Monogalactosyl diacylglycerol, a replicative DNA polymerase inhibitor, from spinach enhances the anti-cell proliferation effect of gemcitabine in human pancreatic cancer cells

Akasaka, Hiroaki

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Monogalactosyl diacylglycerol, a replicative DNA polymerase inhibitor, from spinach enhances the anti-cell proliferation effect of gemcitabine in human pancreatic cancer cells

ホウレンソウ由来の複製型DNAポリメラーゼ阻害剤モノガラクトシル・ジアシルグリセロール（MGDG）とゲムシタビン（GEM）のヒト膵癌細胞における抗細胞増殖活性の併用効果

赤坂　浩亮，　佐々木　良平，　吉田　賢史，　高山　いずみ，
山口　十四文，　吉田　弘美，　水品　善之

神戸大学大学院医学研究科医科学専攻
放　射　線　腫　瘍　学
（指導教員：佐々木良平特命教授）

赤　坂　　浩　亮

Key words: Monogalactosyl diacylglycerol (MGDG), Gemcitabine (GEM), DNA polymerase, Enzyme inhibitor, Anticancer, Cell proliferation, Apoptosis

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Monogalactosyl diacylglycerol, a replicative DNA polymerase inhibitor, from spinach enhances the anti-cell proliferation effect of gemcitabine in human pancreatic cancer cells

Hiroaki Akasaka[a], Ryohei Sasaki[a], Kenji Yoshida[a], Izumi Takayama[a], Toyofumi Yamaguchi[b], Hiromi Yoshida[c], Yoshiyuki Mizushina[c, d, *]

[a] Division of Radiation Oncology, Kobe University Graduate School of Medicine, Chuo-ku Kobe, Hyogo 650-0017, Japan

[b] Department of Life & Health Sciences, Teikyo University of Science, Adachi-ku, Tokyo 120-0045, Japan

[c] Laboratory of Food & Nutritional Sciences, Faculty of Nutrition, Kobe Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan

[d] Cooperative Research Center of Life Sciences, Kobe Gakuin University, Chuo-ku, Kobe, Hyogo 650-8586, Japan

Running title: Anticancer cell proliferative effect of GEM & MGDG

* Corresponding author at: Laboratory of Food & Nutritional Sciences, Faculty of Nutrition, Kobe Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan. Tel: +81-78-974-1551 (ext.3232); Fax: +81-78-974-5689.

E-mail: mizushin@nutr.kobegakuin.ac.jp (Y. Mizushina)

Abbreviations: pol, DNA-dependent DNA polymerase (E.C. 2.7.7.7); MGDG, monogalactosyl diacylglycerol; GEM, gemcitabine; GEM-TP, gemcitabine-5'-triphosphate; GEM-DP, gemcitabine-5'-diphosphate; GEM-MP, gemcitabine-5'-monophosphate; dTTP, 2'-deoxythymidine-5'-triphosphate; dNTP, 2'-deoxynucleotide-5'-triphosphate; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; FBS, fetal bovine serum; ssDNA, single-stranded DNA; IC_{50}, 50% inhibitory concentration; LD_{50}, 50% lethal dose.

Keywords:

Monogalactosyl diacylglycerol (MGDG)

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ABSTRACT

[BACKGROUND] Gemcitabine (GEM) is used to treat various carcinomas and represents an advance in pancreatic cancer treatment. In the screening for DNA polymerase (pol) inhibitors, a glycoglycerolipid, monogalactosyl diacylglycerol (MGDG), was isolated from spinach.

[METHODS] Phosphorylated GEM derivatives were chemically synthesized. *In vitro* pol assay was performed according to our established methods. Cell viability was measured using MTT assay.

