In vivo antitumor effect of liposomes with sialyl Lewis X including monogalactosyl diacylglycerol, a replicative DNA polymerase inhibitor, from spinach

YOSHIYUKI MIZUSHINA[1,2], TAKAHIKO HADA[3] and HIROMI YOSHIDA[1,2]

[1]Department of Nutritional Science, Laboratory of Food and Nutritional Sciences, Nishi-ku, Kobe, Hyogo 651-2180; [2]Cooperative Research Center of Life Sciences, Kobe-Gakuin University, Chuou-ku, Kobe, Hyogo 650-8586; [3]Hada Giken Co., Ltd., Yamaguchi-shi, Yamaguchi 753-0047, Japan

Received April 2, 2012; Accepted May 3, 2012

DOI: 10.3892/or.2012.1893

Abstract. The glycoglycerolipid monogalactosyl diacylglycerol (MGDG) isolated from spinach selectively inhibits the activities of replicative DNA polymerase species and suppresses the growth of human cancer cell lines, while not affecting normal human cells. Liposomes, carrying surface-bound sialyl Lewis X (SLX) and containing MGDG (SLX-Lipo-MGDG) and the fluorescent dye Cy5.5, were administered intravenously to mice bearing HT-29 human colon adenocarcinoma tumors and liposome distribution observed using fluorescence imaging equipment *in vivo*. In an *in vivo* antitumor assay on nude mice bearing HT-29 solid tumors, SLX-Lipo-MGDG was shown to be a stronger and more promising suppressor of solid tumors than MGDG alone. These results suggest that spinach MGDG could be developed into an anticancer compound, SLX-Lipo-MGDG could serve as an effective clinical anticancer drug and that these liposomes may be useful tools as the basis for active targeting drug delivery systems.

Introduction

Cancer is a major public health problem around the world, and epidemiologic and animal studies have indicated that vegetables and fruits with chemopreventive natural products, alone or in a mixture, are associated with reducing the risk of developing cancer (1-3). DNA polymerase [DNA-dependent DNA polymerase (pol), E.C. 2.7.7.7][1] catalyzes deoxyribonucleotide addition to the 3'-hydroxyl terminus of primed double-stranded DNA molecules (4). DNA replication, recombination and repair in eukaryotes are key systems in which pols have important maintenance roles (5). Pol inhibitors can thus be employed as anticancer chemotherapy agents because they in turn inhibit cell proliferation and, based on this idea, we have been screening for mammalian pol inhibitors from natural phytochemical products in vegetables and fruits for over 15 years.

The human genome encodes at least 15 DNA pols that conduct cellular DNA synthesis (6,7). Eukaryotic cells contain 3 replicative pols (α, δ and ε), 1 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] (8,9). Pols have a highly conserved structure, with their overall catalytic subunits showing little variance among species; conserved enzyme structures are usually preserved because they perform important cellular functions that confer evolutionary advantages. On the basis of sequence homology, eukaryotic pols can be divided into four main families, termed A, B, X and Y (9). Family A includes mitochondrial pol γ as well as pols θ and ν. Family B includes the three replicative pols α, δ and ε and also pol ζ. Family X comprises pols β, λ and μ as well as TdT; and last, family Y includes pols η, ι and κ in addition to REV1. Focusing on replicative pol inhibition supposes a concurrent antitumor effect, because replicative pols, such as B-family pols, are essential for cancer cell growth. As a result of this screening, we found that glycoglycerolipids from a fern and an alga potently inhibited eukaryotic pol activities (10,11).

In higher plants, particularly in chloroplasts, the thylakoid membrane contains major glycoglycerolipids, such as monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol and sulfoquinovosyl diacylglycerol (12). It is known that glycoglycerolipids are present in vegetables, fruits and grains (13,14), and it has been found here that spinach was the best glycoglycerolipid source, with the highest MGDG content, among the vegetables tested (15).

In this study, attention was focused on spinach MGDG (Fig. 1) and its antitumor effect on nude mice bearing solid

Correspondence to: Dr Yoshiyuki Mizushina, Department of Nutritional Science, Laboratory of Food and Nutritional Sciences, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan
E-mail: mizushin@nutr.kobegakuin.ac.jp

Abbreviations: pol, DNA-dependent DNA polymerase (E.C. 2.7.7.7); MGDG, monogalactosyl diacylglycerol; SLX, sialyl Lewis X; SLX-Lipo-MGDG, SLX-liposome containing MGDG; DDS, drug delivery system; PBS, phosphate-buffered saline; dTTP, 2'-deoxythymidine-5'-triphosphate; dNTP, 2'-deoxynucleotide-5'-triphosphate; FBS, fetal bovine serum; IC_{50}, 50% inhibitory concentration; LD_{50}, 50% lethal dose; HSA, human serum albumin

Key words: monogalactosyl diacylglycerol, DNA polymerase, enzyme inhibitor, liposome, sialyl Lewis X, antitumor

human tumors. In *in vivo* mouse experiments, the characteristically fat-soluble MGDG was difficult to solubilize in water and results were thus liable to fluctuate relative to the degree of MGDG solubilization. To solve this problem, liposomes were prepared which had sialyl Lewis X (SLX) bound to their surfaces and containing spinach MGDG (SLX-Lipo-MGDG). Liposomes are used as a drug delivery system (DDS) in medicine due to their unique properties, including the ability to carry both hydrophobic and hydrophilic molecules. To deliver cargo molecules at the sites of action, their lipid bilayers can fuse with other bilayers, such as cell membranes, thus delivering their liposome contents. By generating liposomes in a solution of one or more drugs, some of which would normally be unable to diffuse through the membrane, a medicine can be indiscriminately packaged inside the liposomes. Thus, liposomes, incorporating various substances, such as spinach MGDG, and delivering them to a diseased region, have already been used in practice (16,17). However, these DDSs control the effective liposome distribution to target cells by adjusting their particle size and surface electric charge in a passive manner, lacking cell-type specificity (18-20). In addition, trials thus far, providing liposomes with an active targeting ability by surface-binding various ligands, such as antibodies, transferrin, folic acid or monosaccharides, have had few successes (20,21). In this research group, the specific recognition and binding between lectin and sugar chains had been noticed and used for conferring active targeting ability to liposomes (22,23). Much research has been done on animal lectins, such that the molecular recognition mechanisms of sugar chains by lectins have become clear. Based on lectin primary structures, they are classified into fourteen types, which include C-type lectin, galectin, I-type lectin, P-type lectin and pentraxin (24-26). Among these, the mutual recognition of E-selectin, classified into the C-type lectin and SLX has been best clarified in an inflammation model. In a tumor inflammation region, the vascular endothelial cells are activated by inflammatory cytokines, IL-1β and TNF-α, and E-selectin then expressed on the cell surface, such that the sugar chain SLX on leucocyte surfaces then causes these cells to roll along on the vascular endothelial cells via gentle binding with E-selectin; this phenomenon is called 'rolling'. Leucocytes, rolling down through vascular endothelium gaps caused by inflammation, thus migrate into the tissues from the blood vessels; leucocytes appear to accumulate specifically in inflammation regions by such mechanisms (27,28).

