Inhibitory effects of low molecular weight polyphenolics from *Inonotus obliquus* on human DNA topoisomerase activity and cancer cell proliferation

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Abstract. Low molecular weight (LMW) polyphenolics containing a polyhydroxylated benzyl moiety are abundant in medicinal plants. In the present study, we report on the activities of seven LMW polyphenolics isolated from *Inonotus obliquus*, a medicinal mushroom. The isolated compounds included caffeic acid (CA), 3,4-dihydroxybenzalacetone (DBL), gallic acid, syringic acid, protocatechuic acid, 3,4-dihydroxybenzaldehyde and 2,5-dihydroxyterephthalic acid. We analyzed their inhibitory effects on DNA polymerase (pol) and DNA topoisomerase (topo), and their effects on human cancer cell growth. All isolated compounds inhibited human topo II activity; the most potent were DBL and CA, which contain a catechol propanoid moiety. CA and DBL inhibited the activity of human topo I, whereas other compounds had no effect. No compound modulated the activities of 11 mammalian pol species or other DNA metabolic enzymes, including T7 RNA polymerase, mouse IMP dehydrogenase (type II), T4 polynucleotide kinase and bovine deoxyribonuclease I. CA and DBL markedly suppressed the proliferation of human colon HCT116 carcinoma cells with an LD_{50} of 70.0 and 49.4 μM, respectively, and halted the cell cycle in the G2/M phase. The suppressive effect of these compounds on cancer cell growth correlated with their ability to inhibit topo II. These results suggest that CA- and DBL-dependent decreases in cell proliferation are due to the inhibition of cellular topo II. The mechanism of action of these catechol propanoid compounds and the implication for their use as anticancer agents are discussed.

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Abbreviations: LMW, low molecular weight; CA, caffeic acid; DBL, 3,4-dihydroxy-benzalacetone; GA, gallic acid; SA, syringic acid; PCA, protocatechuic acid; DB, 3,4-dihydroxy-benzaldehyde; DTA, 2,5-dihydroxy-terephthalic acid; pol, DNA polymerase (EC 2.7.7.7); topo, DNA topoisomerase; dsDNA, double-stranded DNA; dTTP, 2'-deoxythymidine 5'-triphosphate; dNTP, 2'-deoxyribonucleotide 5'-triphosphate; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EtBr, ethidium bromide; IMP, inosine-5'-monophosphate; ssDNA, single-stranded DNA; DS, Discovery Studio; IC_{50}, 50% inhibitory concentration; LD_{50}, 50% lethal dose; Tm, melting temperature; ClogP, calculated log P

Key words: low molecular weight polyphenolics, *Inonotus obliquus*, caffeic acid, 3,4-dihydroxybenzalacetone, DNA topoisomerase, enzyme inhibitor, cytotoxicity, cell cycle arrest, anticancer agents

Introduction

Cancer is a major public health problem worldwide. Epidemiological and animal studies have indicated that the consumption of fruit and vegetables containing chemopreventive natural products, alone or in combination with others, is associated with a reduced risk of cancer development (1,2). For >15 years, many studies have screened natural phytochemical products in vegetables and fruits for inhibitors of DNA metabolic enzymes, primarily mammalian DNA polymerases (pols) and human DNA topoisomerases (topos).

Pols (DNA-dependent DNA polymerases, E.C. 2.7.7.7) catalyze deoxyribonucleotide addition to the 3'-hydroxyl terminus of primed double-stranded DNA (dsDNA) molecules (3). The human genome encodes at least 15 pols that have functions in cellular DNA synthesis (4,5). Eukaryotic cells contain three replicative pols (α, δ and ε), one mitochondrial pol (γ), and at least 11 non-replicative pols [β, ζ, η, θ, ι, κ, λ, μ, ν, terminal deoxynucleotidyl transferase (TdT) and REV1] (6,7). Pols have a highly conserved structure and their overall catalytic subunits show little variance among species. Conserved enzyme structures are normally preserved over time

due to the fact that they perform important cellular functions that confer evolutionary advantages. On the basis of sequence homology, eukaryotic pols may be divided into four main families: A, B, X and Y (6). Family A includes mitochondrial pol γ in addition to pols θ and ν. Family B includes pol ζ and the three replicative pols α, δ and ε. Family X comprises TdT and pols β, λ and μ. Family Y includes pols η, ι and κ, in addition to REV1.

