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Diallyl sulfides: Selective inhibitors of family X DNA polymerases from garlic (*Allium sativum* L.)

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Abstract

Diallyl sulfides, organosulfur compounds isolated from garlic (*Allium sativum* L.), selectively inhibit the activities of mammalian family X DNA polymerases (pols), such as pol β, pol λ and terminal deoxynucleotidyl transferase (TdT), *in vitro*. The purified fraction (i.e., Sample-A) consisted of diallyl trisulfide, diallyl tetrasulfide and diallyl pentasulfide (molecular ratio: 5.3:3:1). Commercially purchased diallyl sulfides also inhibited the activities of family X pols, and the order of their effect was as follows: Sample-A > diallyl trisulfide > diallyl disulfide > diallyl monosulfide, suggesting that the number of sulfur atoms in the compounds might play an important structural role in enzyme inhibition. The suppression of human cancer cell (promyelocytic leukaemia cell line, HL-60) growth had the same tendency as the inhibition of pol X family among the compounds. Diallyl sulfides were suggested to bind to the pol β-like region of family X pols.

Keywords: Diallyl sulfides; Garlic; DNA polymerase β (pol β); DNA polymerase λ (pol λ); Terminal deoxynucleotidyl transferase (TdT); Family X of DNA polymerases (pol X); Enzyme inhibitor; Cytotoxicity; Pol β-like region

1. Introduction

Naturally-occurring micronutrients in food have been found to have chemopreventive effects, perhaps supporting in part the conclusions from epidemiologic studies that the consumption of fruits and vegetables reduces cancer risk (Ames, 1983; Wargovich, 1988a, 1988b; Wattenberg, 1983, 1985). Our research focused on extract of garlic (*Allium sativum* L.) compounds, because Egyptian records dating to about 1550 BC mention garlic as a remedy for a variety of diseases (Block, 1985), and because garlic has been demonstrated to possess antibiotic, fungicidic, anti-helminthic, anti-thrombotic as well as anti-cancer and anti-carcinogenic properties (Block, 1992; Dausch & Nixon, 1990).

Abbreviations: pol, DNA-directed DNA polymerase (E.C. 2.7.7.7); TdT, terminal deoxynucleotidyl transferase (E.C. 2.7.7.31); dsDNA, double-stranded DNA; BRCT, BRCA1 C-terminus; NLS, nuclear localization signal; HhH, helix-hairpin-helix; dTTP, 2′-deoxythymidine 5′-triphosphate; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; DMSO, dimethyl sulfoxide; IC50, 50% inhibitory concentration; LD50, 50% lethal dose; NP-40, Nonidet P-40.

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In this report, we screened the natural products of garlic extract, which inhibit eukaryotic DNA polymerase (pol) activity and human cancer cell growth, for the development of anti-cancer functional foods and drugs.

Pol catalyses the addition of deoxyribonucleotides to the 3′-hydroxyl terminus of primed double-stranded DNA (dsDNA) molecules. The human genome encodes 14 pols to conduct cellular DNA synthesis (Bebenek & Kunkel, 2004). Eukaryotic cells reportedly contain three replicative types; pols α, δ, and ε, mitochondrial pol γ, and at least 12 repair types: pols β, δ, ε, ζ, η, θ, ι, κ, λ, μ, σ and REV1 (Friedberg, Feaver, & Gerlach, 2000). Pols are classified based on amino acid sequence homology into four families, A, B, X and Y (Burgers et al., 2001; Ohmori et al., 2001). Family X pols (pol X) include pols β, λ, μ, and σ and terminal deoxynucleotidyl transferase (TdT). The pols of this family have evolved as nucleotidyl transferases, to catalyse DNA polymerisation in a distributive manner (Aravind & Koonin, 1999).

We report newly-found compounds from garlic that selectively inhibit the activities of family X pols, such as pols β, λ and TdT. The natural components were diallyl sulfides, which are organosulfur compounds. To our knowledge, there have been no reports about such natural inhibitors specific to X family pols.

Here, we consider the structure–activity relationship in the inhibition by diallyl sulfides of the pol X family and human cancer cell growth, and discuss the inhibitory action of the compounds and its relation to the enzyme structure of the pols.

2. Materials and methods

2.1. Materials

Nucleotides and chemically synthesised DNA template-primers, such as poly(dA), poly(rA), and oligo(dT)$_{12–18}$ and [^{3}H]-dTTP (2′-deoxythymidine 5′-triphosphate, 43 Ci/mmol) were purchased from GE Healthcare Bio-Science Corp. (Amersham, UK). Diallyl sulfides, such as diallyl monosulfide, diallyl disulfide and diallyl trisulfide, were purchased from Sigma–Aldrich (St. Louis, MO). The other organosulfur compounds, such as allicin (diallyl disulfide oxide) and L(+)-alliin (*S*-allyl-L-cysteine sulfoxide), were purchased from LKT Laboratories, Inc. (St. Paul, MN). All other reagents were of analytical grade and purchased from Nacalai Tesque, Ltd. (Kyoto, Japan). Fresh garlic was purchased from Kobe Cooperative Shop (Kobe, Japan). HL-60, a human promyelocytic leukaemia cell line (IFO 050022), was supplied by the Health Science Research Resources Bank (Osaka, Japan).

2.2. Enzymes

Pols from mammal, fish, insect and plant were purified as described in our previous report (Mizushina et al., 2006). Calf TdT, *Taq* pol, T4 pol, and T4 polynucleotide kinase were purchased from Takara (Tokyo, Japan). The Klenow fragment of pol I from *Escherichia coli* and human immunodeficiency virus type-1 (HIV-1) reverse transcriptase were purchased from Worthington Biochemical Corp. (Freehold, NJ). T7 RNA polymerase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (La Jolla, CA).