In light of these observations, the results of this study are discussed in terms of the observed properties of SLX-Lipo-MGDG and their possible use in *in vivo* experiments, including clinical anticancer treatments.

Materials and methods

Materials. Dried spinach (*Spinacia oleracea* L.) was purchased from Kodama Foods Co., Ltd. (Hiroshima, Japan). SLX-Lipo-MGDG with and without contained Cy5.5 solution in distilled phosphate-buffered saline (PBS) were custom-produced by Katayama Chemical Industries Co., Ltd. (Osaka, Japan) using the improved cholate dialysis method with some modifications (29). A chemically synthesized DNA template, poly(dA), was purchased from Sigma-Aldrich (St. Louis, MO, USA) and a

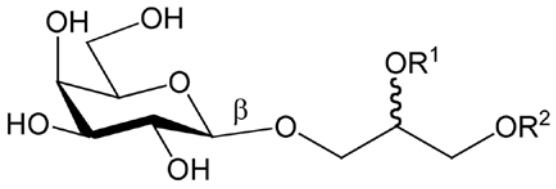

Figure 1. Chemical structure of MGDG. R^1 to R^2, acyloxy groups (fatty acids).

customized oligo$(dT)_{18}$ DNA primer produced by Sigma-Aldrich Japan K.K. (Hokkaido, Japan). The radioactive nucleotide [^{3}H]-deoxythymidine 5'-triphosphate (dTTP, 43 Ci/mmol) was obtained from MP Biomedicals LLC (Solon, OH, USA). All other reagents were analytical grade from Nacalai Tesque, Inc. (Kyoto, Japan).

Enzymes. Pols with high activities from mammals, a fish (cherry salmon), an insect (fruit fly) and plants (cauliflower and rice) were purified according to our previous report (30). Calf TdT, *Taq* pol, T4 pol and T4 polynucleotide kinase were purchased from Takara Bio (Tokyo, Japan). The Klenow fragment of pol I from *E. coli* was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). T7 RNA polymerase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (La Jolla, CA, USA).

Pol assays. The reaction mixtures for pol α, pol β, plant pols, and prokaryotic pols have been described previously (31,32); those for pol γ and for pols δ and ε were as described by Umeda *et al* (33) and Ogawa *et al* (34), respectively; for pols η, ι and κ the same as for pol α; and for pols λ and μ the same as for pol β. For pol reactions, poly(dA)/oligo$(dT)_{18}$ (A/T, 2/1) and 2'-deoxythymidine-5'-triphosphate (dTTP) were used as the DNA template-primer substrate and nucleotide (dNTP, 2'-deoxynucleotide-5'-triphosphate) substrate, respectively. For TdT reactions, oligo$(dT)_{18}$(3'-OH) and dTTP were used as the DNA primer and nucleotide substrate, respectively.

MGDG was dissolved in distilled DMSO at various concentrations and sonicated for 30 sec and 4 μl aliquots mixed with 16 μl of each enzyme (0.05 units) in 50 mM Tris-HCl at pH 7.5 containing 1 mM dithiothreitol, 50% glycerol (by vol), and 0.1 mM EDTA, and held at 0˚C for 10 min. These inhibitor-enzyme mixtures in 8 μl volumes were added to 16 μl of enzyme standard reaction mixture and incubated at 37˚C for 60 min, except for *Taq* pol, which was incubated at 74˚C for 60 min. Activity without inhibitor was considered 100% and relative activity determined for each inhibitor concentration. One unit of pol activity was defined as the amount of each enzyme that catalyzed incorporation of 1 nmol dNTP (specifically dTTP) into synthetic DNA template-primers in 60 min, at 37˚C and under normal reaction conditions (31,32).

Other enzyme assays. Activities of 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 Tamiya-Koizumi *et al* (35), Nakayama and Saneyoshi (36), Soltis and Uhlenbeck (37), and Lu and Sakaguchi (38), respectively.

Cell culture and cell viability assessment. Human cancer cells, including lung cancer cells A549 (JCRB0076), acute lymphoblastoid leukemia cells BALL-1 (JCRB0071), cervical cancer cells HeLa (JCRB9010), promyelocytic leukemia cells HL60 (JCRB0085) and gastric cancer cells NUGC-3 (JCRB0822), were supplied by the Health Science Research Resources Bank (Osaka, Japan). Human colon adenocarcinoma cancer cells HT-29 (ATCC no. HTB-38) were obtained from American Type Culture Collection (Manassas, VA, USA). Normal human cells, human umbilical vein endothelial cells HUVEC (CS-ABI-375) and human dermal fibroblast HDF (CS-2FO-101), were purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan).

A549, HeLa and HUGC-3 cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). BALL-1 and HL60 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1.6 mg/ml $NaHCO_3$. HT-29 cells were maintained in McCoy's 5A medium supplemented with 10% FBS, sodium bicarbonate (2 g/l) and streptomycin (100 μg/ml). HUVEC and HDF cells were cultured according to the manufacturer's instructions (Dainippon Sumitomo Pharma Co., Ltd.). MGDG cytotoxicity was investigated by inoculating ~$5x10^3$ cells/well in 96-well microtiter plates and the addition of spinach MGDG solution in DMSO at various concentrations. After incubation for 48 h at 37˚C in a humidified atmosphere of 5% CO_2/95% air, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution was added to a final 0.5 mg/ml in PBS for 3 h (39), after which time the medium was discarded and the cells lysed in acidified 2-propanol. The A570 was measured in a microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA).