Topos are nuclear enzymes that alter the DNA topology required for the replication, transcription, recombination and segregation of daughter chromosomes (8). Eukaryotic cells have Type I and Type II topos. Topo I catalyzes the passage of the DNA strand through a transient single-strand break in the absence of a high-energy cofactor. Topo II, by contrast, catalyzes the passage of DNA double strands through a transient double-strand break in the presence of ATP.

Due to their antiproliferative and cytotoxic effects, selective inhibitors of pols and topos are considered to be useful as anticancer, antiviral, antiparasitic and birth control agents (9-11). In screening for these enzyme inhibitors, we focused on low molecular weight (LMW) polyphenolics isolated from Chaga, a medicinal mushroom [*Inonotus obliquus* (persoon) Pilat] (12), including caffeic acid (CA, 3,4-dihydroxycinnamic acid, compound 1), 3,4-dihydroxybenzalacetone (DBL, compound 2), and hydroxyl benzoic acid derivatives such as gallic acid (GA, 3,4,5-trihydroxybenzoic acid, compound 3) and their derivatives (Fig. 1). *Inonotus obliquus* is used as a folk medicine in countries such as Korea, Japan and Russia; in Russia it is also used as a source of anticancer medicine (13). The anticancer activities of *Inonotus obliquus* extract and its components have been examined *in vitro* (14,15). LMW phenolic compounds such as CA, ferulic acid and GA are among the major phenolic compounds derived from fruits, vegetables, grains and coffee. These diet-associated phenolic compounds are often described as potential antioxidants and, consequently, as inhibitors of deleterious oxidative processes associated with cardiovascular and inflammatory diseases, and cancer (16,17). Phenolic compounds potentially act as chemopreventive and/or chemotherapeutic agents (16-18). Specifically, these LMW polyphenolics exhibit antioxidant properties in addition to biological activity towards several tumor cells, as their growth inhibitory effects are markedly dependent on their structural characteristics (19).

The purpose of this study was to discover novel bioactivities among seven LMW polyphenolic compounds isolated from *Inonotus obliquus* as shown in Fig. 1. We investigated whether these compounds inhibit enzymes involved in DNA metabolism, such as pols and topos, or whether they block the replication of the colorectal cancer cell line HCT116. We identified two catechol propanoid compounds, CA and DBL, that have possible anticancer activity.

Materials and methods

Materials. CA, GA, 3,4-dihydroxybenzaldehyde (DB, compound 6) and 2,5-dihydroxyterephthalic acid (DTA, compound 7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Syringic acid (SA, 4-hydroxy-3,5-dimethoxybenzoic acid, compound 4) and protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid, compound 5) were purchased from WAKO Chemical Co. Ltd. (Tokyo, Japan). DBL was synthesized and kindly provided by Dr Yutaka Nakamura of the Synthetic Organic Chemistry Lab, Niigata University of Pharmacy and Applied Life Sciences (Niigata, Japan). All compounds were primarily isolated and purified from *Inonotus obliquus* (12), and their chemical structures are shown in Fig. 1. The compounds, purified using HPLC, were of analytical grade. A chemically synthesized DNA template, poly(dA), was purchased from Sigma-Aldrich and a customized oligo$(dT)_{18}$ DNA primer was purchased from Sigma-Aldrich Japan K.K. (Hokkaido, Japan). Radioactive nucleotide [^{3}H]-labeled 2'-deoxythymidine-5'-triphosphate (dTTP; 43 Ci/mmol) was obtained from Moravek Biochemicals Inc. (Brea, CA, USA). Supercoiled pBR322 plasmid dsDNA was obtained from Takara Bio, Inc. (Kyoto, Japan). All other reagents such as buffers were of analytical grade and were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Enzymes. Pol α was purified from calf thymus by immuno-affinity column chromatography, as described by Tamai *et al* (20). Recombinant rat pol β was purified from *Escherichia coli* (*E. coli*) JMpβ5, as described by Date *et al* (21). The human pol γ catalytic gene was cloned into pFastBac. The histidine-tagged enzyme was expressed using the Bacto-Bac HT Baculovirus Expression system according to the manufacturer's instructions (Life Technologies, Frederick, MD, USA) and was purified using ProBond resin (Invitrogen Japan, Tokyo, Japan) (22). Human pols δ and ε were purified by nuclear fractionation of human peripheral blood cancer cells (MOLT-4) using the second subunit of pol δ and ε-conjugated affinity column chromatography, respectively (23). A truncated form of human pol κ (residues 1-511) tagged with His_6 at its C-terminal was expressed in *E. coli* cells and purified as described by Kusumoto *et al* (24). A recombinant mouse pol ι tagged with His_6 at its C-terminal was expressed by *E. coli* and purified by Ni-NTA column chromatography. A truncated form of pol κ (residues 1-560) with six His-tags attached at the C-terminus was overproduced in *E. coli* and purified as described by Ohashi *et al* (25). Recombinant human His-pol λ was overexpressed in *E. coli* and purified according to a method described by Shimazaki *et al* (26). Recombinant human His-pol μ was overexpressed in *E. coli* BL21 and purified by Glutathione Sepharose™ 4B column chromatography (GE Healthcare Bio-Science Corp., Piscataway Township, NJ, USA) following the same method as for pol λ (26). Calf TdT, T7 RNA polymerase, T4 polynucleotide kinase and bovine pancreas deoxyribonuclease I were purchased from Takara Bio, Inc. Purified human placenta topos I and II were purchased from TopoGen Inc. (Port Orange, FL, USA).

