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Some anti-chronic inflammatory compounds are DNA polymerase λ-specific inhibitors

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Abstract

We previously reported that a phenolic compound, petasiphenol, was a selective inhibitor of DNA polymerase λ (pol λ) *in vitro* [Biochemistry 41 (2002) 14463]. We found here that another phenolic compound, curcumin (diferuloylmethane), which is known as an anti-chronic inflammatory agent and is structurally quite similar to petasiphenol, was also a potent pol λ inhibitor. The IC_{50} values of petasiphenol and curcumin were 7.8 and 7.0 μM, respectively. Curcumin, as well as petasiphenol, did not influence the activities of replicative DNA polymerases, such as α, γ, δ, and ε, but also showed no effect even on the pol β activity belonging to the X family. Curcumin could prevent the growth of human NUGC-3 cancer cells with LD_{50} values of 13 μM, and halted them at the G2/M phase in the cell cycle, whereas petasiphenol suppressed the cell growth at 66 μM and arrested the cells at the G1 phase. These data showed that curcumin and petasiphenol were slightly different functionally. We also previously reported that novel anti-inflammatory terpeno benzoic acids and triterpenoids were inhibitors of mammalian DNA polymerases [Biochem. Biophys. Acta 1475 (2000) 1; Biochem. Biophys. Acta 1596 (2002) 193]. They could also efficiently inhibit the pol λ activity, although they influenced the other polymerase species to the same extent, suggesting that there may be a physiological relationship between pol λ inhibition and anti-12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation. Expectedly, petasiphenol also showed an anti-12-*O*-tetradecanoylphorbol-13-acetate-induced inflammatory effect in mice. This finding may provide clues to investigating the molecular mechanism of inflammation.

Keywords: Petasiphenol; Curcumin; DNA polymerase λ; Enzyme inhibitor; Anti-inflammation

1. Introduction

Eukaryotic cells reportedly contain three replicative DNA polymerases (pol α, δ, and ε), mitochondrial DNA polymerase (pol γ), and at least 13 repair types of DNA polymerases (pol β, δ, ε, ζ, η, θ, κ, λ, μ, σ, ϕ, pol 1-like I, and pol 1-like II) [1,2]. We searched for natural compounds that selectively inhibit each of these eukaryotic DNA polymerases, and then reported on an interesting compound that selectively inhibits only the pol λ activity [3]. The natural compound was a phenolic compound, petasiphenol, produced from a higher plant, a Japanese vegetable (*Petasites japonicus*), and the selectivity to pol λ was extremely high. Then, while looking through the

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Abbreviations: pol, DNA polymerase (E.C. 2.7.7.7); dTTP, 2′-deoxythymidine 5′-triphosphate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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Sigma Reagent Catalog, we found that a phenolic compound, curcumin (diferuloylmethane), which is known as an anti-chronic inflammatory agent and an anti-oxidative compound, is structurally similar to petasiphenol. The compound, curcumin, was a yellow substance from the root of the plant *Curcuma longa* Linn. Expectedly, and in a sense, curcumin was also a potent pol λ-selective inhibitor.

Pol λ is a recently described eukaryotic DNA polymerase belonging to the pol X family [4] 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 region (residues 1–35), a BRCA1 C-terminus (BRCT) domain (residues 36–132), a proline–serine-rich region (residues 133–243), and a pol β-like core region (residues 244–575). 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 [5]. The pol X family also contains terminal deoxynucleotidyl transferase (TdT) and pol μ [1], both having a BRCT domain. Nevertheless, the phenolic compounds could not inhibit the activities of pol β and TdT, although the effect on pol μ has not been tested yet. As described previously [3], petasiphenol did not inhibit the activity of the truncated domain, including the pol β-like core, in which the BRCT motif was deleted in its N-terminal region. We therefore concluded that petasiphenol inhibited the pol λ activity indirectly by acting at the BRCT domain side in pol λ, and could not recognize the BRCT domain structure of TdT, suggesting that the three-dimensional structure of the BRCT domain of pol λ differs from that of TdT [3].