Measurement of particle size and zeta-potential. Particle size and zeta-potential were measured at 25˚C using a Zetasizer Nano-S90 (Malvern Instruments, Ltd., Worcestershire, UK) and with the liposome solution diluted 50 times with distilled PBS.

Animal experiments. All animal studies were approved by the Kobe-Gakuin University Animal Committee according to the guidelines for the 'Care and Use of Laboratory Animals' of the University. Animals were anesthetized with pentobarbital before undergoing cervical dislocation. Five-week-old specific pathogen-free female Balb/c nu/nu mice (nude mice) were provided by Japan SLC, Inc. (Shizuoka, Japan), fed a standard diet (MF, Oriental Yeast Co., Ltd., Osaka, Japan) with water *ad libitum*, and maintained under a 12-h light/dark cycle and at 25˚C room temperature.

Production of tumor-bearing mice and SLX-Lipo-MGDG liposome distribution assessment in vivo. HT-29 cells at $5x10^6$ cells/mouse were subcutaneously inoculated to the right femoral region of nude mice (male, 7 week of age) and used for experiments 10 days later. For anesthesia, 200 μl of pentobarbital (Nembutal) solution, diluted 10 times with saline, was administered into the peritoneal cavity. Then, 100, 150 or 200 μl of SLX-Lipo-MGDG (20 mg/kg) containing Cy5.5 in PBS was administered through the tail vein. Using eXplore Optix (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) (Gallant *et al*, OSA BIOMED Meeting WD2, 2004), the Cy5.5 fluorescent signal was monitored in the tumor region (right femoral region) of the same mouse at 0, 24, 48 and 96 h after injection (excitation and emission, 680 and 700 nm, respectively).

Assessment of antitumor activity in vivo. HT-29 cells at $5x10^6$ cells/mouse were subcutaneously inoculated into nude mice and the resulting tumor-bearing mice divided randomly into four groups and treatment started with the SLX-Lipo in PBS, MGDG (4 or 20 mg of MGDG/kg) in PBS, SLX-Lipo-MGDG (4 or 20 mg of MGDG/kg) in PBS or vehicle control (PBS only) 5 days after implantation, when tumor volume [length x $(width)^2$ x 0.5] was 80-90 mm^3. These groups (n=6) were administered ~200 μl of treatment solution into the tail vein three times at 6-day intervals.

Results

Isolation of pol inhibitor MGDG from spinach. Vegetables were screened for eukaryotic pol inhibitors and the ethanol extract from dried spinach (*Spinacia oleracea* L.) was found to inhibit the activities of replicative pol species. The spinach ethanol solution was diluted to 70% ethanol and subjected to Diaion HP-20 (Sigma-Aldrich) column chromatography. The eluted solution of 95% ethanol (v/v) was evaporated to dryness, the residue redissolved in chloroform, and the resulting solution subjected to silica gel (PSQ60B, Fuji Silysia, Tokyo, Japan) column chromatography. After washing the column with chloroform/ethyl acetate (1/1, v/v), the column was eluted with ethyl acetate and the eluate purified using Sep-Pak C_{18} (Waters Corp., Tokyo, Japan) column chromatography eluted with methanol. The active fractions were evaporated, yielding the purified material which was ~98% of the chemical purity that can be obtained by normal-phase silica gel (Shiseido Co., Ltd., Tokyo, Japan) high performance liquid chromatography coupled with evaporative light scattering detector, eluted with chloroform/methanol (1/1, v/v).

The obtained purified compound was a yellow oily material that was analyzed by nuclear magnetic resonance, mass spectroscopy and optical rotation, and the chemical structure characterized as MGDG (Fig. 1) (data not shown). The acyloxy groups of MGDG (R^1 and R^2; Fig. 1) have various lengths and numbers of double bonds such that a molecular weight could not be determined.

Effects of the purified MGDG on the activities of DNA polymerases and other DNA metabolic enzymes. The effects of spinach MGDG on pols from various species and other DNA metabolic enzymes are shown in Table I. This compound selectively inhibited the activities of calf pol α, and human pols γ, δ and ε, with the inhibitory effect ranked pol ε>pol δ>pol α>>pol γ. The inhibitory effect on B-family pols α, δ and ε was stronger than on A-family pols, such as pol γ, with 50% inhibitory concentrations (IC_{50} values) observed at doses of 8.0-16.5 μg/ml and 26.3 μg/ml, respectively by family. On the other hand, MGDG did not influence the activities of the X-family pols, rat pol β, human pol λ and calf TdT, and the Y-family pols, human pol η, mouse pol ι and human pol κ, which suggested that MGDG was a selective inhibitor among mammalian pol species. This compound also

Table I. IC_{50} values of spinach MGDG on the activities of various pols and other DNA metabolic enzymes.

Enzyme	IC_{50} values (μg/ml)
Mammalian DNA polymerases	
A-Family	
Human DNA polymerase γ	26.3±1.4
B-Family	
Calf DNA polymerase α	16.5±1.1
Human DNA polymerase δ	14.9±1.0
Human DNA polymerase ε	8.0±0.6
X-Family	
Rat DNA polymerase β	>200
Human DNA polymerase λ	>200
Human DNA polymerase μ	>200
Calf terminal deoxynucleotidyl transferase	>200
Y-Family	
Human DNA polymerase η	>200
Mouse DNA polymerase ι	>200
Human DNA polymerase κ	>200
Fish DNA polymerases	
B-Family	
Cherry salmon DNA polymerase δ	18.2±1.1
Insect DNA polymerases	
B-Family	
Fruit fly DNA polymerase α	17.8±1.2
Fruit fly DNA polymerase δ	17.6±1.1
Fruit fly DNA polymerase ε	13.0±0.7
Plant DNA polymerases	
B-Family	
Cauliflower DNA polymerase α	>200
X-Family	
Rice DNA polymerase λ	>200
Prokaryotic DNA polymerases	
E. coli DNA polymerase I	>200
Taq DNA polymerase	>200
T4 DNA polymerase	>200
Other DNA metabolic enzymes	
Calf primase of DNA polymerase α	>200
T7 RNA polymerase	>200
T4 polynucleotide kinase	>200
Bovine deoxyribonuclease I	>200

MGDG incubated with each pol (0.05 units) and other DNA metabolic enzymes; 1 unit of pol activity defined as amount of enzyme that catalyzed incorporation of 1 nmol of dNTP into synthetic DNA template-primers in 60 min at 37˚C under each enzyme's normal reaction conditions; enzyme activity in absence of inhibitor was taken as 100%; data, mean ± SE (n=3).

inhibited the activities of fish pol δ, and insect pols α, δ and ε, all B-family, at almost the same concentrations that inhibited mammalian pols, with IC_{50} values of 13.0-18.2 μg/ml.