[RESULTS] Phosphorylated GEMs inhibition of mammalian pol activities assessed, with the order of their effect ranked as: GEM-5'-triphosphate (GEM-TP) > GEM-5'-diphosphate > GEM-5'-monophosphate > GEM. GEM suppressed growth in the human pancreatic cancer cell lines BxPC-3, MIAPaCa2 and PANC-1 although phosphorylated GEMs showed no effect. MGDG suppressed growth in these cell lines based on its selective inhibition of replicative pol species. Kinetic analysis showed that GEM-TP was a competitive inhibitor of pol α activity with nucleotide substrates, and MGDG was a noncompetitive inhibitor with nucleotide substrates. GEM combined with MGDG treatments revealed synergistic effects on the inhibition of DNA replicative pols α and γ activities compared with GEM or MGDG alone. In cell growth suppression by GEM, pre-addition of MGDG significantly enhanced cell proliferation suppression, and the combination of these compounds was found to induce apoptosis. In contrast, GEM-treated cells followed by MGDG addition did not influence cell growth.

[CONCLUSIONS] GEM/MGDG enhanced the growth suppression of cells based on the inhibition of pol activities.

[GENERAL SIGNIFICANCE] Spinach MGDG has great potential for development as an anticancer food compound and could be an effective clinical anticancer chemotherapy in combination with GEM.

1. Introduction

Cancer is a major, worldwide, public health problem, and epidemiologic and animal studies have indicated that vegetables and fruits with chemopreventive natural products, alone or in mixtures, are associated with reducing the risk of developing cancer [1–3]. DNA polymerase (DNA-dependent DNA polymerase [pol], E.C. 2.7.7.7) catalyzes deoxyribonucleotide addition to the 3'-hydroxyl terminus of primed double-stranded DNA molecules [4]. As pols play important maintenance roles in key eukaryotic systems, such as DNA replication, recombination and repair [5], pol inhibitors can be employed as anticancer chemotherapy agents because they inhibit cell proliferation. Based on pol inhibitors' strategic effects, 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 over time 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. The focus is on replicative pol inhibition as it supposes a concurrent antitumor effect because replicative pols, such as B-family pols, are essential for the cell division required for cancer cell growth. As a result of this laboratory's ongoing screening

from natural materials and compounds, glycoglycerolipids from a fern and an alga have been identified that potently inhibit 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 possesses the best glycoglycerolipid source, with the highest MGDG content, among the vegetables tested [15].

Cytidine analogs, such as gemcitabine (GEM, 2',2'-difluoro-2'-deoxycytidine, dFdC) are widely used to treat a variety of cancers and remains in standard therapy for pancreatic cancer in adjuvant and palliative settings [16–18]. However, the GEM response rate is very low in pancreatic cancer, with only an 18% 1-year survival rate [19], which is attributed primarily to the lack of early detection and frequent metastases of primary tumors into lymph nodes and surrounding organs, such as liver and stomach [20–22]. In human cells, GEM must be metabolized by phosphorylation and catalyzed by deoxycytidine kinase to GEM-5'-monophosphate (GEM-MP), which can subsequently be phosphorylated sequentially to the di- and triphosphate forms, GEM-5'-diphosphate (GEM-DP) and GEM-5'-triphosphate (GEM-TP), respectively. Studies using LC/MS/MS have shown that GEM penetrates into cells and that clinically relevant levels of GEM was intracellularly phosphorylated for 24 h, with the converted GEMs, such as GEM-TP, pooling in the cells [23].

In this study, we focused our attention on evaluating human various cancer cell proliferation effects caused by a combination of GEM (Fig. 1A) or its phosphorylated compounds, such as GEM-TP (Fig. 1B), with spinach MGDG (Fig. 1C). Furthermore, the most effective timing of addition of these compounds was examined. In light of the results, their implications are discussed in terms of the observed properties of GEM and MGDG based

on mammalian replicative pol inhibition as well as better treatment outcomes for human pancreatic cancer.