2.3. Pols and TdT assays

The reaction mixtures for pol α, pol β, plant pols and prokaryotic pols were described previously (Mizushina, Yoshida, Matsukage, & Sakaguchi, 1997; Mizushina et al., 1996). Those for pols γ, δ and ε were as described by Ogawa, Murate, Suzuki, Nimura, and Yoshida (1998). The reaction mixtures for pols η, ι and κ were the same as for pol α, and the reaction mixture for intact or truncated pol λ was the same as for pol β. The substrates of the pols used were poly(dA)/oligo(dT)$_{12–18}$ and dTTP, as the DNA template-primer and dNTP (2′-deoxyribonucleoside 5′-triphosphate) substrate, respectively. The substrates of calf TdT used were oligo(dT)$_{12–18}$ (3′-OH) and dTTP, as the DNA primer and dNTP substrate, respectively. The substrates of HIV-1 reverse transcriptase used were poly(rA)/oligo(dT)$_{12–18}$ and dTTP, as the template-primer and dNTP substrate, respectively.

The inhibitory compounds were dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s. The sonicated samples (4 μl) were mixed with 16 μl of each enzyme (final amount, 0.05 units) 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* pol, which was incubated at 74 °C for 60 min. Activity without the inhibitor was considered to be 100%, and the remaining activity at each concentration of inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalysed the incorporation of 1 nmol of dNTP (i.e., dTTP) into synthetic DNA template-primers (i.e., poly(dA)/oligo(dT)$_{12–18}$, $A/T = 2/1$) in 60 min at 37 °C, under normal reaction conditions for each enzyme (Mizushina et al., 1996; Mizushina et al., 1997).

2.4. Other enzyme assays

Activities of calf primase of pol α, T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured in standard assays, according to the manufacturer's specifications, as described by Lu and Sakaguchi (1991), Nakayama and Saneyoshi (1985), Soltis and Uhlenbeck (1982), Tamiya-Koizumi et al. (1997), respectively.

2.5. Investigation of cytotoxicity on cultured cells

To investigate the effects of the compounds on cultured cells, a human cancer cell line, HL-60, derived from a cancer

patient, was used. The cells were routinely cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 100 μg/ml streptomycin, 100 unit/ml penicillin, and 1.6 mg/ml NaHCO3. The cells were cultured at 37 °C in standard medium in a humidified atmosphere of 5% CO_2 and 95% air. The cytotoxicity of the compound was investigated as follows. High concentrations (10 mM) of the compounds were dissolved in DMSO and stored. Approximately 1×10^4 cells per well were inoculated in 96-well microplates, and then the compound stock solution was diluted to various concentrations and applied to each well. After incubation for 24 h, the survival rate was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983).

3. Results

3.1. Isolation of pol inhibitors from garlic

We screened food materials for eukaryotic pol inhibitors and found that the ethanol extract from garlic (*A. sativum* L.) inhibited the activities of rat pol β and human pol λ, but did not inhibit calf pol α activity. The purification scheme is shown in Fig. 1. The fresh garlic (1 kg) was homogenised and extracted with 2 l of hot water. The tissue cake was freeze dried (100 g), and extracted with 1 l of ethanol. The extracted solution was subjected to Diaion HP-20 column chromatography (5.0 × 40 cm), a hydrophobic type of chromatography. The eluted solution of