Table II. LD_{50} values of spinach MGDG on the growth of human cancer and normal cell lines.

Human cultured cell line	LD_{50} values (μg/ml)
Cancer cells	
A549 (lung cancer)	36.5±3.0
BALL-1 (acute lymphoblastoid leukemia)	28.9±2.4
HeLa (cervix cancer)	35.3±2.9
HL60 (promyelocytic leukemia)	27.4±2.2
HT-29 (colon adenocarcinoma)	25.9±2.2
NUGC-3 (stomach cancer)	35.6±2.9
Normal cells	
HDF (human dermal fibroblasts)	>200
HUVEC (human umbilical vein endothelial cells)	>200

Human normal and cancer cells incubated with MGDG for 48 h; viability determined by MTT assay; results calculated as percentage of value obtained from untreated cells; data, mean ± SE (n=5).

Table III. Particle size and formation of SLX-Lipo-MGDG.

Property	SLX-Lipo-MGDG
Particle size[a]	
0 mg/ml MGDG	222±8.7 nm
0.4 mg/ml MGDG	241±9.5 nm
2 mg/ml MGDG	285±11.6 nm
Absorbance (680 nm)	2.9
Lipid	0.93 mg/ml
Photodynamic inactivation	0.287
Protein	2.95 mg/mi
Zeta potential	-40 mV

[a]Data, mean ± SE (n=50).

In contrast, MGDG exhibited no effect on the plant pols, cauliflower pol α and rice pol λ, and the prokaryotic pols, *E. coli* pol I, *Taq* pol or T4 pol (Table I); the three-dimensional structures of eukaryotic pols are likely to differ greatly from prokaryotic pols. When activated DNA (bovine deoxyribonuclease I-treated DNA) and dNTPs were used as the DNA template-primer substrate and nucleotide substrate, respectively, in place of synthesized DNA [poly(dA)/oligo$(dT)_{18}$ (A/T=2/1)] and dTTP, respectively, the inhibitory effects of these compounds did not change (data not shown).

This MGDG had minimal influence on the activity of other DNA metabolic enzymes, such as primase of calf pol α, T7 RNA polymerase, T4 polynucleotide kinase or bovine deoxyribonuclease I. Collectively, these results suggested that this compound selectively inhibited the activity of animal family A and B pols, such as pols α, γ, δ and ε.

Effects of MGDG on cultured human cancer and normal cells. As pols conduct cellular DNA synthesis (4-6) and are

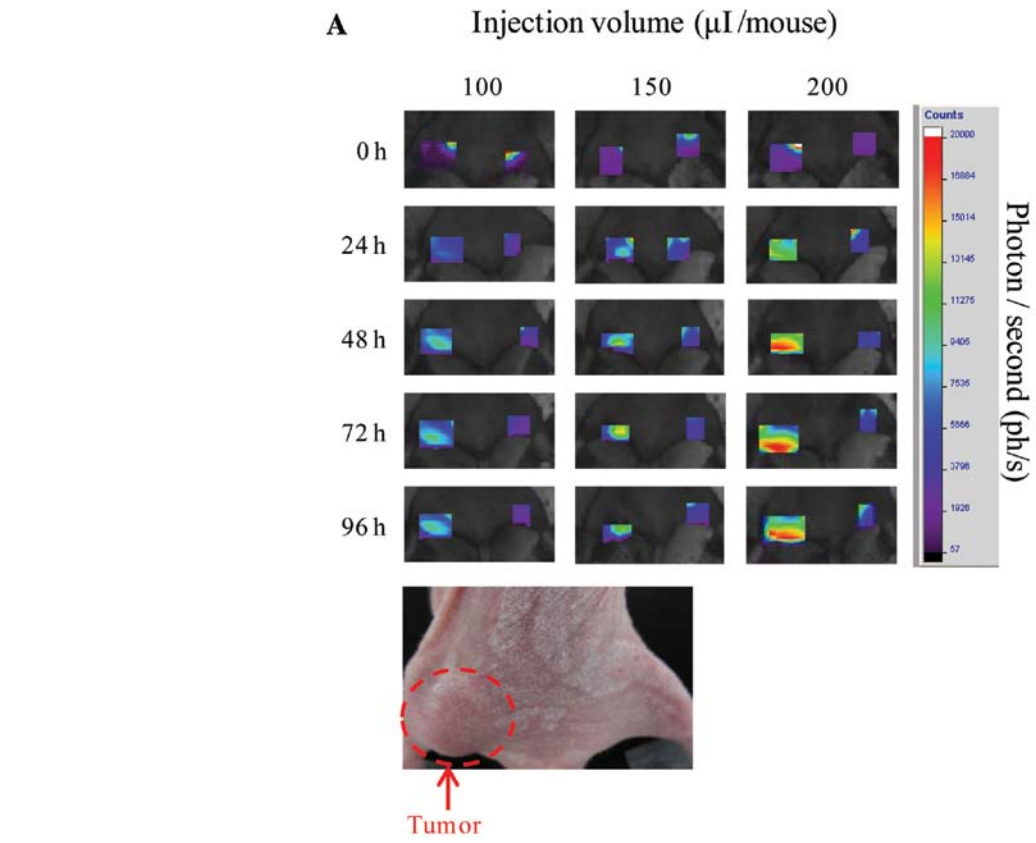

Figure 2. Accumulation of SLX-Lipo-MGDG containing Cy5.5 to tumor regions in a tumor-bearing mouse. (A) SLX-Lipo-MGDG (2 mg/ml) with Cy5.5 administered to tail vein (100, 150 or 200 μl/mouse); HT-29 solid tumor region (right femoral region) of same mouse observed by eXplore Optix (Ex, 680 nm; Em, 700 nm) at 0, 24, 48, 72 and 96 h post-injection; data normalized. (B) time-response curves of SLX-Lipo-MGDG containing Cy5.5 accumulation; data, quantified Cy5.5 fluorescent signal of (A); data, mean ± SE (n=3).

essential for DNA replication, repair and subsequent cell division, inhibition of these enzymes will lead to cell death, particularly under proliferative conditions. Thus, pol inhibitors can be considered potential agents for cancer chemotherapy and, thus, the effect of MGDG on cell growth was investigated in eight human cell lines.