2. Materials and methods

2.1. Materials

GEM (Fig. 1A) was purchased from Eli Lilly Japan K.K. (Kobe, Japan). Dried spinach (*Spinacia oleracea* L.) was purchased from Kodama Foods Co., Ltd. (Hiroshima, Japan). Calf thymus DNA was purchased from Sigma-Aldrich, Inc. (St. Louis, Inc., MO, USA), and four 2'-deoxynucleotide-5'-triphosphates (dNTPs), including 2'-deoxyadenine-5'-triphosphate, 2'-deoxycytidine-5'-triphosphates (dCTP), 2'-deoxyganine-5'-triphosphate and 2'-deoxythymidine-5'-triphosphate (dTTP), from GE Healthcare Life Sciences, Ltd. (Uppsala, SE). Radioactive [^{3}H]-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).

2.2. Chemical synthesis of phosphorylated GEM

N^4-benzoylgemcitabine [24] was converted to N^4-benzoylgemcitabine 5'-diphosphate and 5'-triphosphate by selective phosphorylation with phosphoryl chloride in triethyl phosphate [25], followed by further phosphorylation using a phosphorimidazolidate method [26]. GEM-MP, GEM-DP and GEM-TP were obtained by treatment of the corresponding N^4-benzoates with 1 M NH_4OH at room temperature for 1 d. When analyzed by high performance liquid chromatography (HPLC) equipped with a TSKgel DEAE-2SW column, (4.6 mm × 25 cm, Tosoh Bioscience LLC, King of Prussia, PA, USA), elution using 0.15 M potassium phosphate buffer at pH 6.95 and containing 20% CH_3CN, and spectrophotometric detection at 270 nm, the purities of GEM-MP, GEM-DP and GEM-TP were confirmed to be greater than 99, 96 and 98%, respectively.

2.3. Isolation of MGDG from spinach.

Dried spinach was extracted with ethanol and the extract diluted to 70% aqueous ethanol and then subjected to Diaion HP-20 (Sigma-Aldrich, Inc.) column chromatography eluted with 95% aqueous ethanol. The eluted solution was evaporated to dryness, the residue redissolved in chloroform, and the resulting solution subjected to silica gel (PSQ60B, Fuji Silysia Chemical Ltd., 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., Milford, MA, USA) column chromatography eluted with methanol. The MGDG fraction was evaporated, yielding purified MGDG at ~98% of the chemical purity that can be obtained by normal-phase silica gel (Shiseido Co., Ltd., Tokyo, Japan) HPLC coupled with an evaporative light scattering detector (M&S Instruments Inc., Osaka, Japan) and eluted with chloroform/methanol (1/1, v/v).

2.4. Pol assays

Mammalian pols with high activities were purified according to our previous report [27], and standard pol reaction mixtures for pols α and β have been previously described [28]; those for pol γ and for pols δ and ε described by Umeda *et al.* [29] and Ogawa *et al.* [30], respectively; for pols η, ι and κ the same as for pol α; and for pol λ, μ and TdT the same as for pol β. For pol reactions, activated DNA, in the form of bovine deoxyribonuclease I-treated calf thymus DNA, and four dNTPs containing [^{3}H]-dTTP were used as the DNA template-primer substrate and nucleotide substrate, respectively.

The protocol for pol inhibitory assay was shown in Supplemental Fig. 1. MGDG was dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. Then, 4 μL aliquots of the solutions were 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. GEM or a phosphorylated GEM was dissolved in distilled phosphate buffered saline (PBS) at various concentrations, and 4 μL aliquots mixed with 16 μL of the standard pol reaction mixture containing the DNA template-primer/nucleotide substrates and held at 0 °C for 10 min. These MGDG-enzyme mixtures in 8 μL volumes were then added to 20 μL of GEM-pol reaction mixture, and incubated at 37 °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 into synthetic DNA template-primers in 60 min, at 37 °C and under normal reaction conditions [28].