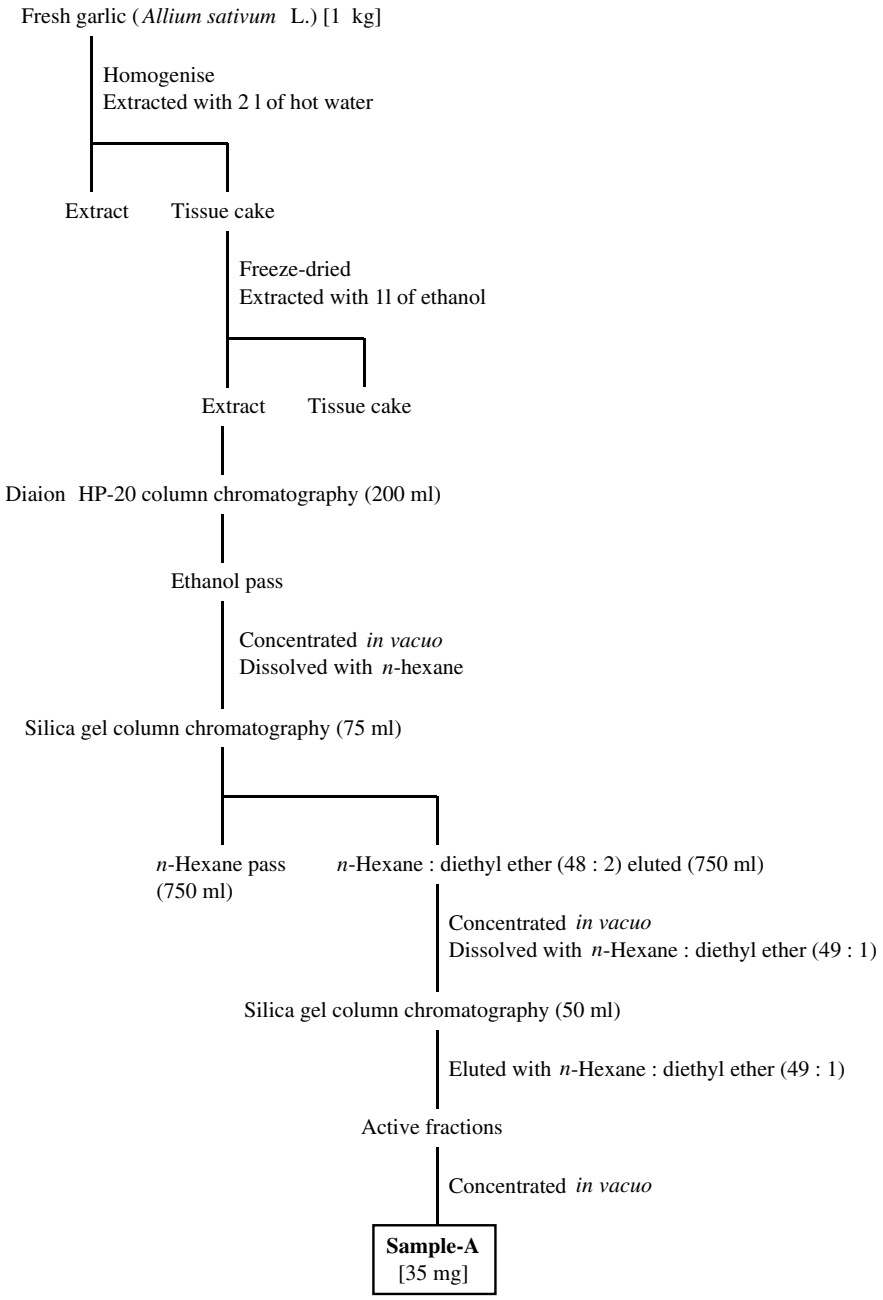

Fig. 1. Purification method of the fraction of mammalian DNA polymerase inhibitor (Sample-A) from garlic (*Allium sativum* L.).

ethanol was evaporated and dissolved in *n*-hexane, and the solution was applied to first silica gel column chromatography (PSQ 60B, Fuji Silysia, 5.0 × 15 cm). After the column was washed with *n*-hexane, the fraction eluted with *n*-hexane: diethyl ether (v/v 48:2) was collected. The active elution was purified by second silica gel column chromatography (PSQ 60B, 2.5 × 20 cm, Fuji Silysia Chemical, Ltd., Durham, NC) using *n*-hexane:diethyl ether (v/v 49:1). The active fractions were evaporated, and the purified material (Sample-A) was obtained (35 mg).

3.2. Determination of the structure of the pol inhibitor

^{1}H NMR spectra were recorded on a Bruker DRX600 (Bruker Daltronics, Inc., Billerica, MA). ^{13}C NMR spectra were recorded on a Bruker DXR400. Chemical shifts were reported in δ, parts per million (ppm), relative to TMS as an internal standard or calibrated using residual undeuterated solvent as an internal reference. IR spectra were recorded on a JASCO FT/IR-410 spectrometer (JASCO International Co., Ltd., Tokyo, Japan). Electrospray ionization high resolution mass spectra (ESI-HRMS) were obtained on an API QSTAR Pulsar i spectrometer (Applied Biosystems, Foster City, CA). The chemical structure of the purified fraction from garlic (i.e., Sample-A) was characterised by NMR, IR and MS. Sample-A contained three diallyl sulfides (compounds **1**–**3**) as an inseparable mixture, and the ratio of compounds **1**–**3** was estimated to be 5.3:3:1 by the ^{1}H NMR spectrum. The molecular formulae of these compounds were established to be $C_6H_{10}S_3$, $C_6H_{10}S_4$, and $C_6H_{10}S_5$, respectively, by high resolution mass spectrometry. The ^{1}H NMR spectrum of the mixture revealed the presence of three symmetric allyl moieties ($CH_2CH{=}CH_2$) [**1**: δ 3.50 (2H, d, $J = 7.3$ Hz), 5.22 (2H, m) and 5.89 (1H, m); **2**: δ 3.60 (2H, d, $J = 7.4$ Hz), 5.22 (2H, m) and 5.89 (1H, m); **3**: δ 3.62 (2H, d, $J = 7.3$ Hz), 5.22 (2H, m) and 5.89 (1H, m)]. The ^{13}C NMR spectrum of the mixture showed signals derived from the allyl moieties [**1**: δ 41.7, 119.6, 132.7; **2**: δ 42.1, 119.6, 132.7; **3**: δ 42.3, 119.9, 132.2]. The IR spectrum showed the absorption of the vinyl moiety (1634, 985, 923 cm^{-1}). The spectral data of compounds **1**–**3** were identical to those reported by other groups (Block et al., 1988; Higuchi, Tateshita, & Nishimura, 2003; Hu et al., 2002).

Compounds **1**–**3**. IR (neat): 2927, 1634, 1463, 1216, 985, 923, 668.

Diallyl trisulfide (**1**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.50 (2H, d, $J = 7.3$ Hz), 5.22 (2H, m), 5.89 (1H, m). ^{13}C NMR (100 Mz, $CDCl_3$) δ 41.7, 119.1, 132.7. ESI-HRMS: calculated for $C_6H_{10}S_3Na$ 200.9836, found 200.9878.

Diallyl tetrasulfide (**2**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.60 (2H, d, $J = 7.4$ Hz), 5.22 (2H, m), 5.89 (1H, m). ^{13}C NMR (100 Mz, $CDCl_3$) δ 42.1, 119.6, 132.5. ESI-HRMS: calculated for $C_6H_{10}S_4Na$ 232.9557, found 232.9563.