MGDG suppressed the growth of all six human cancer cell lines tested, including A549, BALL-1, HeLa, HL60, HT-29 and NUGC-3, and showed the strongest growth inhibitory effect on HT-29 cells, with a 50% lethal dose (LD_{50} value) of 25.9 μg/ml (Table II). Effective suppression of cell growth involved concentrations similar to those for inhibition of mammalian pols α, γ, δ and ε by MGDG, suggesting that the cause of MGDG's influence in cancer cells may be its effect on pol activities, particularly the replicative pols α, δ and ε. The cytotoxic dose was almost the same or ~2-fold higher than the enzyme inhibitory concentrations (the range of LD_{50} and IC_{50} values for MGDG were 25.9-36.5 μg/ml and 8.0-26.3 μg/ml, respectively, Tables I and II), suggesting that MGDG could penetrate human cancer cells and inhibit nuclear and mitochondrial replicative pol activities.

In comparison, MGDG did not influence the growth of normal human cells HUVEC and HDF with a 48-h incubation (Table II), suggesting that MGDG could be a potent and selective anticancer chemotherapeutic agent.

Properties of SLX-Lipo-MGDG. The contents and properties of SLX-Lipo-MGDG prepared by the improved cholate dialysis method are shown in Table III. The particle size of SLX-Lipo (without MGDG), SLX-Lipo-MGDG (0.4 mg/ml) and SLX-Lipo-MGDG (2 mg/ml) showed an almost uniform distribution and mean particle size of 222-285 nm. The zeta-potential, showing the electric charge of liposome membrane surface, was negatively charged at -40 mV. After storage at 4˚C for 6 months, the particle size distribution was almost the same as right after preparation, demonstrating the stability of these liposomes. From electron microscopic observations, nearly all liposomes appeared spherical and 200-300 nm in size (data not shown).

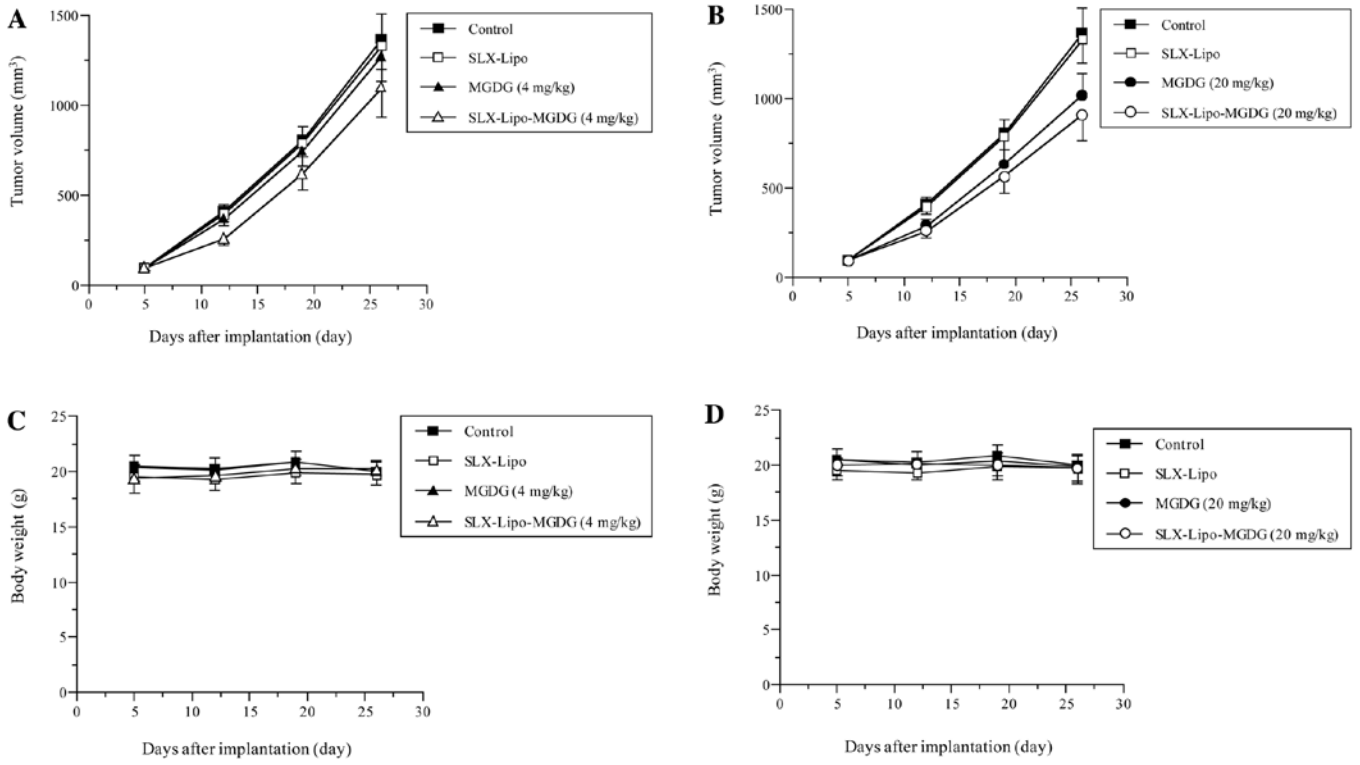

Figure 3. *In vivo* antitumor effects of MGDG and SLX-Lipo-MGDG. Nude mice bearing HT-29 solid tumors administered with PBS (control), SLX-Lipo, MGDG and SLX-Lipo-MGDG at a doses of (A) 4 mg and (B) 20 mg MGDG/kg; data, means ± SE (n=6). Body weight change of nude mice bearing HT-29 solid tumors administered with PBS (control), SLX-Lipo, MGDG and SLX-Lipo-MGDG at a doses of (C) 4 mg and (D) 20 mg MGDG/kg; data, means ± SE (n=6)..