Aside from the phenolic compounds, we also reported previously that novel anti-12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammatory natural compounds, a novel terpeno benzoic acid (i.e. myrsinoic acid A (MAA)) and novel triterpenoids (i.e. tormentic acid (TA) and euscaphic acid (EA)), were inhibitors of mammalian pol α and pol β [6,7]. Although tumor promoters are compounds that promote tumor formation [8], these compounds were well known to also cause inflammation and were generally used as artificial inflammation inducers for the screening of anti-inflammatory agents [9]. Tumor promoter-induced inflammation could be distinguished from acute inflammation, which is exudative and is accompanied by fibroblast proliferation and granulation. To search for new types of anti-inflammatory compounds, we used TPA. TPA could not only cause inflammation, but influences cell proliferation and has physiological effects on cells [10]. Therefore, the new anti-inflammatory agents were expected to suppress mammalian cell proliferation related to the action of TPA, and the major mode of action must be related to this suppressive effect. Since curcumin was a typical anti-TPA-induced inflammatory agent and a pol λ inhibitor, we also tested if terpeno benzoic acids and triterpenoids could influence the pol λ activity. They could inhibit the pol λ activity, although to the same extent as their inhibitory effects on pol α and pol β. The purpose of this report is to elucidate a newly found pol λ-specific inhibitor and its inhibitory effect on TPA-induced inflammation.

2. Materials and methods

2.1. Materials

Petasiphenol was purified from a Japanese vegetable, *P. japonicus*, as described previously [3]. Curcumin was purchased from Sigma. Petasiphenol and curcumin are curcuminoids which are structurally related compounds, and these compounds comprise phenolic yellowish pigments. An anti-inflammatory terpeno benzoic acid, MAA (5-geranyl-4-hydroxy-5-(3′-methyl-2′-butenyl)-benzoic acid), was purified from the methanolic extract of *Myrsine seguinii* as described previously [6], and anti-inflammatory triterpenoids, TA and EA, were purified from the fresh leaves of *Rubus sieboldii* as described previously [7]. The chemical structures of these compounds are shown in Fig. 1. Nucleotides and chemically synthesized template-primers, such as poly(dA), oligo(dT)$_{12–18}$, and [^{3}H]2′-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mM) were purchased from Amersham Pharmacia Biotech. TPA was purchased from Sigma. All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries.

2.2. Enzymes

DNA polymerase α (pol α) was purified from calf thymus by immuno-affinity column chromatography [11]. Recombinant rat pol β and human pol λ were purified from *Escherichia coli* as described by Date *et al*. [12] and Shimazaki *et al*. [13], respectively. The human pol γ catalytic gene was cloned into pFastBac. Histidine-tagged enzyme was expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier' manual (LIFE TECHNOLOGIES) and purified using ProBond resin (Invitrogen Japan). pol δ and pol ε were purified from calf thymus [14] and HeLa cells [15], respectively, as described previously. pol I (α-like) and pol II (β-like) from a higher plant, cauliflower inflorescence, were purified according to the methods outlined by Sakaguchi *et al*. [16]. The Klenow fragment of pol I and human immunodeficiency virus type-1 (HIV-1) reverse transcriptase were purchased from Worthington Biochemical Corp. T7 RNA polymerase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems. Taq DNA polymerase, T4 DNA

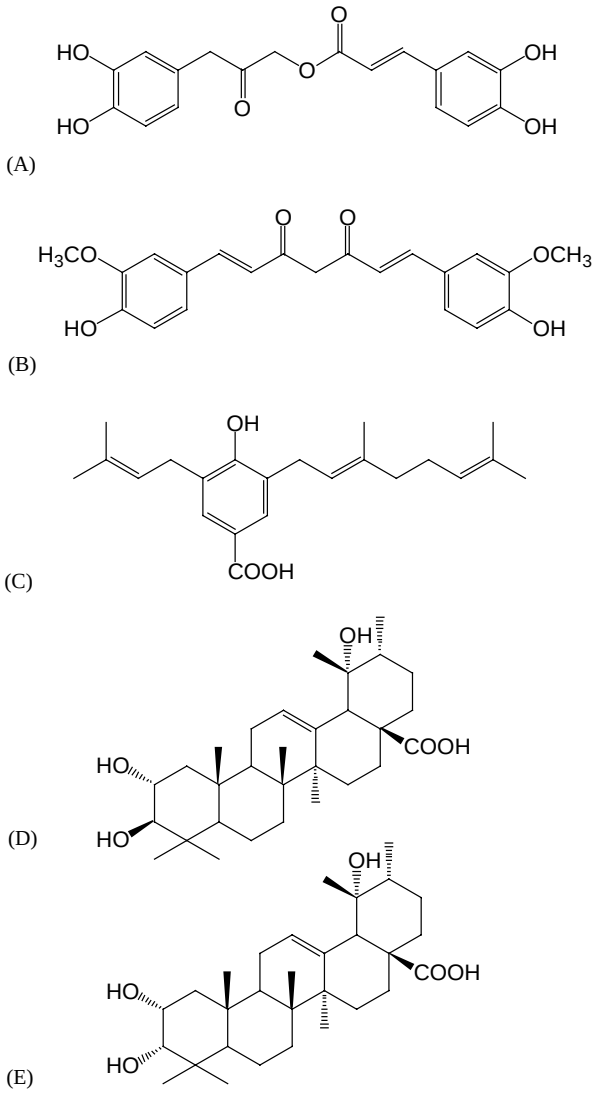

Fig. 1. Chemical structures of the compounds: (A) petasiphenol, (B) curcumin, (C) myrsinoic acid A (MAA, 5-geranyl-4-hydroxy-5-(3′-methyl-2′-butenyl)-benzoic acid), (D) tormentic acid (TA), (E) euscaphic acid (EA).

polymerase, and T4 polynucleotide kinase were purchased from Takara.