Diallyl pentasulfide (**3**). ^{1}H NMR (400 MHz, CDCl3) δ 3.62 (2H, d, $J = 7.3$ Hz), 5.22 (2H, m), 5.89 (1H, m). ^{13}C NMR (100 Mz, CDCl3) δ 42.3, 119.9, 132.2. ESI-HRMS: calculated for $C_6H_{10}S_5Na$ 264.9278, found 264.9230.

From the above data, Sample-A was identified as three compounds (**1**–**3**) of diallyl sulfides (Fig. 2A), which are the major organosulfur compounds and flavor components of garlic (Block et al., 1988; Higuchi et al., 2003; Hu et al., 2002).

3.3. Effects of Sample-A and organosulfur compounds on the activities of mammalian DNA polymerases α, β and λ

First, we prepared commercially available diallyl sulfides, diallyl monosulfide (compound **4**), diallyl disulfide (compound **5**) and diallyl trisulfide (compound **1**), and other organosulfur compounds present in garlic, such as allicin (compound **6**) and alliin (compound **7**) (Fig. 2B). Diallyl tetrasulfide (compound **2**) and diallyl pentasulfide (compound **3**), which were components of Sample-A, could not be commercially obtained as reagents. The inhibitory activity of calf pol α, rat pol β and human pol λ against 200 μM solutions of each compound was investigated (Fig. 3). In mammalian pols, pol α and pols β and λ were used as representative replicative pol and repair/recombina-

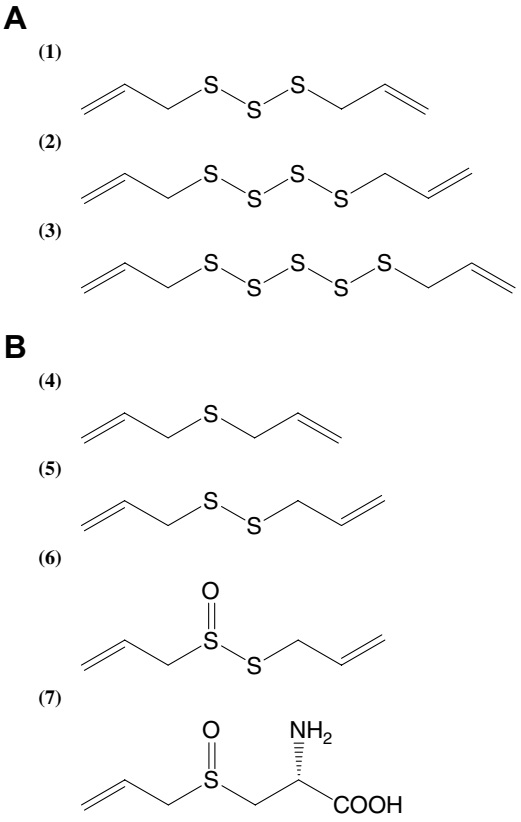

Fig. 2. Structures of organosulfur compounds from garlic. (A) The three diallyl sulfides comprising Sample-A. Compound **1**: diallyl trisulfide, compound **2**: diallyl tetrasulfide, and compound **3**: diallyl pentasulfide. (B) Commercially purchased reagents of Sample-A (i.e., diallyl sulfides)-related compounds. Compound **4**: diallyl monosulfide, compound **5**: diallyl disulfide, compound **6**: allicin (diallyl disulfide-oxide), and compound **7**: L(+)-alliin (*S*-allyl-L-cysteine sulfoxide).

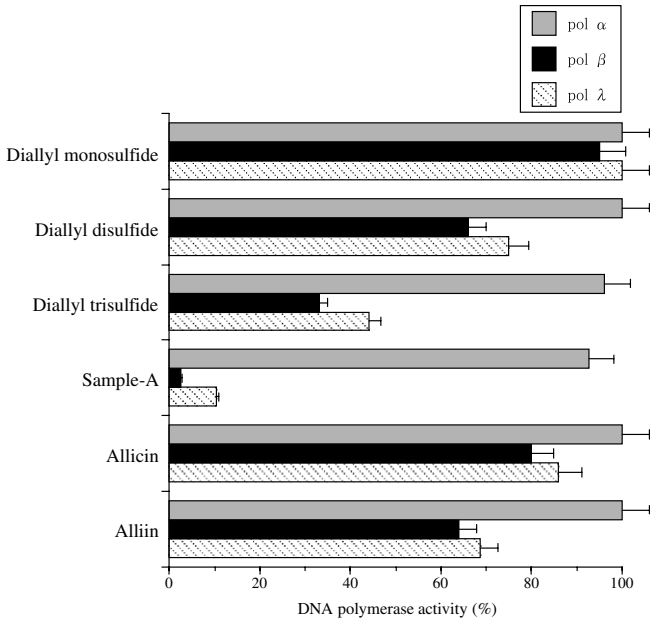

Fig. 3. Effect of organosulfur compounds from garlic on the activities of mammalian pols. Two-hundred μM of each compound was incubated with calf pol α, rat pol β and human pol λ (0.05 units each). Pol activities were measured as described in Section 2. Enzymatic activity in the absence of the compound was taken as 100%. Data are shown as the mean ± SEM of four independent experiments.