Measurement of pol activity. Reaction mixtures for calf pol α and rat pol β have been previously described by Mizushima *et al* (27,28); those for pol γ and for pols δ and ε were as described by Umeda *et al* (22) and Ogawa *et al* (29), respectively. Reaction mixtures for pols η, ι and κ were the same as those for pol α and the mixtures for pols λ, μ and TdT were the same as those for pol β. For the pol reactions, poly(dA)/oligo$(dT)_{18}$ (A/T, 2/1) and dTTP were used as the DNA template-primer substrate and nucleotide (dNTP;

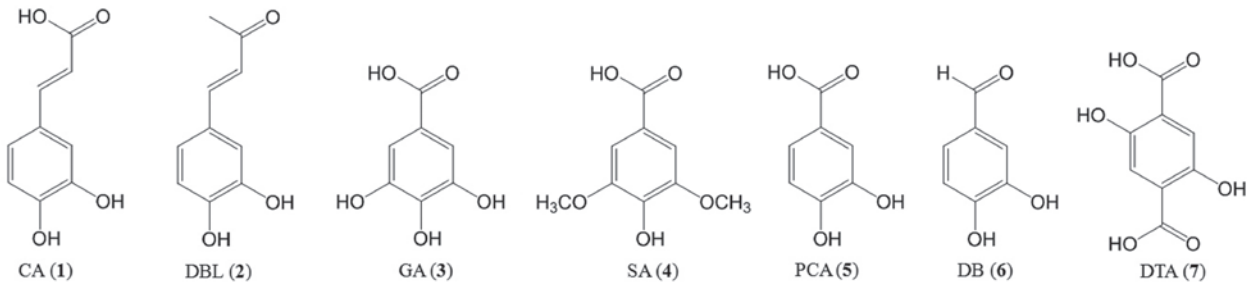

Figure 1. Structures of *Inonotus obliquus* low molecular weight polyphenolic compounds. (1) Caffeic acid (CA, 3,4-dihydroxycinnamic acid); (2) 3,4-dihydroxy-benzalacetone (DBL); (3) gallic acid (GA, 3,4,5-trihydroxy-benzoic acid); (4) syringic acid (SA, 4-hydroxy-3,5-dimethoxy-benzoic acid); (5) protocatechuic acid (PCA, 3,4-dihydroxy-benzoic acid); (6) 3,4-dihydroxy-benzaldehyde (DB); (7) 2,5-dihydroxy-terephthalic acid (DTA).

2'-deoxynucleoside-5'-triphosphate) substrate, respectively. For TdT reactions, oligo$(dT)_{18}$ (3'-OH) and dTTP were used as the DNA primer substrate and nucleotide substrate, respectively.

The test compounds 1-7 were dissolved in various concentrations of distilled DMSO and sonicated for 30 sec. Subsequently, 4-μl aliquots were mixed with 16 μl of each enzyme (0.05 units) in 50 mM Tris-HCl at pH 7.5 that contained 1 mM dithiothreitol, 50% glycerol (by vol) and 0.1 mM EDTA. The mixtures were maintained at 0˚C for 10 min. Next, 8 μl of each inhibitor-enzyme mixture was added to 16 μl of the enzyme standard reaction mixture and incubated at 37˚C for 60 min. The activity of samples without inhibitors was considered to be 100%, and the activity was determined for each inhibitor concentration relative to the uninhibited activity. One unit of pol activity was defined as the amount of each enzyme that catalyzed the incorporation of 1 nmol dTTP into synthetic DNA template-primers in 60 min at 37˚C under normal reaction conditions (27,28).