2.3. DNA polymerase assays

The reaction mixtures for pol α, pol β, plant and prokaryotic DNA polymerases were described previously [17,18], and those for pol γ, δ and ε were as described by Ogawa *et al*. [19]. The reaction mixture for pol λ was the same as that for 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 phenolic compounds were dissolved in 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 U) in 50 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol, and 0.1 mM EDTA, and kept at 0° 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° for 60 min, except for Taq DNA polymerase which was incubated at 74° 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 deoxyribonucleoside 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° under the normal reaction conditions for each enzyme [17,18].

2.4. Other enzyme assays

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*. [20], Nakayama and Saneyoshi [21], Soltis and Uhlenbeck [22], and Lu and Sakaguchi [23], respectively.

2.5. Investigation of cytotoxicity on cultured cells

For investigation of the *in vivo* effects of the phenolic compounds, a human gastric cancer cell line, NUGC-3, a human B cell acute lymphoblastoid leukemia cell line, BALL-1, and a human T cell acute lymphoblastic leukemia cell line, MOLT-4, were obtained from Health Science Research Bank. The cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 units/mL penicillin, and 1.6 mg/mL $NaHCO_3$. The cells were routinely cultured at 37° in standard medium in a humidified atmosphere of 5% CO_2/95% air. The cytotoxicity of the compounds was investigated as follows: High concentrations (10 mM) of the compounds were dissolved in DMSO and stocked. Approximately 5×10^3 cells per well were inoculated in 96-well microplates, then the compound stock solution was diluted to various concentrations, and applied to each well. After incubation for 48 hr, the survival rate was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [24].

2.6. Cell cycle analysis

The cellular DNA content for cell cycle analysis was determined as follows: Aliquots of 3×10^5 NUGC-3 cells were inoculated into a 35-mm dish, and incubated with medium containing the phenolic compounds for 48 hr.

Then, the cells were collected by trypsinization and washed with cold PBS three times by centrifugation. The cells were fixed with 10 mL of PBS containing 70% (v/v) ethanol, and stored at 4°. DNA was stained with DAPI staining solution for at least 10 min at room temperature in the dark. The fluorescence intensity of the 8000 stained cells was measured by flow cytometry (Partec cell counter analyzer, CCA model). The cell cycle distribution was analyzed with the MULTICYCLE software program (version 3.11; Phoenix Flow Systems).

2.7. Anti-inflammatory assay

Mouse inflammatory test was performed according to the Gschwendt' method [25]. This experiment complied with the regulations concerning animal experimentation and the care of experimental animals of the Faculty of Agriculture, Shinshu University. Briefly, a methanol solution of the test compound (500 μg/20 μL) was applied to the inner part of the mouse ear. Thirty minutes after applying the test compound, a TPA solution (0.5 μg/20 μL acetone) was applied to the same part of the ear. To the other ear of the same mouse, methanol and a TPA solution were applied as a control. After 7 hr, a disk (6 mm diameter) was obtained from the ear and weighed. The inhibitory effect (IE) is presented as the ratio of the weight increase of the ear disks: IE = [(TPA only) − (tested compound plus TPA)]/[(TPA only) − (vehicle)] × 100.

3. Results

3.1. Effect of phenolic compounds on the activities of DNA polymerases and other DNA metabolic enzymes

As described previously, a phenolic compound, petasiphenol (Fig. 1A), was a potent inhibitor selective to pol λ [3]. The agent, not a commercial chemical, was purified from a higher plant, a Japanese vegetable (*P. japonicus*). This means mass production of petasiphenol is very difficult. On the other hand, we also described the three-dimensional relationship between DNA polymerase inhibitors and DNA polymerase structure [3,26,27]. Curcumin (diferuloylmethane) (Fig. 1B), a commercial chemical, is the same phenolic compound as petasiphenol, and it is known as an anti-chronic inflammatory agent and an anti-oxidative compound which could potently and selectively inhibit human pol λ activity (Table 1). Figure 2 shows the inhibition dose–response curves of these phenolic compounds against human pol λ. Petasiphenol and curcumin were effective at inhibiting the pol λ activity, and the inhibition was dose-dependent, with 50% inhibition observed at doses of 7.8 and 7.0 μM, respectively (Fig. 2). These compounds had no influence at all on the activities of not only replicative DNA polymerases, such as calf pol α, calf pol δ, and human pol ε, mitochondrial DNA polymerase, such as pol γ, but also repair-related DNA polymerase, such as rat pol β (Table 1). The