2.5. Cell culture and cell viability assessment

Human pancreatic cancer cell lines, such as BxPC-3, MIAPaCa2 and PANC-1, and human other cancer cell lines, such as lung (A549), prostate (DU145 and PC3), cervix (HeLa), hepatocellular liver (HepG2) and breast (MCF-7), were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI1640 medium supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL) and 1.6 mg/mL $NaHCO_3$. A human normal cell line, primary human dermal fibroblast cells (PHDFCs) and its complete medium kit were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). GEM, phosphorylated GEMs and MGDG cytotoxicities were investigated by inoculating ~1×10^3 cells/well in 96-well microtiter plates and the addition of these compounds to various concentrations. After incubation for 72–96 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 0.6 mg/mL in Milli-Q purified water for 2

h [31], after which time the medium was discarded and the cells lysed in DMSO. The A_{540} was then measured in a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA).

2.6. Assessment of apoptotic cells

The induction of apoptosis by the test compounds was evaluated by a DNA fragmentation assay using the APO-Direct™ Assay Staining kit (BD Bioscience Pharmingen, San Diego, CA, USA). This assay is a single-step method for labeling DNA breaks with fluorescein isothiocyanate-2'-deoxyuridine-5'-triphosphate (FITC-dUTP) followed by analysis using flow cytometry (FACS Calibur, Becton, Dickinson & Co., Franklin Lakes, NJ, USA). MIAPaCa2 cells were treated with GEM (50 nM) alone, MGDG (70 μM) alone, or a GEM-MGDG combination for 24 h. Cultured cells were harvested by trypsinization, washed with PBS, fixed in 1% paraformaldehyde for 15 min, and then in 70% ethanol overnight at -20°C. Fixed cells were next combined with a DNA labeling solution containing conjugated FITC for 30 min. The stained cells were then resuspended in PBS and subjected to flow cytometry analysis. The resulting data were analyzed with CellQuest software (Becton, Dickinson & Co.) and apoptotic cells quantitated as a percentage of the total number of cells.

3. Results

3.1. Effects of GEM and phosphorylated GEMs on mammalian pol activities

Initially, GEM (Fig. 1A) and its phosphorylated compounds, such as GEM-MP, GEM-DP and GEM-TP (Fig. 1B), were chemically synthesized and their *in vitro* biochemical action investigated. Next, the inhibition of four mammalian pols, calf pol α, rat pol β, human pol γ and human pol κ, by each compound at 10 μM was investigated. Pols α, β, γ and κ were used as representative of the B, X, A and Y families of pols, respectively [6–8]. Among these compounds, GEM-TP was the strongest inhibitor of these mammalian pols, while GEM had

no effect (Fig. 2). The inhibitory effect of these four compounds on mammalian pols was ranked as follows: GEM-TP > GEM-DP > GEM-MP > GEM; therefore, the degree of GEM phosphorylation appeared important for pol inhibition. The inhibitory effect of GEM-TP on the activities of pols α and γ, which are DNA replicative pols in the nucleus and mitochondria, respectively, was stronger than that of pols β and κ, which are DNA repair-related pols. Table 1 shows the 50% inhibitory concentrations (IC_{50}'s) of GEM-TP on the activities of 11 mammalian pols, showing a 4-fold stronger inhibition of B-family pols than X and Y-families of pols. It is interesting that GEM-TP is an effective inhibitor of DNA replicative pols, such as pols α, δ and ε, causing a 50% inhibition of these pols at 3.4–3.8 μM.

3.2. Effects of the purified spinach MGDG on the activities of mammalian pols

MGDG, a yellow oily material, was purified from dried spinach (*Spinacia oleracea* L.), and the chemical structure analyzed by nuclear magnetic resonance, mass spectroscopy, and optical rotation. Its structure was characterized (Fig. 1C) [12], and the two acyl groups of MGDG found to be mainly palmitoleic acid (C16:1) and α-linolenic acid (C18:3).

The effects of the purified spinach MGDG on mammalian pols are shown in Table 1. This compound selectively inhibited the activities of calf pol α, and human pols γ, δ and ε, with inhibitory effects ranked as: 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 pol γ, with IC_{50} observed at doses of 10.7–22.0 μM and 35.1 μM, respectively by family. On the other hand, MGDG did not influence the activities of X-family pols rat pol β, human pol λ, human pol μ and calf TdT, and Y-family pols human pol η, mouse pol ι and human pol κ, which suggested that MGDG was a selective inhibitor of DNA replicative pols, such as A and B-families of pols, among mammalian pol species.

