shown in Figure 6A.

Docking Simulation of DNA Polymerase λ and Petasiphenol. According to the homology modeling of the BRCT domain described above, the three-dimensional binding structure between the BRCT domain of human pol λ and petasiphenol was also studied. The N-terminal BRCT domain of pol λ (residues 36−132) was assumed to form three α-helices and four β-sheets [Figure 6A (a)]. The three-dimensional position of the α-helices in the BRCT domain of pol λ was different from that of human XRCC1, and the three-dimensional position of the β-sheets in the BRCT domain of pol λ was the same as that of human XRCC1 (Figure 1A). The petasiphenol-binding site of the modeled BRCT domain of pol λ was refined to use software Insight II/Binding Site Analysis (Accelrys Inc.). A cavity having a space (i.e., the grid size, site of open size, and site of cutoff size were 0.8, 8, and 20 Å, respectively) that a petasiphenol molecule can bind was searched for on the surface of the protein. The number of final docking positions was set to 5, although only one promising position was finally identified. Petasiphenol did not inhibit the activity of terminal deoxynucleotidyl transferase (TdT), which has a BRCT domain (Table 1). The BRCT domain sequence of pol λ was compared with that of TdT, and the conserved sequence region between pol λ and TdT was estimated. The cavity farthest away from the conserved region was determined as the petasiphenol-binding site. As a result, the petasiphenol-binding region in the BRCT domain of pol λ is assumed to consist of the two loops (residues 74−81 and 84−107) between the β-sheet (residues 82−83).

As shown in Figure 6C (a), petasiphenol could be mapped to one face of the BRCT domain of pol λ. The loops was