Table 1
IC_{50} values of petasiphenol, curcumin, and anti-inflammatory compounds on the activities of various DNA polymerases and other DNA metabolic enzymes

Enzyme	IC_{50} value (μM)				
	Petasiphenol	Curcumin	Myrsinoic acid A	Tormentic acid	Euscaphic acid
Mammalian DNA polymerases					
Calf DNA polymerase α	>100	>100	22 ± 1.5	37 ± 2.1	61 ± 2.6
Rat DNA polymerase β	>100	>100	11 ± 1.0	46 ± 2.5	98 ± 3.0
Calf DNA polymerase γ	>100	>100	25 ± 2.0	40 ± 2.3	72 ± 2.8
Calf DNA polymerase δ	>100	>100	24 ± 1.8	34 ± 2.0	52 ± 2.5
Human DNA polymerase ε	>100	>100	18 ± 1.5	28 ± 1.8	47 ± 2.1
Human DNA polymerase λ	7.8 ± 1.2	7.0 ± 1.0	9.5 ± 1.0	21 ± 2.0	35 ± 1.9
Plant DNA polymerases					
Cauliflower DNA polymerase I (α-like)	>100	>100	>100	>100	>100
Cauliflower DNA polymerase II (β-like)	>100	>100	>100	>100	>100
Prokaryotic DNA polymerases					
Escherichia coli DNA polymerase I (Klenow fragment)	>100	>100	>100	>100	>100
Taq DNA polymerase	>100	>100	>100	>100	>100
T4 DNA polymerase	>100	>100	>100	>100	>100
Other DNA metabolic enzymes					
Calf DNA primase of DNA polymerase α	>100	>100	>100	>100	>100
Calf terminal deoxynucleotidyl transferase	>100	>100	>100	>100	>100
HIV-1 reverse transcriptase	>100	>100	>100	>100	>100
T7 RNA polymerase	>100	>100	>100	>100	>100
T4 polynucleotide kinase	>100	>100	>100	>100	>100
Bovine deoxyribonuclease I	>100	>100	>100	>100	>100

These compounds were incubated with each enzyme (0.05 U). The enzymatic activity was measured as described in Section 2. Enzyme activity in the absence of the compound was taken as 100%.

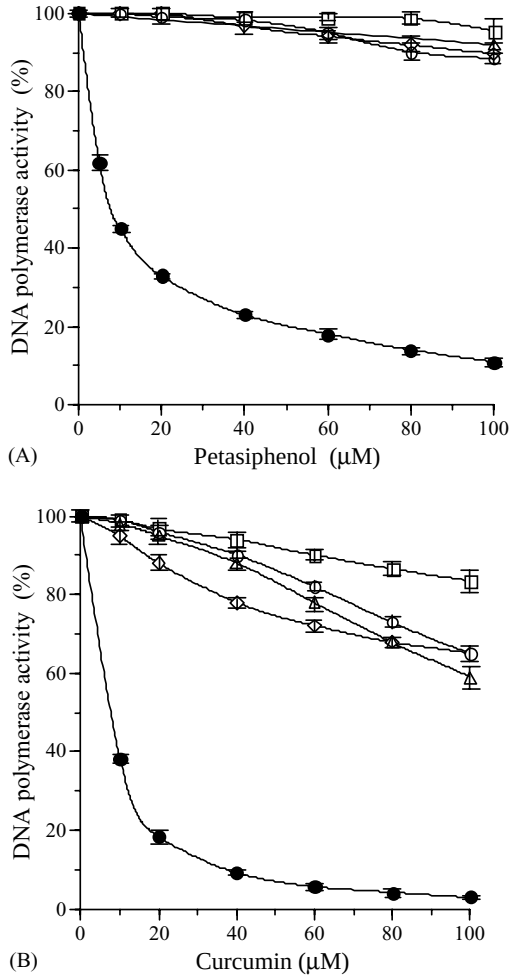

Fig. 2. Inhibition of mammalian DNA polymerase activities by phenolic compounds. Calf pol α (open circle), rat pol β (open square), calf pol δ (open triangle), human pol ε (open diamond), and human pol λ (closed circle) were preincubated with the indicated concentrations of petasiphenol (A) and curcumin (B). The enzymatic activity was measured as described in previous reports [17,18]. Enzyme activity (0.05 U) without the compound was taken as 100%. Data are shown as means ± SEM of three independent experiments.