Measurement of topo activity. The catalytic activity of topo I was determined by detecting supercoiled plasmid DNA (Form I) in its nicked form (Form II) (30). The topo I reaction was performed in a 20-μl reaction mixture that contained 10 mM Tris-HCl (pH 7.9), pBR322 DNA (250 ng), 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol, 2 μl of one of the seven test compounds dissolved in DMSO and 2 units of topo I. The catalytic activity of topo II was analyzed in the same manner, except the reaction mixture contained 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, supercoiled pBR322 DNA (250 ng) and 2 units of topo II (30). The reaction mixtures were incubated at 37˚C for 30 min, followed by digestion with 1% sodium dodecyl sulfate (SDS) and 1 mg/ml proteinase K. Following digestion, 2 μl loading buffer consisting of 5% sarkosyl, 0.0025% bromophenol blue and 25% glycerol was added. To study the binding of enzymes to DNA based on mobility shifts, the same procedure was followed, but the SDS denaturation and proteinase K digestion steps were omitted. The mixtures were subjected to 1% agarose gel electrophoresis in Tris/borate/EDTA buffer. Agarose gel was stained with ethidium bromide (EtBr), and the DNA band shifts from Form I to Form II by topos I and II were detected using an enhanced chemiluminescence detection system (Perkin Elmer Life Sciences Inc., Waltham, MA, USA). Zero-D scan (Version 1.0, M&S Instruments Trading Inc., Osaka, Japan) was used for densitometric quantitation.

Other enzyme assays. Standard assays were used according to the manufacturer's instructions to measure the activities of T7 RNA polymerase, mouse inosine-5'-monophosphate (IMP) dehydrogenase (type II), T4 polynucleotide kinase, and bovine deoxyribonuclease I, as described by Nakayama and Saneyoshi (31), Mizushina *et al* (32), Soltis and Uhlenbeck (33), and Lu and Sakaguchi (34), respectively.

Cell culture and measurement of cancer cell viability. Human colon carcinoma cell line HCT116 was obtained from the American Type Culture Collection (Manassas, VA, USA). HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in a humid atmosphere of 5% CO_2/95% air. For the cell viability assay, cells were plated at $1x10^4$ into each well of a 96-well microplate with 10 and 100 μM of one of the test compounds (1-7). Cell viability was determined by WST-1 assay (35).

Cell cycle analysis. Cellular DNA content for cell cycle analysis was determined as follows: aliquots of $3x10^5$ HCT116 cells were added to a 35-mm dish and incubated with a medium containing 70.0 μM CA and 49.4 μM DBL, based on the LD_{50} values, for 24 h. Cells were then washed with ice-cold PBS three times by centrifugation, fixed with 70% (v/v) ethanol and stored at -20˚C. DNA was stained with PI {3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium di iodide} staining solution for a minimum of 10 min at room temperature in the dark. The intensity of the fluorescence was measured using a FACSCanto flow cytometer in combination with FACSDiVa software (Becton-Dickinson Co., Franklin Lakes, NJ, USA).

Computational analysis. The molecular structures of compounds were constructed using Discovery Studio (DS) 3.5 modeling software (Accelrys Inc., San Diego, CA, USA). Energy minimization was achieved using minimization and dynamics protocols within the DS. Calculations were performed using the Chemistry at HARvard Macromolecular Mechanics (CHARMm) force-field. The calculated logP (ClogP) values and pKa values of the test compounds 1-7 were obtained from the calculated properties in SciFinder

Table I. IC_{50} values of *Inonotus obliquus* LMW polyphenolic compounds 1-7 for the activities of mammalian pols, topos and other DNA metabolic enzymes.

	IC_{50} values (μM)						
Enzyme	CA (1)	DBL (2)	GA (3)	SA (4)	PCA (5)	DB (6)	DTA (7)
Mammalian pols							
Family A							
Human pol γ	>200	>200	>200	>200	>200	>200	>200
Family B							
Calf pol α	>200	>200	>200	>200	>200	>200	>200
Human pol δ	>200	>200	>200	>200	>200	>200	>200
Human pol ε	>200	>200	>200	>200	>200	>200	>200
Family X							
Rat pol β	>200	>200	>200	>200	>200	>200	>200
Human pol λ	>200	>200	>200	>200	>200	>200	>200
Human pol *μ*	>200	>200	>200	>200	>200	>200	>200
Calf TdT	>200	>200	>200	>200	>200	>200	>200
Family Y							
Human pol η	>200	>200	>200	>200	>200	>200	>200
Mouse pol ι	>200	>200	>200	>200	>200	>200	>200
Human pol κ	>200	>200	>200	>200	>200	>200	>200
Mammalian topos							
Human topo I	150±15	130±15	>200	>200	>200	>200	>200
Human topo II	15±2.0	10±1.5	50±4.0	175±17	80±7.0	150±15	170±16
Other DNA metabolic enzymes							
T7 RNA polymerase	>200	>200	>200	>200	>200	>200	>200
Mouse IMP dehydrogenase (type II)	>200	>200	>200	>200	>200	>200	>200
T4 polynucleotide kinase	>200	>200	>200	>200	>200	>200	>200
Bovine deoxyribonuclease I	>200	>200	>200	>200	>200	>200	>200