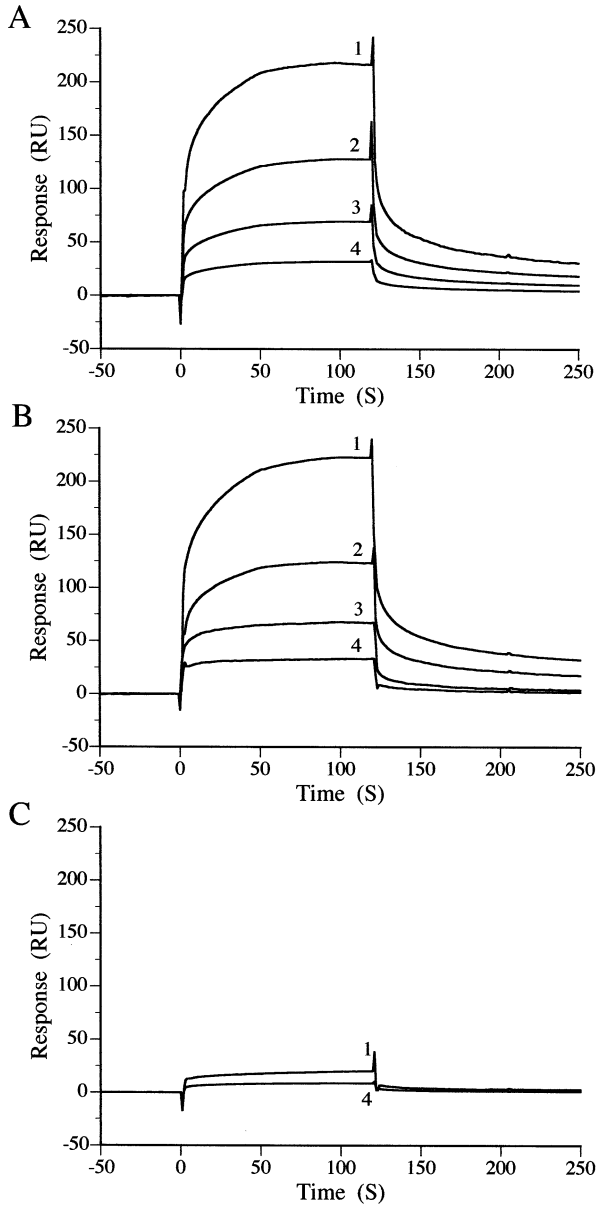

FIGURE 4: BIAcore analysis of binding of petasiphenol to immobilize the N-terminal BRCT domain and the C-terminal domain of pol λ: (A) full-length pol λ (residues 1−575, 63.4 kDa); (B) the N-terminal BRCT domain of pol λ (residues 36−132, 12 kDa); (C) pol λ except for the BRCT domain (residues 133−575, 47.4 kDa). Binding to petasiphenol was detected by the surface plasmon resonance signal (BIAcore; see Materials and Methods) and is indicated in response units. Four different concentrations of petasiphenol (curve 1, 20 μM; curve 2, 10 μM; curve 3, 5 μM; curve 4, 2.5 μM) were injected over the proteins of pol λ for 120 s at 20 μL/min and dissociated for 130 s at 20 μL/min. The background resulting from injection of running buffer alone was subtracted from the data before plotting.

significantly moved following the binding of petasiphenol by the flexible docking procedure in the Affinity program within the Insight II modeling software. In this domain, Gln76, Arg93, and Arg99, which are hydrophilic amino acids, were significantly moved. The hydroxyl groups (−OH) and ketone groups (−C═O) of petasiphenol may, therefore, show a preference for binding to the hydrophilic residue of Gln76, Arg93, and Arg99, and on the other side, the benzene groups may be absorbed to the hydrophobic amino acids in the loops (Figure 6B,C). The petasiphenol-interacted amino acid residues and their binding energies are indicated in Table 3. In the energy-minimized docking simulation, the binding energies between NH_2 of Gln76, NH_2^+ of Arg93, or NH_2^+ of Arg99 and the hydrophilic groups in petasiphenol were −9.400, −3.652, or −4.642 kcal/mol by hydrogen bond, respectively, and the binding force consisted of coulomb force (−7.904, −2.220, or −3.186 kcal/mol, respectively) and van der Waals forces (−1.496, −1.432, or −1.656 kcal/mol, respectively) (Table 3). The distances between the three hydroxyl groups of petasiphenol and the hydrophilic residues of Gln76, Arg93, and Arg99 were 1.69, 1.79, and 2.00−2.04 Å, respectively (Figure 6C). The binding energy between the other hydrophilic amino acids (i.e., Asp90, Glu92, Ala94, Arg96, Gln102, and Pro104) and petasiphenol was −11.284 kcal/mol, and the binding energy between the benzene backbone of petasiphenol and the hydrophobic amino acids (i.e., Ile83, Leu95, Leu98, Leu100, and Leu103) was −15.342 kcal/mol (Table 3). The Connolly surface of the loops and the three-dimensional position of petasiphenol is indicated in Figure 6D. On the BRCT domain of pol λ, petasiphenol was smoothly intercalated into the pocket of the loops, and the residues around the amino acid site consisting of hydrogen bonds (i.e., Gln76, Arg93, and Arg99) appear to be important for petasiphenol binding.

DISCUSSION

As described in this report, we found a potent inhibitor specific to DNA polymerase λ (pol λ) from a Japanese vegetable, *P. japonicus*, and characterized its biochemical properties. This is the first report of the finding and characterization of a pol λ-specific inhibitor in mammalian DNA polymerases. The natural compound was found to be petasiphenol, a bio-antimutagen isolated from the same plant, *P. japonicus*, by Iriye et al. (*11*). Petasiphenol could inhibit only the activity of DNA polymerase λ (pol λ) in the range of 5−50 μM. Since the polymerase species specificity was extremely high, petasiphenol could be useful as a pol λ-specific inhibitor in studies to determine the precise roles of pol λ. Petasiphenol must inhibit the pol λ activity indirectly by acting at the BRCT domain side in pol λ. It is possible that petasiphenol might inhibit other cellular proteins containing the BRCT domain, for example, terminal deoxynucleotidyl transferase (TdT). Petasiphenol, however, did not influence the TdT activity at all (Table 1). Petasiphenol could not recognize the BRCT domain structure of TdT, suggesting that the three-dimensional structure of the BRCT domain of pol λ is different from that of TdT, and subsequently, petasiphenol could selectively inhibit the pol λ activity.