IC_{50} values in Table 1 did not change when the DNA template-primer was activated DNA (data not shown). Both compounds had no inhibitory effect on higher plant cauliflower pol I (α-like) and pol 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 HIV-1 reverse transcriptase, T7 RNA polymerase, human DNA topoisomerase I and II, T4 polynucleotide kinase, and bovine deoxyribonuclease I (Table 1). Like petasiphenol, curcumin was also a selective pol λ inhibitor *in vitro*.

3.2. Mode of DNA polymerase λ inhibition by petasiphenol and curcumin

Next, to elucidate the mechanism of inhibition of phenolic compounds, such as petasiphenol and curcumin, on pol λ, the extent of inhibition as a function of DNA template-primer or dNTP substrate concentrations was studied (Table 2). 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 non-competitive with both the DNA template (K_m was unchanged at 2.46 μM) and the dNTP substrate (K_m was unchanged at 1.18 μM). 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. Similarly, curcumin inhibition of pol λ activity was non-competitive with the DNA template and dNTP substrate. In the case of the DNA template, the apparent K_m was unchanged at 2.46 μM, while its V_{max} decreased from 83.3 to 11.4 pmol/hr, in the presence of 20 μM curcumin. The K_m for the dNTP substrate was 1.18 μM, whereas a 6-fold decrease in V_{max} was observed in the presence of 20 μM curcumin. The K_i values, obtained from Dixon plots, were found to be 2.9 and 3.6 μM for the DNA template and dNTP substrate, respectively. Since the K_i values of curcumin were lower than those of petasiphenol, the inhibitory effect on pol λ activity by curcumin was stronger than that of petasiphenol, although the inhibition of pol λ by these phenolic compounds showed the same inhibitory mode. The inhibition by these compounds 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 these compounds exhibited the same inhibition mode as with the synthesized DNA template-primer (data not shown). These results suggested that these phenolic compounds did not directly bind to either the DNA template binding site or the dNTP substrate binding site of pol λ.

Pol λ has been recently identified as a new family member of pol β. Petasiphenol bound to the N-terminal region, including the BRCT domain of pol λ directly, and subsequently, inhibited the DNA polymerase activity of the pol β-like core of pol λ [4,13]. Petasiphenol did not only inhibit the activity of pol β (Table 1 and our previous report [3]), but also the activity of calf TdT, which is also an X family enzyme with a BRCT domain, suggesting that petasiphenol did not always recognize the BRCT domain structure [3]. Since the inhibition spectrum by curcumin in Tables 1 and 2 is quite similar to that by petasiphenol, the action of curcumin as an inhibitor might be almost the same.

3.3. Effect of anti-inflammatory activity

Aside from the pol λ-inhibitory effect of curcumin, curcumin was well known as an inhibitor of chronic inflammation using TPA, but the molecular action mode remained unclear [28]. TPA cannot only cause inflammation, but influences cell proliferation and has physiological

Table 2
Kinetic analysis of the inhibition by phenolic compounds on the activities of DNA polymerase λ, as a function of the DNA template-primer dose and the dNTP substrate concentration

Compound	Substrate	Compound concentration (μM)	K_m (μM)	V_{max} (pmol/hr)	K_i (μM)	Inhibitory mode
Petasiphenol	DNA template-primer[a]	0	2.46	83.3	3.8	Non-competitive
		5		52.6		
		10		33.9		
		20		13.2		
	dNTP substrate[b]	0	1.18	52.6	4.5	Non-competitive
		5		29.4		
		10		19.2		
		20		9.09		
Curcumin	DNA template-primer[a]	0	2.46	83.3	2.9	Non-competitive
		5		33.3		
		10		20.4		
		20		11.4		
	dNTP substrate[b]	0	1.18	52.6	3.6	Non-competitive
		5		24.4		
		10		15.2		
		20		8.70		

[a] poly(dA)/oligo(dT)$_{12–18}$ (=2/1).
[b] dTTP.

effects on cells, because TPA is a tumor promoter [10]. Therefore, the anti-inflammatory agents were expected to suppress mammalian cell proliferation related to the action of TPA. Since pol λ is a repair-related polymerase [29], the result that the molecular target of curcumin was pol λ is well-matched. If so, the pol λ inhibitor could be one of the inhibitors of chronic inflammation. Therefore, we tested whether petasiphenol, as well as curcumin, could also be an inhibitor of chronic inflammation using TPA.