Accumulation of SLX-Lipo MGDG containing Cy5.5 using in vivo imaging analysis. The HT-29 tumor region of Balb/c nude mouse was observed at 0, 24, 48, 72 and 96 h after administration using eXplore Optix. SLX-Lipo-MGDG (2 mg/ml) containing Cy5.5 accumulated significantly at the tumor region, increasing gradually until 48 h (Fig. 2A). Accumulations from 200 μl injections were greater than those of 100 and 150 μl injections and were thus dependent on injected amounts (Fig. 2B). On the other hand, accumulation of Lipo-MGDG with Cy5.5, with no SLX on the liposome surfaces, indicated no accumulation (data not shown). To examine overall fluorescence distribution, the whole body was scanned 96 h after injection and the liver, bladder and tumor regions exhibited the strongest fluorescence signals, which were analyzed in terms of fluorescence lifetime. In the tumor regions, only Cy5.5 bound to human serum albumin (HSA; lifetime, 1.8 ns) was detected, while in the liver and bladder, both free (lifetime, 1.5 ns) and HSA-bound Cy5.5 was identified (lifetime, 1.8 ns, data not shown). In addition, leucocyte rolling was observed on blood vessel endothelium in the tumor region by scanning fluorescent microscope, indicating that SLX-Lipo-MGDG was recognized by E-selectin expressed during tumor growth and, as a result, it accumulated in the tumor region. According to previous reports, the E-selectin expression pattern on human umbilical vein endothelial cells, induced by lipopolysaccharides, was not uniform but formed partial and high density aggregates on the cells (40,41). From these results, it was concluded that SLX-Lipo-MGDG bound partially, not uniformly, to the vascular endothelium.

Effect of SLX-Lipo MGDG on antitumor activity in vivo. At 5 days after HT-29 cell implantation, nude mice bearing solid tumors were intravenously injected with PBS (control), SLX-Lipo alone (0 mg MGDG/kg body weight), 4 or 20 mg/kg MGDG alone or 4 or 20 mg/kg SLX-Lipo-MGDG at 6-day intervals for a total of three injections. As the mouse body weights were ~20 g, 4 and 20 mg of MGDG/kg were equivalent to the administration of ~200 μl of 0.4 and 2 mg/ml MGDG, respectively. Tumor volumes of the groups administered the control and SLX-Lipo increased time-dependently, at 1370 and 1271 mm^3, respectively, by 26 days after the implantation (Fig. 3); therefore, SLX-Lipo alone showed no effect on tumor growth. SLX-Lipo-MGDG at 4 mg/kg significantly suppressed tumor growth by 12 days compared to the control and SLX-Lipo groups, and tumor volume decreased 20.3% by 26 days (Fig. 3A). In contrast, 4 mg/kg of MGDG alone exhibited barely any tumor suppression, suggesting that the DDS of MGDG in SLX-Lipo must be very important for an effective antitumor impact. Both 20 mg/kg of MGDG and SLX-Lipo-MGDG (20 mg/kg) suppressed tumor formation in a time-dependent manner, and the tumor volume decreases were 25.5 and 33.6%, respectively, at 26 days compared to the control (Fig. 3B). Thus, as MGDG inhibited the activities of replicative pols, this compound might also suppress tumor activity.

In the present study, none of the nude mice showed any significant loss of body weight throughout the experimental period (Fig. 3C and D), and it was also noted that the main visceral organs, such as the liver, lung, kidney, spleen, heart, stomach, small intestine, large intestine, pancreas and testes of all groups showed no toxic or degenerative histological

appearance (data not shown). These observations suggested that SLX-Lipo-MGDG did not have detectable side effects, such as animal death or evident toxicity, loss of body weight and/or major organ damage, in these mice and that this liposome system should be of great interest as a DDS candidate for anticancer treatment.

Discussion

MGDG is a non-nutrient compound found in vegetables, grains and fruits and, although its content varies among these plants (13), it is ingested daily in food. MGDG's chemical structure includes two acyloxy groups consisting of two fatty acid molecules (R^1 and R^2; Fig. 1). In the present study, spinach MGDG was rich in *n*-3 α-linolenic acid (26.3% of total fatty acids in spinach MGDG, data not shown). MGDG ingested from wheat flour includes non-*n*-3 fatty acids, such as *n*-6 linoleic acid, and saturated fatty acids (42), and it appears that the fatty acyl component influences the antitumor effects. Therefore, the present findings suggest that researchers should observe and pay attention to the lipid content and fatty acid composition in MGDG studies.

In this study, MGDG, isolated from a vegetable, spinach (*Spinacia oleracea* L.), was found to a selective inhibitor of mammalian pols α, γ, δ and ε, while having no effect on other mammalian pols, such as repair-related pols β, η, ι, κ, λ, μ and TdT (Table I). This MGDG prevented cell growth in 6 human cancer cell lines but had no effect on normal human cell proliferation (Table II). As the MGDG LD_{50} values on human cancer cell growth were almost the same or within 2-fold of MGDG's IC_{50} values on pol activities, this inhibition was concluded to be mostly led by effects on pol functions and, therefore, MGDG was able to penetrate cancer cells and reach the nucleus and mitochondria, thus inhibiting mammalian pols α, γ, δ and ε activities and leading to human cancer cell growth suppression. The mechanism of selective cell growth suppression between cancer and normal cell lines by MGDG remains unclear, and it may be considered that the expression amounts and activities of pols α, δ and ε, which are nuclear DNA replicative pols, as well as pol γ, which is a mitochondrial DNA replicative pol, in cancer cells are higher than in normal cells and, thus, MGDG could only inhibit cancer cell proliferation.