Although the biochemical function of pol λ is unclear as yet, pol λ appears to work in a manner similar to that of pol β (*31*). Pol β, which is widely known to have roles in the short-patch base excision repair (BER) pathway (*31*−*36*), plays an essential role in the neural development (*37*). Recently, pol λ was found to contain 5′-deoxyribose 5-phosphate (dRP) lyase activity but no apurinic/apyrimidinic (AP) lyase activity (*35*) and to be able to substitute pol β in in vitro base excision repair (BER), suggesting that pol λ also participates in BER. Northern blot analysis indicated that the transcripts of pol β were abundantly expressed in the testis, thymus, and brain in rats (*38*), but pol λ was efficiently transcribed mostly in the testis (*12*). The reason the testis and thymus require the pol β activity has been suggested;

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                 ....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ... .|....| ....|....| ....|....| ..
                36   40        50        60        70        80        90        100       110       120       130
Pol lambda      EAEEW LSSLRAHVVR TGIGRARAEL FEKQIVQHGG QLCPAQGPGV THIVVDEGMD YERALRLLRL PQ-LPPGAQLV KSAWLSLCLQ ERRLVDVAGF SI
XRCC1           ELPDF FQGKHFFLYG EFPGDERRKL -IRYVTAFNG ELEDYMSDRV QFVITAQEWD PS-----FEE ALMDNPSLAFV RPRWIYSCNE KQKLLPHQLY GV
                *                 * * *         * *       *         *                 *   *   *  *     *
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FIGURE 5: The BRCT domain amino acid sequence alignments between human DNA polymerase λ and human XRCC1.