Using the mouse inflammatory test described in Section 2, we examined the anti-inflammatory activities of these phenolic compounds. An application of TPA (0.5 μg) on the mouse ear induced edema, the weight increase of an ear disk 7 hr after application being 241%. As expected, curcumin completely inhibited inflammation at an applied dose of at least 200 μg (IE: 83%) (Table 3). Interestingly, pretreatment of petasiphenol (500 μg) also showed a remarkable reduction in TPA-induced inflammation (IE: 42%), indicating that petasiphenol possesses anti-inflammatory activity.

Table 3
Anti-inflammatory activities of petasiphenol and curcumin in the mouse ear inflammation test

Compound	Inhibitory effect (%) (±SE)	
	200 μg/ear	500 μg/ear
Petasiphenol	9 (±1.00)*	42 (±1.26)*
Curcumin	83 (±1.32)*	83 (±1.00)*

SE: shown in parentheses. A sample (200 or 500 μg) was applied on one mouse ear and, after 30 min, TPA (0.5 μg) was applied to both ears of the mouse. The edema was evaluated after 7 hr, inhibitory effect being expressed as the percentage ratio of the edema. Five mice were used for each experiment.
* Significantly different, $P < 0.05$, by the Student's t-test.

To confirm if there is a relationship between inhibition of pol λ and anti-chronic inflammation or not, we tested the effect of other anti-inflammatory compounds on pol λ. We reported previously that novel anti-inflammatory compounds, a terpeno benzoic acid, such as MAA (Fig. 1C) and triterpenoids, such as TA (Fig. 1D) and EA (Fig. 1E), were inhibitors of pol α and pol β [6,7]. We tested the inhibition spectrum of them more precisely this time. As

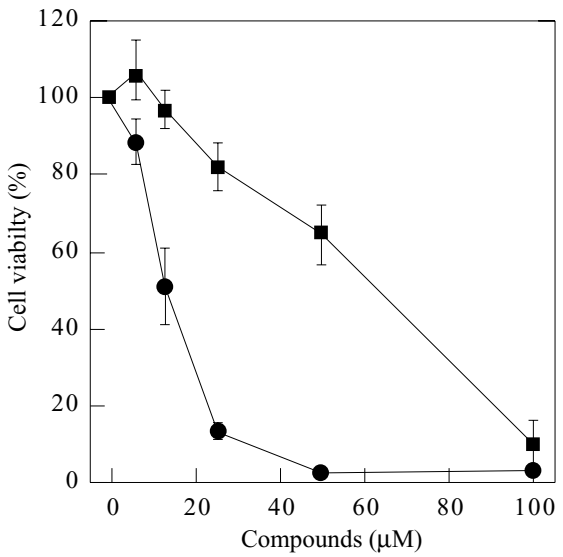

Fig. 3. Human stomach cancer cell growth inhibition by phenolic compounds. Dose–response curves of growth inhibition of the human stomach cancer cell line, NUGC-3, by petasiphenol (closed square) and curcumin (closed circle). The assays were carried out under the conditions described in Section 2 with the compounds at the indicated concentrations. The survival rate was determined by MTT assay [24]. Data are shown as means ± SEM of three independent experiments.

shown in Table 1, they could also inhibit the activity of pol λ, although they inhibited all the polymerases tested. The inhibition of pol λ by MAA, TA, or EA was the strongest. On the other hand, they did not influence the activities of plant and prokaryotic DNA polymerases and other DNA metabolic enzymes. These results led us to speculate that TPA-induced inflammation may contain a process requiring pol λ.

3.4. Cell growth inhibitory properties

Next, to test if the *in vivo* function of petasiphenol and curcumin is in the same molecular basis, we investigated their cytotoxicity and the effect on the cell cycle. As shown in Fig. 3, these phenolic compounds showed a potent growth inhibitory effect against the NUGC-3 human stomach cancer cell line. The concentrations of petasiphenol and curcumin required for the LD_{50} were 66 and 13 μM, respectively. We also tried flow cytometry analysis. The cell cycle distribution was analyzed with the MULTICYCLE software program (version 3.11; Phoenix Flow System). As shown in Fig. 4, the NUGC-3 cells were arrested in G1 phase (14.9% increase of G1 phase and 4.3% decrease of G2/M phase) by 66 μM petasiphenol with an incubation for 48 hr (Fig. 4). Petasiphenol was suggested to inhibit the cell growth by blocking the primary steps of DNA replication. On the other hand, the cells were arrested in G2/M phase (25.6% increase of G2/M phase and 0.4% decrease of G1 phase) by 13 μM curcumin with an incubation for 48 hr (Fig. 4). Curcumin has been reported as a cyclin-dependent kinases (CDKs) inhibitor with the arrest of G2/M phase [30], therefore, curcumin might be more influence the CDKs inhibition than pol λ inhibition. The effect was dependent on the incubation time (data not shown). HeLa (human carcinoma of cervix) cells were also halted at G1 phase and G2/M