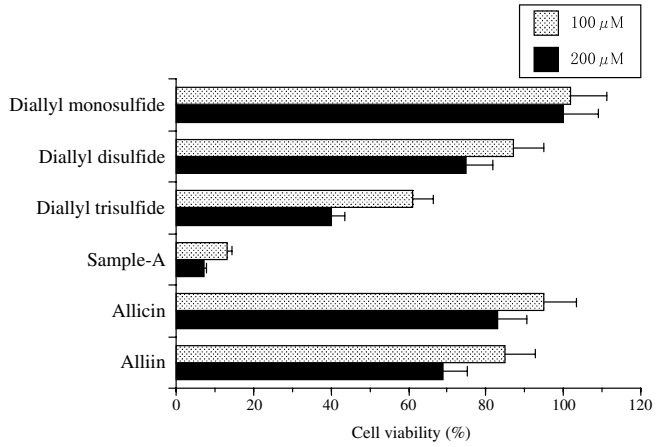

Fig. 4. Effect of organosulfur compounds from garlic on human cancer cell growth. Each compound (100–200 μM) was added to the culture of a promyelocytic leukaemia cell line HL-60, and incubated for 24 h. The rate of viability was determined by MTT assay (Mosmann, 1983). Cell viability of cancer cells in the absence of the compound was taken as 100%. Data are shown as the mean ± SEM of five independent experiments.

tion-related pols, respectively, (Bebenek & Kunkel, 2004; Friedberg et al., 2000). The molecular concentration of Sample-A was calculated by the ratio of compounds **1**–**3** (5.3:3:1, respectively). Sample-A had the strongest inhibitory effect on pols β and λ of the tested compounds, diallyl trisulfide was the second strongest, diallyl disulfide showed moderate inhibition, and diallyl monosulfide had no influence. These results suggested that the effect of the diallyl sulfides ranked as follows: diallyl pentasulfide > diallyl tetrasulfide > diallyl trisulfide > diallyl disulfide > diallyl monosulfide. The inhibitory effects of allicin and alliin had the same tendency as that of diallyl disulfide, suggesting that the number of sulfur atoms of the compound might be important for the inhibition of pols β and λ. On the other hand, no organosulfur compounds affected the activity of pol α. When activated DNA (i.e., DNA digested by bovine deoxyribonuclease I) was used as the DNA template-primer, instead of poly(dA)/oligo(dT)$_{12-18}$ ($A/T = 2/1$), the mode of inhibition by these compounds did not change (data not shown).

3.4. Effects of Sample-A and organosulfur compounds on cultured human cancer cells

Pols have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. The organosulfur compounds (i.e., compounds **1**–**7**) could therefore be useful in chemotherapy and we investigated the cytotoxic effect of Sample-A and the five purchased compounds against a human promyelocytic leukaemia cell line, HL-60.

As shown in Fig. 4, 100–200 μM solutions of Sample-A had the strongest growth inhibitory effect on this cancer cell line of the tested compounds, diallyl trisulfide and diallyl disulfide were the second and third strongest, and diallyl monosulfide had no effect. The suppression of cell growth had the same tendency as the inhibition of mammalian pols β and λ among the compounds, suggesting that the cause of the cancer cell influence might be the activity of pols, especially repair- and recombination-related pols. The cytotoxic dose was approximately 3.5- to 5-fold higher than the enzyme inhibitory concentrations (i.e., LD50 and IC50 values of Sample-A were 48 and 9.7–34.5 μM, respectively) (Fig. 3).

Therefore, we concentrated our efforts on Sample-A (mixture of compounds **1**–**3**), which is the purified mixture of diallyl sulfides from garlic, and diallyl trisulfide (compound **1**), which is a commercially purchased fine chemical reagent, in the latter part of this study.

3.5. Effects of Sample-A on the activities of DNA polymerases and other DNA metabolic enzymes

As shown in Table 1, Sample-A and diallyl trisulfide inhibited the activities of pols β, λ and calf TdT, and the inhibitory effect of pol β was the strongest of these enzymes. The compound had no influence at all on the activities of nuclear replicative pols such as calf pol α, human pol δ and human pol ε, human mitochondrial replicative pol γ, or repair-related pols such as human pols η, ι and κ. Given that the pol A family includes pol γ, the pol B family includes pols α, δ and ε, the pol X family includes pols β, λ and TdT, and the pol Y family includes pols η, ι and κ (Aravind & Koonin, 1999; Burgers et al., 2001; Ohmori et al., 2001), diallyl sulfides did not inhibit the activities of family A, B and Y pols. Sample-A and diallyl trisulfide had no inhibitory effect on fish (i.e., cherry salmon) pol δ,

Table 1
IC50 values of Sample-A and diallyl trisulfide on the activities of various DNA polymerases and other DNA metabolic enzymes

Enzyme	IC50 value (μM)	
	Sample-A	Diallyl trisulfide
Mammalian DNA polymerases		
Calf DNA polymerase α	>1000	>1000
Rat DNA polymerase β	9.7 ± 0.8	115 ± 9.5
Human DNA polymerase γ	>1000	>1000
Human DNA polymerase δ	>1000	>1000
Human DNA polymerase ε	>1000	>1000
Human DNA polymerase η	>1000	>1000
Human DNA polymerase ι	>1000	>1000
Human DNA polymerase κ	>1000	>1000
Human DNA polymerase λ		
Full-length (residues 1–575)	34.5 ± 2.8	146 ± 12
Del-1 (residues 133–575)	16.3 ± 1.5	122 ± 7.6
Del-2 (residues 245–575)	9.5 ± 0.8	116 ± 9.5
Calf terminal deoxynucleotidyl transferase	37.1 ± 3.2	144 ± 11
Fish DNA polymerases		
Cherry salmon DNA polymerase δ	>1000	>1000
Insect DNA polymerases		
Fruit fly DNA polymerase α	>1000	>1000
Fruit fly DNA polymerase δ	>1000	>1000
Fruit fly DNA polymerase ε	>1000	>1000
Plant DNA polymerases		
Cauliflower DNA polymerase I (α like)	>1000	>1000
Cauliflower DNA polymerase II (β like)	>1000	>1000
Prokaryotic DNA polymerases		
E. coli DNA polymerase I (Klenow fragment)	>1000	>1000
Taq DNA polymerase	>1000	>1000
T4 DNA polymerase	>1000	>1000
Other DNA metabolic enzymes		
Calf primase of DNA polymerase α	>1000	>1000
HIV-1 reverse transcriptase	>1000	>1000
T7 RNA polymerase	>1000	>1000
T4 polynucleotide kinase	>1000	>1000
Bovine deoxyribonuclease I	>1000	>1000