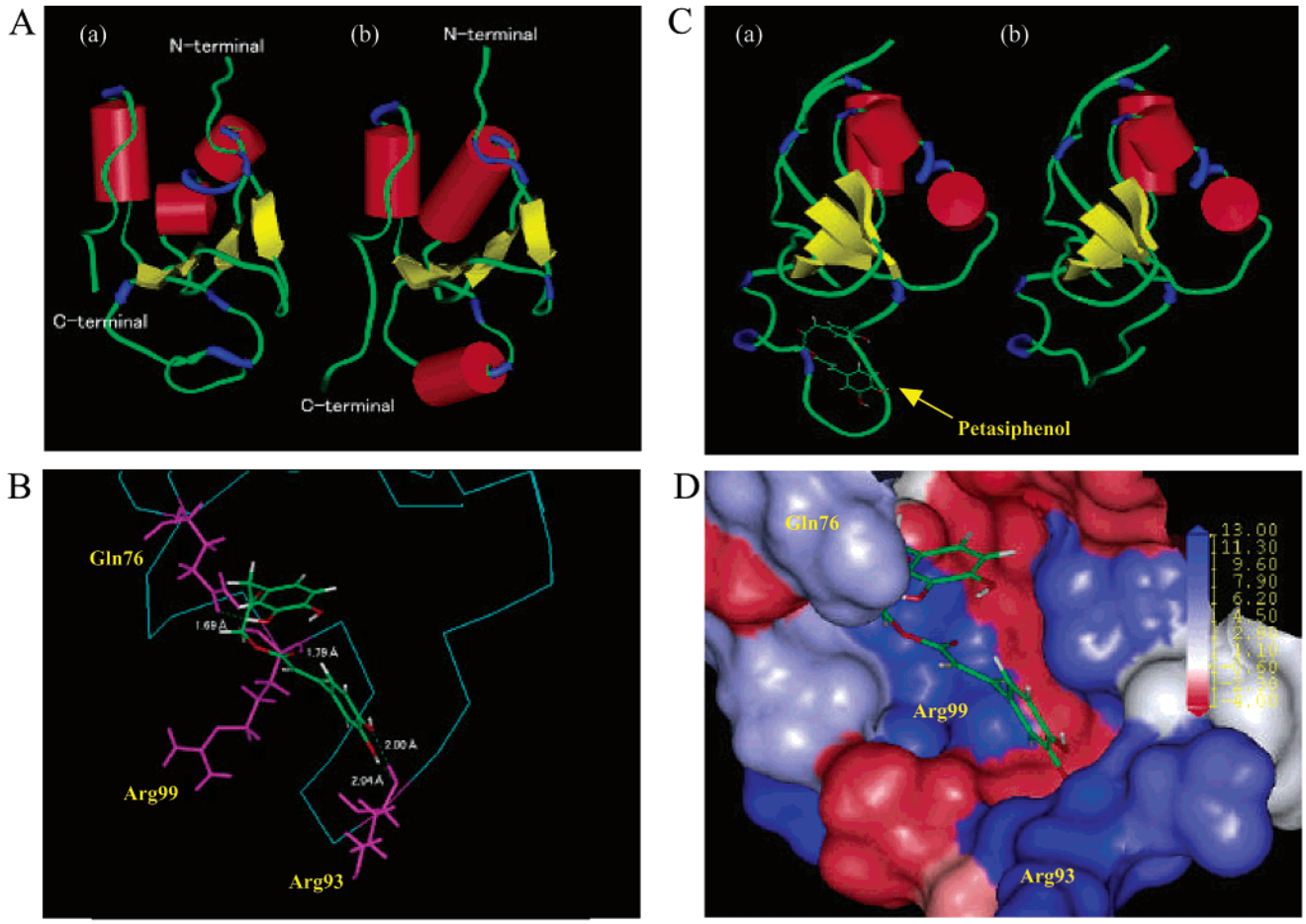

FIGURE 6: Docking simulation of the petasiphenol interaction interface on the N-terminal BRCT domain of human pol λ. (A) The homology-modeled structure of the BRCT domain (97 amino acids, residues 36−132) of human pol λ (a) and the X-ray crystallized structure of the BRCT domain of human XRCC1 (b). (B) Interaction between petasiphenol and the BRCT domain of pol λ. (C) Flexible docking simulation of the BRCT domain of pol λ with (a) or without (b) petasiphenol. (D) Connolly surface and hydrophobicity (i.e., blue is high and red is low) of the two loops (residues 74−81 and 84−107) between three β-sheets (residues 71−73, 82−83, and 108−109) in the BRCT domain of pol λ. The three-dimensional structure of the N-terminal BRCT domain of human pol λ consists of three helices, helix 1 (residues 55−58), helix 2 (residues 64−67), and helix 3 (residues 113−120), and four β-sheets (consisting of residues 45−47, 71−73, 82−83, and 108−109). The remainder of the domain consists of six turns (residues 48−54, 59−63, 68−70, 74−81, 84−107, and 110−112) and extended structures. The Cα backbone of the structure of pol λ is shown in green. The carbons, oxygens, and hydrogens of the structure of petasiphenol are indicated in green, red, and white, respectively. The Protein Data Bank accession code for the BRCT domain of human XRCC1 is 1CDZ. This figure was prepared using Insight II/Affinity (Accelrys Inc.).

both organs have DNA repair and recombination systems for meiotic crossing over and immunoglobulin production (*39*, *40*), and the systems require the polymerase. The roles of pol β in the brain are unknown as yet. Therefore, pol λ as well as pol β may also have a role in the testis. Since the DNA repair system at meiotic prophase requires the pol β activity, the system must contain a process similar to BER. The system may also require the pol λ activity, and pol λ may be an essential enzyme for nucleotide excision repair (NER). In this connection, the fact that the molecular target of the bio-antimutagen was a pol λ inhibitor is of great interest. The bio-antimutagen may lead to blockage of the mismatch error in BER, NER, and translesion synthesis of DNA-damaged cells. If so, pol λ might have a much lower fidelity in the DNA synthesis than pol β. However, speculation concerning the biochemistry, the structure, and the function of pol λ should be done later, because not only the crystal and solution structure of pol λ but also the in vivo function is mostly unknown. To determine why a bio-antimutagen is a pol λ-specific inhibitor, we are at present analyzing the structure and function of pol λ using an inhibitor.