Compounds 1-7 were incubated with pols, topos and other DNA metabolic enzymes. Enzyme activity in the absence of the compound was considered to be 100%. Data are presented as the means ± SD of three independent experiments. CA, caffeic acid; DBL, 3,4-dihydroxybenzalacetone; GA, gallic acid; SA, syringic acid, PCA, protocatechuic acid; DB, 3,4-dihydroxybenzaldehyde; DTA, 2,5-dihydroxyterephthalic acid; pol, DNA polymerase (E.C. 2.7.7.7); topo, DNA topoisomerase; IMP, inosine-5'-monophosphate; LMW, low molecular weight; IC_{50}, 50% inhibitory concentration.

Scholar, which were originally calculated using Advanced Chemistry Development (ACD/Lab) Software V8.14 for Solaris (ACD/Labs).

Results

Effect of Inonotus obliquus LMW polyphenolic compounds 1-7 on the activity of mammalian pols. The inhibitory activity of each of the seven LMW polyphenolics from *Inonotus obliquus* toward mammalian pols was investigated using 11 mammalian pol species. These pols belong to the A, B, X and Y families of pols (6,7). Assessment of the relative activity of each pol at 200 μM after the addition of the seven test compounds revealed that none of the compounds had any effect on pol inhibition, as no compound resulted in <90% relative pol activity (Table I). These results suggest that these tested compounds did not affect the activity of any of the 11 mammalian pol species tested *in vitro*. When activated DNA (bovine deoxyribonuclease I-treated DNA) was used as the DNA template-primer substrate instead of synthesized DNA [poly(dA)/oligo$(dT)_{18}$ (A/T = 2/1)] and dNTP was used as the nucleotide substrate instead of dTTP, the inhibitory effects of these compounds did not change (data not shown).

Effect of Inonotus obliquus LMW polyphenolic compounds 1-7 on the activity of human topos I and II. The inhibitory effect of each LMW polyphenolic was examined against human topos I and II, which have ssDNA and dsDNA nicking activity, respectively (8). CA and DBL inhibited topo I nicking activity, and 50% inhibition was observed at a concentration of 150 and 130 μM, respectively (Table I). Therefore, DBL is a more potent topo I inhibitor compared with CA under these conditions. By contrast, the other compounds had no affect on topo I activity, even at a concentration of 200 μM (Table I).

These LMW polyphenolics from *Inonotus obliquus* potently inhibited the nicking activity of topo II, and the inhibition was ranked as DBL > CA >> GA > PCA >> DB > DTA > SA (Table I). Specifically, the compounds may be categorized into three groups as follows: significant topo II inhibitors [CA and DBL with 50% inhibitory concentration (IC_{50}) values of 15 and 10 μM, respectively], moderate topo II inhibitors (GA and PCA with IC_{50} values of 50 and 80 μM, respectively), and weak topo II inhibitors (SA, DB, and DTA with IC_{50} values of 150-175 μM).

Effect of Inonotus obliquus LMW polyphenolic compounds 1-7 on the activity of mammalian pols, topos and other DNA metabolic enzymes. None of the LMW polyphenolics examined affected the activity of other DNA metabolic enzymes such as T7 RNA polymerase, mouse IMP dehydrogenase (type II), T4 polynucleotide kinase and bovine deoxyribonuclease I (Table I). These results indicate that CA and DBL are potent and selective inhibitors of human topos I and II, whereas other compounds should be specifically classified as inhibitors of human topo II.