These compounds were incubated with each enzyme (0.05 units). Enzymatic activity was measured as described in Section 2. Enzyme activity in the absence of the compound was taken as 100%. Data are expressed as the mean ± SD; $n = 4$.

insect (i.e., fruit fly) pols α, δ and ε, plant (i.e., cauliflower) pols I (α-like) and II (β-like), or prokaryotic pols such as the Klenow fragment of *E. coli* pol I, *Taq* pol and T4 pol. The compounds also did not inhibit the activities of other DNA-metabolic enzymes such as primase of calf pol α, HIV-1 reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase or bovine deoxyribonuclease I. These results suggested that diallyl sulfides could selectively inhibit the activity of eukaryotic family X pols, such as pols β and λ and TdT.

3.6. Inhibition of Sample-A on full-length or fragments of human pol λ

Fig. 5 shows the inhibition dose-response curves of Sample-A against intact or truncated human pols λ and

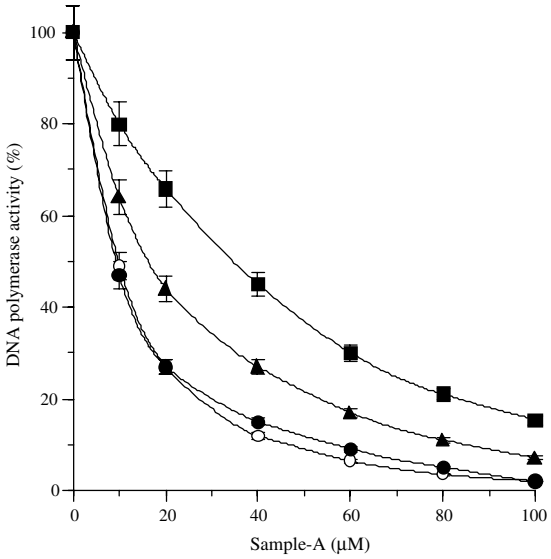

Fig. 5. Dose-response curves of Sample-A (0–100 μM) for family X pols. The enzymes used (0.05 units each) were full-length (residues 1–575) human pol λ (closed square), del-1 (residues 133–575) of human pol λ (closed triangle), del-2 (residues 245–575) of human pol λ (closed circle) and rat pol β (open circle). The activities of pols β and λ were measured as described in the text. Enzymatic activity in the absence of Sample-A was taken as 100%. Data are shown as the mean ± SEM of three independent experiments.

β. Full-length pol λ (residues 1–575) and N-terminal-deleted versions, del-1 pol λ (133–575) and del-2 pol λ (245–575), were prepared. Full-length and fragments of pol λ were dose-dependently inhibited by Sample-A. The inhibitory effect of Sample-A on truncated versions of pol λ such as del-1 and del-2, was stronger than on the full-length enzyme. The inhibition of del-2 pol λ was the strongest, with 50% inhibition observed at 9.5 μM, and this was the same value as for pol β inhibition. The effects seemed to be relatively selective between pol β and del-2 pol λ lacking NLS, the BRCT domain and proline-rich region.

3.7. Effect of interaction of nucleic acid, protein and Sample-A

To determine whether the inhibitor resulted in binding to DNA or enzymes, the interaction of Sample-A with dsDNA was investigated, based on the thermal transition of dsDNA with or without Sample-A. The T_m of dsDNA with an excess amount of Sample-A (200 μM) was measured using a spectrophotometer equipped with a thermoelectric cell holder. In the concentration range used, no thermal transition of T_m was observed, whereas ethidium bromide used as a positive control, a typical intercalating compound, produced clear thermal transition (data not shown). These results indicated that the diallyl sulfides of Sample-A did not intercalate to DNA as a template-primer, and the compound might directly bind to the enzyme and inhibit its activity.

To determine the effects of a non-ionic detergent on the binding of Sample-A to X family pols, such as pols β, λ and TdT, Nonidet P-40 (NP-40) was added to the reaction

mixture at a concentration of 0.05% or 0.1%. In the absence of Sample-A, the activities of these enzymes were not affected by addition of NP-40, and we designated the activities in these cases as 100%. The inhibitory effect of Sample-A at 20 μM was not reversed by the addition of 0.1% NP-40 to the reaction mixture (data not shown). These results suggested that Sample-A could bind to and interact with the hydrophilic region of the enzyme protein. We also tested whether an excess amount of a substrate DNA analogue, poly(rC) (50 μM), or a protein, BSA (200 μg/ml), could prevent the inhibitory effects of Sample-A. If the compound bound to the enzymes by non-specific adhesion, the addition of the nucleic acid and/or protein would be expected to reduce the inhibitory activity. The fact that neither poly(rC) nor BSA influenced the inhibitory effects on Sample-A suggested that the compound could occur selectively or bind to a specific site on the enzymes and not to the nucleic acid.