It has been reported that liposome retention in blood vessels significantly influences their accumulation in tumor regions and this accumulation increases by extending their retention in blood vessels (43). Such extension of blood vessel liposome residence would require 'avoidance of taking into reticuloendothelial system in the liver and the spleen', 'avoidance of the phagocytosis with the macrophage' and 'avoidance of non-specific adsorption with vascular endothelial cells and the cells such as leucocytes' (44). SLX-Lipo-MGDG is negatively charged (Table III), as are vascular endothelial cells and others, such as erythrocytes and leucocytes, and are thus electrically repulsed and do not adsorb non-specifically to such cells. In addition, hydrophilization of the liposome surface can prevent opsonin protein adsorption from blood plasma as well as macrophage phagocytosis and, consequently, extend liposome retention in blood stream.

The experiments here using MGDG *in vivo* were relatively difficult to perform because of its solubility. With the use of liposomes with SLX, high concentrations of MGDG (SLX-Lipo-MGDG) and particle sizes of 200-300 nm (Table III), this problem was easily solved and thus should prove helpful in the pharmaceutical application of MGDG. Indeed, liposomes stabilized with emulsifiers, such as phospholipids, have been receiving considerable attention as DDSs (28,44), and as drug carriers, these liposomes have many appealing properties, such as biodegradability and biocompatibility. Nanoparticulate systems using nanosomes (the lower nanometer range of liposome size) have been developed for pharmaceutical use and their application as drug carriers for anticancer therapies has recently attracted a great deal of attention. In the present results, SLX-Lipo-MGDG containing Cy5.5 was found to accumulate specifically and efficiently in murine-carried human tumor regions (Fig. 2), showing an affinity for E-selectin and excellent blood vessel retention. The antitumor effect *in vivo* of SLX-Lipo-MGDG was stronger than that of MGDG alone (Fig. 3). From the present findings, it was concluded that, by selecting appropriate sugar chains and densities, liposomes carrying sugar chains, such as SLX, could deliver MGDG, an effective replicative pol inhibitor, to the specific and desired (disease) region in the body, indicating that these liposome types might be useful as active-targeting DDSs.

Acknowledgements

We are grateful for the donation of calf pol α by Dr M. Takemura of Tokyo University of Science (Tokyo, Japan); rat pol β and human pols δ and ε by Dr K. Sakaguchi of Tokyo University of Science (Chiba, Japan); human pol γ by Dr M. Suzuki of Nagoya University School of Medicine (Nagoya, Japan); mouse pol η and human pol ι by Dr F. Hanaoka and Dr C. Masutani of Osaka University (Osaka, Japan); human pol κ by Dr H. Ohmori of Kyoto University (Kyoto, Japan); and human pols λ and μ by Dr O. Koiwai of Tokyo University of Science (Chiba, Japan). We thank M. Hirai of Katayama Chemical Industries Co., Ltd., for preparing SLX-Lipo-MGDG with and without Cy5.5. Y.M. acknowledges Grant-in-Aids from Grant-in-Aid for Scientific Research (C) (No. 24580205) from MEXT (Ministry of Education, Culture, Sports, Science and Technology, Japan), Takeda Science Foundation (Japan), and the Nakashima Foundation (Japan). This research was supported by Adaptable and Seamless Technology Transfer Program through Target-driven R&D (A-STEP), Japan Science and Technology Agency (JST) and the MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2012-2016.

References

1. Terry P, Giovannucci E, Michels KB, Bergkvist L, Hansen H, Holmberg L and Wolk A: Fruit, vegetables, dietary fiber, and risk of colorectal cancer. J Natl Cancer Inst 93: 525-533, 2001.
2. Surh YJ: Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 3: 768-780, 2003.
3. Liu RH: Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr 134: S3479-S3485, 2004.
4. Kornberg A and Baker TA: DNA replication. W.D. Freeman and Co., New York, NY, Chapter 6, 2nd edit. pp197-225, 1992.
5. DePamphilis ML: DNA replication in eukaryotic cells. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996.
6. Hübscher U, Maga G and Spadari S: Eukaryotic DNA polymerases. Annu Rev Biochem 71: 133-163, 2002.
7. Bebenek K and Kunkel TA: Functions of DNA polymerases. Adv Protein Chem 69: 137-165, 2004.