We recently found that solanapyrone A, a plant phytotoxin, isolated from the causal fungus of early potato blight, *Alternaria solani*, is also a potent and selective inhibitor of both pol β and λ (*10*). Both polymerases also belong to the pol X family. Another pol X family enzyme is a terminal deoxynucleotidyl transferase (TdT), but solanapyrone A did not influence the TdT activity. Solanapyrone A appeared to inhibit the polymerases by acting with a common part in the catalytic core side of both pol β and λ, which is not present in TdT. In pol β, the catalytic core domain is in the

Table 3: Binding Energy of Petasiphenol and DNA Polymerase λ[a]

petasiphenol interacting amino acid	energy (kcal/mol)		
	coulomb	van der Waals	total
Gln76	−7.904	−1.496	−9.400
Ile83	−0.012	−1.754	−1.766
Asp90	1.092	−5.320	−4.228
Glu92	1.176	−2.458	−1.282
Arg93	−2.220	−1.432	−3.652
Ala94	−0.084	−0.784	−0.868
Leu95	0.944	−3.532	−2.588
Arg96	0.882	−2.548	−1.666
Leu98	−2.326	−5.736	−8.062
Arg99	−3.186	−1.656	−4.842
Leu100	−0.218	−1.092	−1.310
Gln102	0.036	−2.146	−2.110
Leu103	0.306	−1.922	−1.616
Pro104	1.084	−2.216	−1.130

[a] All amino acids in the BRCT domain of human pol λ which could interact with petasiphenol are indicated. The binding energy was calculated by the flexible docking procedure in the Affinity program within the Insight II modeling software.

31 kDa domain, but not in the 8 kDa domain, which is the DNA template-binding domain (*41*, *42*). Solanapyrone A was found to directly bind to the 8 kDa domain of pol β but not to the 31 kDa domain. Solanapyrone A could be mapped to one face of the 8 kDa domain of pol β by the same docking simulation analysis (*10*). In the docking simulation, solanapyrone A was smoothly intercalated into the pocket between helix 3 and helix 4 on the 8 kDa domain, and the residues around the Lys60 site appear to be important for solanapyrone A binding (*10*). The action mode of solanapyrone A in pol λ is expected to be quite similar to that in pol β. On the other hand, petasiphenol acted only to the BRCT domain. In the BRCT domain of pol λ, we could determine the movement following the binding of petasiphenol by the flexible docking procedure in the Affinity program within the Insight II modeling software (Figure 6). On the BRCT domain of pol λ, petasiphenol must smoothly be intercalated into the pocket of the two loops (i.e., residues 74−81 and 84−107). Solanapyrone A may also bind to a pocket with the Lys site between two helices around the DNA template-binding domain of pol λ, because the pol β-like core region of pol λ has a high amino acid identity to pol β. Therefore, by making proper use of both of the agents, petasiphenol and solanapyrone A, the analysis of the crystal and solution structures of both pol β and pol λ will be able to progress.

As described above, another purpose of this study was to screen a useful agent for analyzing the in vivo functions of pol β and pol λ in pol β- and pol λ-rich tissues. We could finally report the properties of petasiphenol with regard to its effect on pol λ. Although pol β was efficiently transcribed in the testis, the thymus, and the brain, pol λ was mainly in the testis (*12*). Since solanapyrone A could inhibit only the activities of both pol β and pol λ, and since petasiphenol could distinguish between pol β and pol λ, petasiphenol and solanapyrone A would be useful for analyzing the function of pol β and pol λ in the testis, especially at the point where homologous chromosomes pair and recombine at meiotic prophase. Higher plants have not pol β but pol λ (Y. Uchiyama et al., in preparation). Moreover, the activity of the plant pol λ is present only in the cells at meiotic prophase (Y. Uchiyama et al. in preparation). These natural compounds may, therefore, act on the plant reproduction system.

Moreover, as speculated, petasiphenol may lead to blockage of the mismatch error in DNA repair synthesis to rescue the cells containing the damaged DNA under clinical radiation therapy or chemotherapy. Petasiphenol could be a useful molecular tool to develop a drug design strategy for cancer chemotherapy agents which help clinical radiation therapy or cancer chemotherapy. We are trying to synthesize both petasiphenol and solanapyrone A chemically for further studies.

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