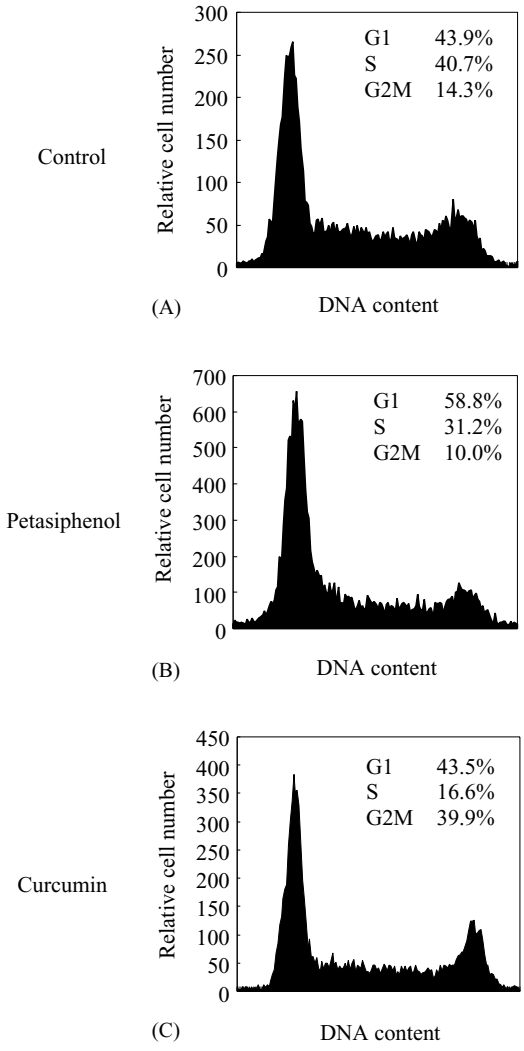

Fig. 4. Flow cytometric analysis of cell cycle perturbation by phenolic compounds. NUGC-3 cells were incubated without (control) (A), with 66 μM petasiphenol (B), or with 13 μM curcumin (C) for 48 hr. DNA was stained with DAPI solution. Fluorescence intensity of the 8000 stained cells was measured by flow cytometry. The cell cycle distribution was analyzed with the MULTICYCLE software program (version 3.11; Phoenix Flow Systems). Cell debris and fixation artifacts were gated out. All experiments were performed five times.

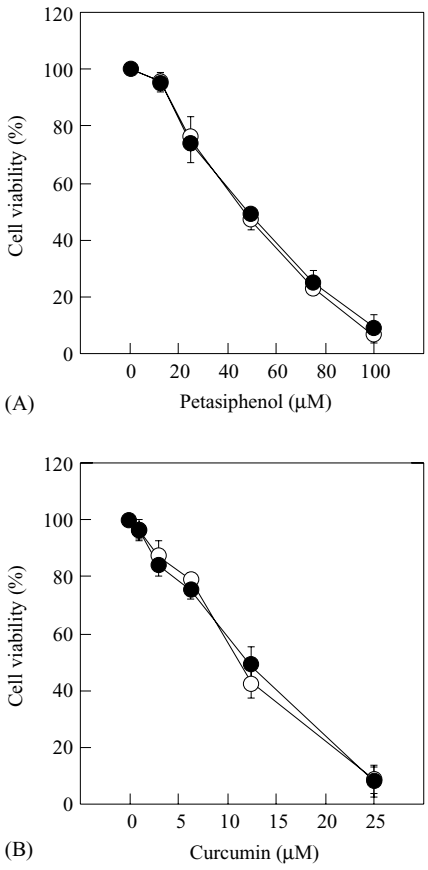

Fig. 5. Human immunological cell growth inhibition by phenolic compounds. Dose–response curves of growth inhibition of the human B cell acute lymphoblastoid leukemia cell line, BALL-1 (open circle), and the human T cell acute lymphoblastic leukemia cell line, MOLT-4 (closed circle), by petasiphenol (A) and curcumin (B). The assays were carried out under the conditions described in Section 2 with the compounds at the indicated concentrations. The survival rate was determined by MTT assay [24]. Data are shown as means ± SEM of three independent experiments.

phase in the cell cycle by petasiphenol and curcumin, respectively (data not shown). The cell growth of the human B cell acute lymphoblastoid leukemia cell line, BALL-1, and a human T cell acute lymphoblastic leukemia cell line, MOLT-4, was also inhibited by these phenolic compounds, indicating that they do not selectively inhibit either of the B cells or the T cells (Fig. 5). The LD_{50} values of petasiphenol and curcumin were approximately 48 and 12 μM, respectively (Fig. 5). These compounds could obviously inhibit the cells derived from lymphocytes as well as the gastric cells (NUGC-3 cells) (Figs. 3 and 5).