Specific assays were performed in order to determine whether the inhibitory activity of these LMW polyphenolics was due to their ability to bind to DNA or to the enzyme. The interaction of these compounds with dsDNA was investigated by studying changes in the thermal transition of the DNA. To accomplish this, the melting temperature (Tm) of dsDNA in the presence of an excess of compound (200 μM) was measured using a spectrophotometer equipped with a thermoelectric cell holder. When a typical intercalating compound, EtBr (15 μM), was used as a positive control, a clear thermal transition (Tm) was observed. However, no such thermal transition was observed when any of the seven LMW polyphenolics were heated with dsDNA (data not shown). We considered whether the inhibitory effects of the seven *Inonotus obliquus* LMW polyphenolics resulted from nonspecific adhesion to human topos or from their selective binding to specific sites. This was investigated by determining whether an excessive amount of nucleic acid [poly(rC)] or protein (BSA) prevented the inhibitory effect of the compounds. Poly(rC) and BSA had little or no effect on the inhibition of topos by the isolated compounds (data not shown), suggesting that all seven LMW polyphenolics selectively bound to the topo enzyme molecule. These findings indicate that the compounds do not act as DNA intercalating agents or as template-primer substrates. Instead, the compounds directly bind to topos and inhibit their activities.

These results suggest that compounds 1-7 are potent and specific inhibitors of human topos. We therefore investigated in more detail whether topo inhibition by these compounds results in decreased human cancer cell proliferation.

Effect of Inonotus obliquus LMW polyphenolic compounds 1-7 on cultured human cancer cells. Topos have recently emerged as important cellular targets for chemical intervention in the development of anticancer agents. The seven LMW polyphenolics examined in this study may be useful in chemotherapy; therefore, we investigated the cytotoxic effect of these compounds against the HCT116 cell line. As shown in Fig. 2, 24 h of treatment with 10 μM of CA and DBL marginally suppressed HCT116 cell growth, whereas treatment with the other compounds at 10 μM did not. On the other hand, 100 μM of CA and DBL markedly suppressed, and GA and PCA moderately suppressed, cell proliferation, whereas SA, DB and DTA suppressed growth weakly or not at all (Fig. 2). The dose of these compounds required for the suppression of cell growth was approximately the same as that for topo II inhibition. The suppression of HCT116 cell growth by CA and DBL was dose-dependent, with LD_{50} of 70.0 and 49.4 μM, respectively. These compounds suppressed the growth of other human cancer cell lines with approximately the same LD_{50} values (data not shown). These LD_{50} values are ~5-fold higher than the IC_{50} values for topo II. This indicates that these catechol propanoid compounds may interact with other cellular components prior to reaching the nucleus. Subsequently, they bind and interact with topo II in order to inhibit its activity and suppress cell growth, although they exhibited rather specific inhibitory action to topo II.

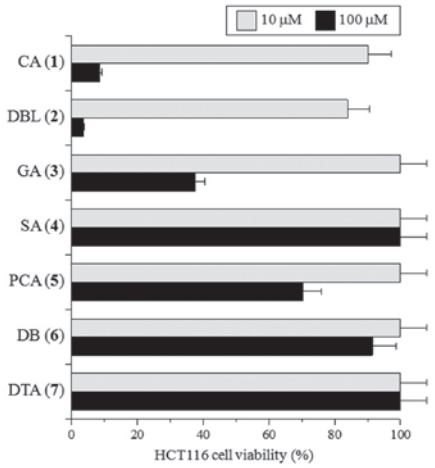

Figure 2. Effect of *Inonotus obliquus* LMW polyphenolic compounds 1-7 on the proliferation of HCT116 growth. Each compound (10 and 100 μM) was added to the cells for 24 h, and growth inhibition was determined using the WST-1 assay (35). Cell growth in the absence of the compound was considered to be 100%. Data are presented as the means ± SD of five independent experiments.

These results suggest that, among the seven compounds tested, CA and DBL are potent inhibitors of human topo II rather than topo I, and were therefore selected for further study.

Effect of CA and DBL on cell cycle progression. We analyzed whether CA and DBL affected the cell cycle distribution of HCT116 cells. The cell-cycle fraction was recorded following 24 h of treatment with the concentration of each compound equal to its LD_{50}. The ratio of cells in all three phases (G1, S and G2/M) of the cell cycle is shown in Fig. 3. Treatment with CA and DBL increased the population of cells in the G2/M phase (1.21- and 1.30-fold, respectively), did not change the proportion of cells in the S phase and decreased the percentage of cells in the G1 phase. Etoposide, a classic topo II inhibitor, arrested cells in the G2/M phase (1.40-fold increase, data not shown). These results suggest that CA and DBL are effective inhibitors of topo II and lead to a blockade of the cell cycle at the G2/M phase.

Table II. Molecular length and width, ClogP and pKa values of the three-dimensional structure of *Inonotus obliquus* LMW polyphenolic compounds 1-7.