8. Takata K, Shimizu T, Iwai S and Wood RD: Human DNA polymerase N (POLN) is a low fidelity enzyme capable of error-free bypass of 5S-thymine glycol. J Biol Chem 281: 23445-23455, 2006.
9. Loeb LA and Monnat RJ Jr: DNA polymerases and human disease. Nat Rev Genet 9: 594-604, 2008.
10. Mizushina Y, Watanabe I, Ohta K, Takemura M, Sahara H, Takahashi N, Gasa S, Sugawara F, Matsukage A, Yoshida S and Sakaguchi K: Studies on inhibitors of mammalian DNA polymerase α and β: sulfolipids from a pteridophyte, *Athyrium niponicum*. Biochem Pharmacol 55: 537-541, 1998.
11. Ohta K, Mizushina Y, Hirata N, Takemura M, Sugawara F, Matsukage A, Yoshida S and Sakaguchi K: Sulfoquinovosyldiacylglycerol, KM043, a new potent inhibitor of eukaryotic DNA polymerases and HIV-reverse transcriptase type 1 from a marine red alga, *Gigartina tenella*. Chem Pharm Bull (Tokyo) 46: 684-686, 1998.
12. Roughan PG and Batt RD: The glycerolipid composition of leaves. Phytochemistry 8: 363-369, 1969.
13. Sugawara T and Miyazawa T: Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection. Lipids 34: 1231-1237, 1999.
14. Yunoki K, Sato M, Seki K, Ohkubo T, Tanaka Y and Ohnishi M: Simultaneous quantification of plant glyceroglycolipids including sulfoquinovosyldiacylglycerol by HPLC-ELSD with binary gradient elution. Lipids 44: 77-83, 2009.
15. Kuriyama I, Musumi K, Yonezawa Y, Takemura M, Maeda N, Iijima H, Hada T, Yoshida H and Mizushina Y: Inhibitory effects of glycolipids fraction from spinach on mammalian DNA polymerase activity and human cancer cell proliferation. J Nutr Biochem 16: 594-601, 2005.
16. Gabison A, Shmeeda H and Barenholz Y: Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies. Clin Pharmacokinet 42: 419-436, 2003.
17. Unezaki S, Maruyama K, Ishida O, Takahashi N and Iwatsuru M: Enhanced tumor targeting of Doxorubicin by ganglioside GM1-bearing long-circulating liposomes. J Drug Target 1: 287-292, 1993.
18. Maruyama K, Yuda T, Okamoto A, Kojima S, Suginaka A and Iwatsuru M: Prolonged circulation time *in vivo* of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly (ethylene glycol). Biochim Biophys Acta 1128: 44-49, 1992.
19. Gabizon A and Papahadjopoulos D: Liposome formulation with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci USA 85: 6949-6953, 1988.
20. Vyas SP, Singh A and Sihorkar V: Ligand-receptor-mediated drug delivery: an emerging paradigm in cellular drug targeting. Crit Rev Ther Drug Carrier Syst 18: 1-76, 2001.
21. Willis M and Forssen E: Ligand-targeted liposomes. Adv Drug Deliv Rev 29: 249-271, 1998.
22. Yamazaki N, Kojima S and Yokoyama H: Biomedical nanotechnology for active drug delivery systems by applying sugar-chain molecular functions. Curr Appl Phys 5: 112-117, 2005.
23. Yamazaki N: Active targeting DDS. Farumashia 42: 125-129, 2006.
24. Ehrhardt C, Kneuer C and Bakowsky U: Selectin-an emerging target for drug delivery. Adv Drug Deliv Rev 56: 527-549, 2004.
25. Dodd RB and Drickamer K: Lectin-like proteins in model organisms: implications for evolution of carbohydrate-binding activity. Glycobiology 11: R71-R79, 2001.
26. Kilpatrick DC: Animal lectins: a historical introduction and overview. Biochim Biophys Acta 1572: 187-197, 2002.
27. Bevilacqua MP, Stengelin S, Gimbrone MA Jr and Seed B: Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science 243: 1160-1165, 1989.
28. Vestweber D and Blanks JE: Mechanisms that regulate the function of the selectins and their ligands. Physiol Rev 79: 181-213, 1999.
29. Hirai M, Minematsu H, Kondo N, Oie K, Igarashi K and Yamazaki N: Accumulation of liposome with Sialyl Lewis X to inflammation and tumor region: application to *in vivo* bioimaging. Biochem Biophys Res Commun 353: 553-558, 2007.
30. Myobatake Y, Takeuchi T, Kuramochi K, Kuriyama I, Ishido T, Hirano K, Sugawara F, Yoshida H and Mizushina Y: Pinophilins A and B, inhibitors of mammalian A-, B-, and Y-family DNA polymerases and human cancer cell proliferation. J Nat Prod 75: 135-141, 2012.
31. Mizushina Y, Tanaka N, Yagi H, Kurosawa T, Onoue M, Seto H, Horie T, Aoyagi N, Yamaoka M, Matsukage A, *et al*: Fatty acids selectively inhibit eukaryotic DNA polymerase activities in vitro. Biochim Biophys Acta 1308: 256-262, 1996.
32. Mizushina Y, Yoshida S, Matsukage A and Sakaguchi K: The inhibitory action of fatty acids on DNA polymerase β. Biochim Biophys Acta 1336: 509-521, 1997.
33. Umeda S, Muta T, Ohsato T, Takamatsu C, Hamasaki N and Kang D: The D-loop structure of human mtDNA is destabilized directly by 1-methyl-4-phenylpyridinium ion (MPP+), a parkinsonism-causing toxin. Eur J Biochem 267: 200-206, 2000.
34. Ogawa A, Murate T, Suzuki M, Nimura Y and Yoshida S: Lithocholic acid, a putative tumor promoter, inhibits mammalian DNA polymerase β. Jpn J Cancer Res 89: 1154-1159, 1998.
35. Tamiya-Koizumi K, Murate T, Suzuki M, Simbulan CG, Nakagawa M, Takamura M, Furuta K, Izuta S and Yoshida S: Inhibition of DNA primase by sphingosine and its analogues parallels with their growth suppression of cultured human leukemic cells. Biochem Mol Biol Int 41: 1179-1189, 1997.
36. Nakayama C and Saneyoshi M: Inhibitory effects of 9-β-D-xylofuranosyladenine 5'-triphosphate on DNA-dependent RNA polymerase I and II from cherry salmon (*Oncorhynchus masou*). J Biochem 97: 1385-1389, 1985.
37. Soltis DA and Uhlenbeck OC: Isolation and characterization of two mutant forms of T4 polynucleotide kinase. J Biol Chem 257: 11332-11339, 1982.
38. Lu BC and Sakaguchi K: An endo-exonuclease from meiotic tissues of the basidiomycete *Coprinus cinereus*: its purification and characterization. J Biol Chem 266: 21060-21066, 1991.
39. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63, 1983.
40. Welply JK, Abbas SZ, Scudder P, Keene JL, Broschat K, Casnocha S, Gorka C, Steininger C, Howard SC and Schmuke JJ: Multivalent sialyl-LeX: potent inhibitors of E-selectin-mediated cell adhesion; reagent for staining activated endothelial cells. Glycobiology 4: 259-265, 1994.
42. Pober JS, Bevilacqua MP, Mendrick DL, Lapierre LA, Fiers W and Gimbrone MA Jr: Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. J Immunol 136: 1680-1687, 1986.
43. Sugawara T and Miyazawa T: Digestion of plant monogalactosyldiacylglycerol and digalactosyldiacylglycerol in rat alimentary canal. J Nutr Biochem 11: 147-152, 2000.
44. Drummond DC, Meyer O, Hong K, Kirpotin DB and Papahadjopoulos D: Optimizing liposome for delivery of chemotherapeutic agents to solid tumors. Pharmacol Rev 51: 691-743, 1999.
45. Yamazaki N, Kodama M and Gabius HJ: Neoglycoprotein–liposome and lectin-liposome conjugates as tools for carbohydrate recognition research. Methods Enzymol 242: 56-65, 1994.