3.8. Mode of inhibition of pol X family by Sample-A

Next, to elucidate the mechanism by which Sample-A inhibited family X pols, such as pols β, λ and TdT, the extent of inhibition as a function of substrate concentration was studied. In kinetic analysis of pols β and λ, 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 Sample-A-induced inhibition of rat pol β activity was competitive, with respect to both the DNA template-primer and the dNTP substrate (Table 2). In the case of the DNA template-primer, the apparent maximum velocity (V_{max}) was unchanged at 111 pmol/h, whereas 48.7 pmol/h of the Michaelis constant (K_m) increased in the presence of 9 μM of Sample-A. The V_{max} for the dNTP substrate was unchanged at 62.5 pmol/h, and the K_m for the dNTP substrate increased from 3.05 to 16.7 μM in the presence of 9 μM of Sample-A. The inhibition constant (K_i) values, obtained from Dixon plots, were found to be 2.61 and 3.68 μM for the DNA template-primer and dNTP substrate, respectively. As shown in Table 2, the inhibition of human full-length-pol λ activity was also competitive with respect to both the DNA template-primer (V_{max} was unchanged at 83.3 pmol/h) and the dNTP substrate (V_{max} was unchanged at 52.6 pmol/h).

In kinetic analysis of TdT, oligo(dT)$_{12–18}$ and dTTP were used as the DNA primer and dNTP substrate, respectively. Double reciprocal plots of the results showed that the Sample-A-induced inhibition of calf TdT activity was competitive with respect to both the DNA primer and the dNTP substrate (Table 2). In the case of the DNA primer, the V_{max} was unchanged at 90.9 pmol/h, whereas 8.47-fold increases in the K_m were observed in the presence of

Table 2
Kinetic analysis of the inhibitory effects of Sample-A on the activities of mammalian DNA polymerases β, λ (full-length, residues 1–575), and terminal deoxynucleotidyl transferase as a function of the DNA template-primer dose and the nucleotide substrate concentration

Enzyme	DNA Substrate	Sample-A (μM)	K_m[a] (μM)	V_{max}[a] (pmol/h)	K_i[b](μM)	Inhibitory mode[a]
Rat Pol β	Template-primer[c]	0	6.74	111	2.61	Competitive
		3	12.5			
		6	21.1			
		9	48.7			
	Nucleotide[d] substrate	0	3.05	62.5	3.68	Competitive
		3	4.76			
		6	8.33			
		9	16.7			
HumanPol λ	Template-primer[c]	0	2.38	83.3	8.00	Competitive
		10	4.55			
		20	7.69			
		30	14.3			
	Nucleotide[d] substrate	0	1.18	52.6	11.2	Competitive
		10	1.92			
		20	3.33			
		30	5.56			
CalfTdT	Primer[e]	0	2.15	90.9	8.62	Competitive
		10	4.88			
		20	9.09			
		30	18.2			
	Nucleotide[d] substrate	0	1.02	55.6	12.1	Competitive
		10	2.00			
		20	2.23			
		30	5.26			

[a] Data obtained from a Lineweaver Burk plot.
[b] Data obtained from a Dixon plot.
[c] poly(dA)/oligo(dT)$_{12–18}$.
[d] dTTP.
[e] oligo(dT)$_{12–18}$.

30 μM of Sample-A. The V_{max} for the dNTP substrate was unchanged at 55.6 pmol/h, and the K_m for the dNTP substrate increased from 1.02 to 5.26 μM in the presence of 0–30 μM of Sample-A. The inhibition constant (K_i) values, obtained from Dixon plots, were found to be 8.62 μM and 12.1 μM for the DNA primer and dNTP substrate, respectively.

The inhibition of pols β and λ by Sample-A had the same kinetic mode as that of TdT, i.e., competitive with respect to both the DNA primer and the dNTP substrate, suggesting that the compound could bind directly to both the DNA primer-binding site and the dNTP substrate-binding site, and then might directly inhibit the DNA polymerisation process. As the K_i values for nucleic acid were smaller than those for the dNTP substrate, the affinity of the diallyl sulfides of Sample-A could be greater for the enzyme-nucleic acid binary complex than for the enzyme-nucleotide substrate complex.

4. Discussion

Geographical differences in the incidence of cancer indicate that diet plays a critical role in carcinogenesis (Hill, 1997; Sinha & Potter, 1997); diet is an important factor in the development of almost 40% of all human neoplasias (Doll & Peto, 1981). Epidemiological studies indicate that dietary factors influence the development of human cancer, and experimental analysis suggests that natural or synthetic constituents of the diet can act as anticancer agents to inhibit human cancer. Among dietary factors, certain phytochemicals, particularly those in the daily diet, have marked cancer chemopreventative properties (Surh, 1998). As described in this report, we used a major vegetable, garlic (*A. sativum* L.) to find natural components having new bioactivity, such as pol inhibitory activity.

The inhibition of mammalian pols by an ethanol extract was evaluated and the active fraction (Sample-A) was characterised and identified as a mixture of three diallyl sulfides (i.e., diallyl trisulfide, diallyl tetrasulfide and diallyl pentasulfide) by chromatographic and spectroscopic means. We are trying to purify the three diallyl sulfides from Sample-A, and the bioactivity of each compound will be addressed in further studies.

Diallyl sulfides could selectively inhibit the activities of family X pols and human cancer cell, a promyelocytic leukaemia cell line (HL-60), growth, and in order of their effects, the diallyl sulfides might be ranked as follows: diallyl pentasulfide > diallyl tetrasulfide > diallyl trisulfide > diallyl disulfide > diallyl monosulfide. These results suggested that the number of sulfur atoms in the compounds might play an important structural role in inhibition. This is the first report showing that the inhibition of repair/recombination-related pols, such as the pol X family, not replicative pols, might be involved in the cytotoxicity of cancer cells.