Factor	CA (1)	DBL (2)	GA (3)	SA (4)	PCA (5)	DB (6)	DTA (7)
Length (Å)	8.0	8.7	6.6	6.6	6.6	5.7	6.9
Width (Å)	4.0	4.0	5.2	5.8	4.0	4.0	5.2
ClogP	0.66±0.28	1.55±0.28	0.53±0.32	1.28±0.33	1.01±0.23	0.93±0.26	3.23±0.37
pKa	4.58±0.10	9.41±0.10	4.33±0.10	4.33±0.10	4.45±0.10	7.61±0.18	2.17±0.10

The molecular length and width of energy-minimized three-dimensional compounds 1-7 were measured using Discovery Studio. CA, caffeic acid; DBL, 3,4-dihydroxybenzalacetone; GA, gallic acid; SA, syringic acid, PCA, protocatechuic acid; DB, 3,4-dihydroxybenzaldehyde; DTA, 2,5-dihydroxyterephthalic acid; LMW, low molecular weight.

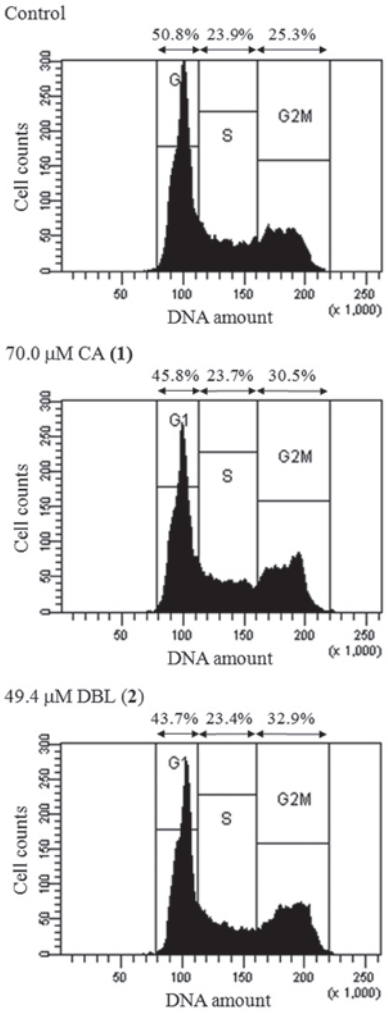

Figure 3. Effect of CA (1) and DBL (2) on the cell cycle. HCT116 cells were cultured for 24 h with or without the LD_{50} concentrations of CA (70.0 μM) and DBL (49.4 μM), and were analyzed using flow cytometry. Cell cycle distribution was calculated as the percentage of cells in the G1, S, and G2/M phases. All experiments were performed three times. CA, caffeic acid; DBL, 3,4-dihydroxybenzalacetone; LD_{50}, 50% inhibitory concentration.

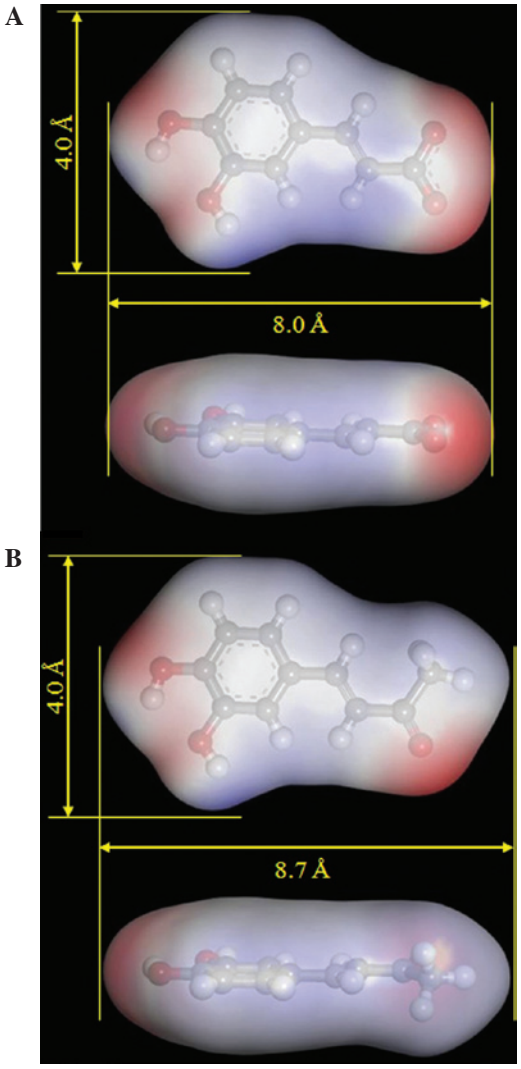

Figure 4. Three-dimensional structure of CA and DBL. (A) CA and (B) DBL. The carbon, hydrogen and oxygen atoms of the stick models are grey, white, and red, respectively. In the electrostatic potentials over molecular surfaces, the blue areas are positively charged, the red areas are negatively charged and white areas are neutral. CA, caffeic acid; DBL, 3,4-dihydroxybenzalacetone.