4. Discussion

We showed here that all five different types of novel anti-chronic inflammatory agent tested (i.e. terpeno benzoic acid, such as MAA [6], triterpenoids, such as TA and EA [7], petasiphenol [3], and curcumin (diferuloylmethane)) could inhibit the activity of pol λ in common, and especially, two phenolic compounds, petasiphenol and curcumin (diferuloylmethane), were pol λ-specific inhibitors. So far, only petasiphenol and curcumin, which have similar chemical structures, are known as pol λ-specific inhibitors. Interestingly, curcumin is known as an anti-chronic inflammatory agent and an anti-oxidative compound [28]. The fact that the major molecular target of curcumin was pol λ is of great interest.

Pol λ is a pol X family DNA polymerase [4]. The pol X family also contains TdT and pol μ [1]. Petasiphenol and curcumin could not inhibit the activities of pol β and TdT, although the effect on pol μ has not been tested yet. Although the biochemical function of pol λ is unclear as yet, pol λ appears to work in a similar manner to pol β [29]. pol β, which is widely known to have roles in the short-patch base excision repair (BER) pathway [31–34], plays an essential role in neural development [35]. Recently, pol λ was found to contain 5′-deoxyribose-5-phosphate (dRP) lyase activity, but no apurinic/apyrimidinic (AP) lyase activity [36] and to be able to substitute pol β in *in vitro* 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 [37], but pol λ was efficiently transcribed mostly in the testis [4]. Bertocci *et al*. reported that mice knocked pol λ down were not only viable and fertile, but also display a normal hypermutation pattern [38]. Therefore, at least pol λ hardly participates in the process, when it lacks, to be lethal.

Petasiphenol and curcumin showed a potent growth inhibitory effect against the NUGC-3 human stomach cancer cell line, the human B cell acute lymphoblastoid leukemia cell line, BALL-1, and the human T cell acute lymphoblastic leukemia cell line, MOLT-4. In the cell cycle analysis, petasiphenol arrested the cells at G1 phase, but the cells were arrested in G2/M phase by curcumin. Petasiphenol and curcumin inhibited the cell growth by blocking the primary and late steps of DNA replication, respectively. Although petasiphenol and curcumin seemed to contain the same molecular basis, their *in vivo* functions may be slightly different to each other. pol λ may have some roles in the cell cycle division, which is perhaps related to the fibroblast proliferation process. On the other hand, since mice knocked pol λ down were viable and fertile [38], the cell cycle effect of petasiphenol and curcumin may be cytostatic.

The other anti-TPA-induced inflammatory agents, MAA [6], TA, and EA [7], originally reported as inhibitors of pol α and β, could also potently inhibit the pol λ activity. MAA, TA, and EA could not inhibit plant and prokaryotic polymerase species and other DNA metabolic enzymes tested. They were not necessarily pol λ-specific inhibitors, but their inhibitory effects were strongest to pol λ.

Except petasiphenol, all these agents were originally found as anti-inflammatory agents to the inflammation caused using tumor promoters, such as TPA. As described in this report, petasiphenol also showed anti-inflammation activity (Table 3). Therefore, the major mode of biochemical and cell biological action must be related to the TPA-induced inflammation-suppressive effect. TPA is a compound that promotes tumor formation [8], and was generally used as an artificial inflammation inducer [9,10]. The TPA-induced inflammation could, therefore, be distinguished from acute inflammation, and is accompanied by fibroblast proliferation and granulation. On the other hand, curcumin could reportedly inhibit benzo(*a*)pyrene-induced DNA adduct formation, development of skin tumors and tumor promotion in mouse skin [39]. Dietary administration of curcumin during the initiation or post-initiation period is also known to inhibit tumorigenesis in the forestomach and intestine of mice [40] and tongue of rats [41]. Huang *et al*. indicated that curcumin not only reduces the number of tumors per mouse and the percentage of mice with tumors, but it also reduces tumor size in the forestomach and intestine [40]. Thus, curcumin is now regarded as a promising chemo-preventive agent for human cancers. These data were suggested that molecular basis of the so-called promotion process in oncogenesis also contain a biochemical process which requires pol λ.

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