Pols β, λ and TdT belong to the pol X family, which includes pol μ, yeast pol IV, mitochondrial pol β, nuclear pol β from protozoans and 20 kDa African swine fever virus pol X (Aravind & Koonin, 1999). Family X pols are composed of an NLS, a BRCT domain, a proline-rich region, and a pol β-like region containing two HhHs and a

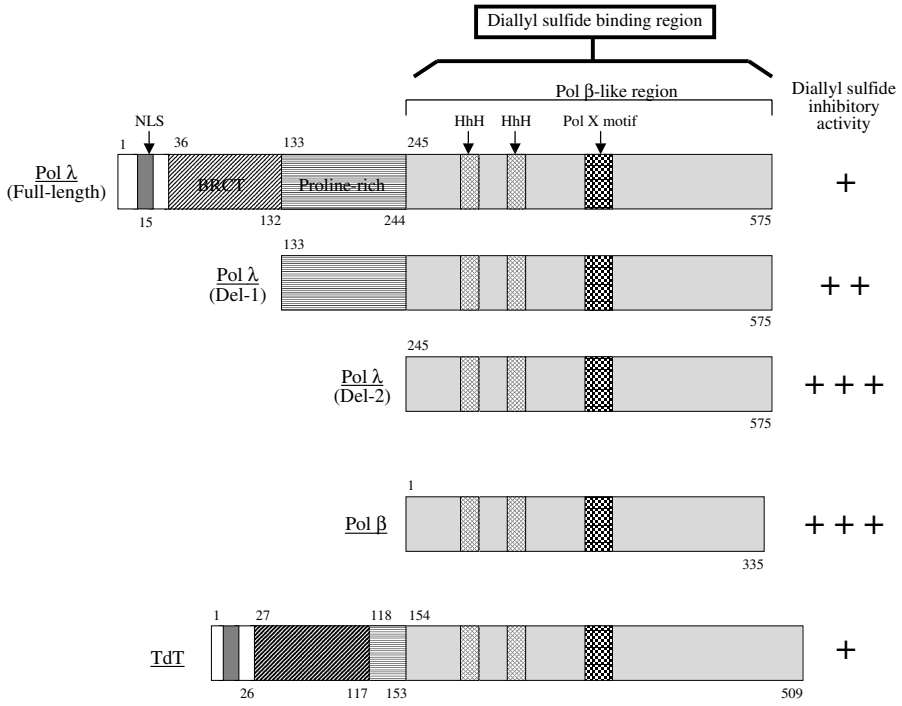

Fig. 6. Schematic representation of pols β, λ and TdT of the family X pols. The NLS (nuclear localization signal), BRCT (BRCA1 C-terminus) domain, proline-rich region, HhH (helix-hairpin-helix) and pol X motif are indicated. The pol β-like region includes two HhHs and a pol X motif. The inhibitory activity of diallyl sulfide (i.e., Sample-A) against these enzymes is indicated below, "+++" is an IC50 value of <10 μM, "++" is an IC50 value of 10–20 μM, and "+" is an IC50 value of 20–40 μM.

pol X motif (Fig. 6). Human pol λ shares 54%, 47% and 30% homology to human pols β and μ, and yeast pol IV, respectively. Sample-A inhibited the activities of pol β, intact and truncated pol λ and TdT, among the eukaryotic pols and other DNA metabolic enzymes tested, with the strongest inhibitory effect on both pol β and del-2 pol λ (residues 245–575), which mainly consisted of a pol β-like region (Fig. 5). The compound did not intercalate into dsDNA, and did not non-specifically interact with proteins and nucleic acids. The Sample-A-induced inhibition of pols β, λ and TdT was competitive with respect to both the DNA template-primer and the dNTP substrate (Table 2), indicating that Sample-A directly binds to the DNA template-primer-binding site and the dNTP substrate-binding site of the enzymes. Since both of these sites are present in the pol β-like region of pols β, λ and TdT (García-Díaz et al., 2000), Sample-A might directly bind to the pol β-like region, which has the activity of DNA polymerisation. There was no reversibility in the inhibition of pols β, λ and TdT by NP-40, indicating that Sample-A might bind to or interact with the hydrophilic region (maybe DNA primer-binding and dNTP-binding regions) of the pol β-like core on the enzyme molecules. Since the inhibitory effect of pol β and del-2 pol λ, consisting only of the pol β-like core region, was approximately 3.6-fold stronger than that of full-length pol λ, an N-terminal part such as the NLS, BRCT domain or proline-rich region of pol λ might prevent binding to the pol β-like region by Sample-A. The inhibitory activity of Sample-A against del-2 pol λ was as strong as that for pol β. This result suggested that the three-dimensional structure of pol β and del-2 pol λ might be quite similar. We are trying to co-crystallize diallyl sulfide and pol β/del-2 pol λ for further study.

As described here, we have succeeded in identifying novel pol inhibitors. Over the last 10 years, we have screened for and reported many new inhibitors of eukaryotic pols (Sakaguchi, Sugawara, & Mizushina, 2002). The overarching aim of this series of studies is to provide an alternative means for examining the roles of pol. These inhibitors can be used as tools and molecular probes to distinguish pols, since they can directly bind to or interact with a target enzyme. Since pol species specificity was extremely high, diallyl sulfides could be useful as pol X family-specific inhibitors in studies to determine the precise roles of pols.

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