Three-dimensional structural simulation of Inonotus obliquus LMW polyphenolic compounds 1-7. To obtain information regarding the molecular basis of the inhibitory properties of compounds 1-7, we performed computational analyses using molecular simulation. The molecular length of the three-dimensional structure of CA and DBL were 8.0 and 8.7 Å, respectively, and other LMW polyphenolics were 5.7-6.9 Å long (Table II); hence, CA and DBL are longer than

the others. The molecular width of the three-dimensional structure of the compounds had the same values (4.0-5.6 Å) (Table II). We focused on the chemical properties of these compounds, including the ClogP value and pKa. ClogP values, which are an indication of hydrophobicity, revealed that CA and DBL were the first and third highest ranking of the compounds ClogP = 1.55 and 1.01, respectively; Table II). This suggests that the structural difference of the propenyl side chain in the 1-position of the polyhydroxybenzene backbone contributes to the hydrophobicity. However, the pKa values of DBL and DB were larger than those for other compounds (pKa = 9.41 and 7.61, respectively; Table II). Thus, the presence of a free carboxylic acid moiety is the major determinant of the pKa value. These data suggest that the molecular length and ClogP value of CA and DBL are important factors that affect their topo II inhibitory activities, as they exhibited marked inhibition of human topo II activity and cancer cell proliferation.

Discussion

We observed that the LMW polyphenolics 1-7 isolated from *Inonotus obliquus* (Fig. 1) inhibited the activity of human topo II (Table I) and suppressed HCT116 cell growth (Fig. 2). In particular, CA and DBL were marked inhibitors of human topo II. The suppression of cell growth correlated with the inhibitory effects of these compounds on topo II, suggesting that topo II is critical for cell survival. To analyze the role of topo II in cancer cell growth and proliferation, we studied the effects of small interfering RNAs (siRNA) on targeting topo II in tumor cells.

CA is found in fruit (36), wine (37,38) and coffee (39). It exerts diverse biological effects and has antibacterial (39), antioxidative (40) and anti-inflammatory (37) properties. The molecular length, width and three-dimensional structure of CA and DBL, from which the energy-minimized compounds were calculated, were compared in Fig. 4. The length and width of CA is virtually identical to that of DBL. There is a potential inhibitor-binding pocket located on the topo II protein surface, with a width and length of ~4.0 Å and 8.0-8.7 Å, respectively, to accommodate the compounds. The pKa values of CA and DBL vary (pKa = 4.58 for CA and pKa = 9.41 for DBL), but these compounds have nearly the same ClogP values (ClogP: CA = 1.01; DBL = 1.55) (Table II). The molecular length, width and hydrophobicity (ClogP and surface area of the functional group negative/positive charges as shown in Fig. 4) of these compounds are important for their bioactivity, rather than their pKa.

Topo II inhibitors such as doxorubicin, amsacrine, ellipticine, saintopin, streptonigrin and terpentecin are DNA intercalating agents that bind the DNA molecule directly and subsequently indirectly inhibit topo II activity. These chemicals inhibit the DNA chain-rejoining reactions catalyzed by topo II by stabilizing a tight topo II protein-DNA complex called the 'cleavable complex'. The possibility that these LMW polyphenolics, in particular CA and DBL, also bind to DNA was examined by measuring the Tm of dsDNA, but none of these compounds were found to bind to dsDNA (data not shown). Therefore, we conclude that these compounds inhibit enzyme activity via direct interaction. Topo inhibitors are categorized into two classes, 'suppressors', which are considered to interact directly with the enzyme, and 'poisons', which stimulate DNA cleavage and intercalation (41). CA and DBL are considered to 'suppress' topo function rather than act as conventional poisons, as the compounds do not appear to stabilize topo II protein-DNA covalent complexes. These compounds may be a new class of topo II inhibitor.

In conclusion, several LMW polyphenolic compounds isolated from the medicinal mushroom *Inonotus obliquus* markedly suppress human cancer cell proliferation with cell cycle arrest associated with the inhibition of cellular topo II activity. CA and DBL should therefore be considered lead compounds in the search for novel cancer chemotherapy agents.

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