3.3. Mode of inhibition of calf pol α by GEM-TP and MGDG

Next, to elucidate the mechanism of GEM-TP and MGDG's selective inhibition for mammalian pol species, the inhibitory mode of these compounds against calf pol α, a representative DNA replicative pol, was investigated. Activated DNA and four dNTPs were used as synthetic DNA template-primer substrate and nucleotide substrate, respectively, for kinetic analysis. The extent of inhibition as a function of the DNA template-primer substrate or nucleotide substrate concentration was measured (Table 2).

The collected data, expressed as Lineweaver-Burk plots (double reciprocal plots), showed that GEM-TP inhibited pol α activity noncompetitively with the DNA template-primer substrate because there was no change in the apparent Michaelis constant (K_m) of 7.80 μM, whereas decreases of 55.6, 27.8, 18.5 and 13.9 pmol/h in maximum velocity (V_{max}) were observed in the presence of GEM-TP at 0, 1, 2 and 3 μM, respectively. On the other hand, pol α inhibition by GEM-TP was competitive with the nucleotide substrate, with the V_{max} unchanged at 29.2 pmol/h and the K_m increased from 1.65 to 11.1 μM in the presence of 0–3 μM GEM-TP. The inhibition constant (K_i), obtained from Dixon plots, was found to be 1.35 μM for the DNA template-primer substrate and 0.67 μM for the nucleotide substrate. As the K_i value for the nucleotide substrate was ~2.0-fold less than that for the DNA template-primer substrate, it was concluded that GEM-TP had a greater affinity for the nucleotide substrate binding site than for the DNA template-primer binding site of the pol α protein.

Pol α inhibition by MGDG was noncompetitive with respect to both the DNA template-primer substrate and the nucleotide substrate. For the DNA template-primer substrate, there was no change in the apparent K_m (7.80 μM), while the V_{max} decreased from 55.6 to 15.2 pmol/h for the DNA template-primer substrate in the presence of 0–15 μM of MGDG. The induced inhibition of pol α activity by MGDG was noncompetitive with respect

to the nucleotide substrate, with the K_m unchanged at 7.80 μM and the V_{max} for the nucleotide substrate 4.97-fold less in the presence of 15 μM of MGDG. From the Dixon plots, the K_i was 5.08 and 6.44 μM for the DNA template-primer substrate and the nucleotide substrate, respectively. Therefore, MGDG had a 1.3-fold greater affinity for the DNA template-primer substrate binding site than for the nucleotide substrate-binding site of the pol α protein- These results suggested that GEM-TP, a dCTP analog, competed with dCTP to interact with the nucleotide substrate binding site of pol α, whereas MGDG bound to another site on pol α.

To confirm the pol α noncompetitive inhibitory mode against DNA template-primer substrate by GEM-TP and MGDG, their binding should be precisely analyzed by gel mobility shift assay as described previously [32]. Mammalian pol α is made up of four subunits, i.e. p180, p68, p54 and p46 [6,33]. The largest subunit, p180, and the smallest subunit, p46, have the catalytic DNA polymerase and DNA primase activities, respectively. The other subunits, p68 and p54, have no known enzyme activities. We constructed the core domain (p110) in which we deleted the amino-terminal (1–329) and the carboxyl-terminal (1280–1465) regions of the largest (p180) subunit of mouse pol α, and then the recombinant proteins were expressed and purified [34]. The core domain of pol α protein (0.2 nmol) was bound to M13 single-stranded DNA (ssDNA, 2.2 nmol; nucleotide), such as DNA template-primer substrate, and were shifted in the gel. When the I/E ratio (the molecular ratios of inhibitor and enzyme) was 100, both GEM-TP and MGDG had no interfered with the complex formation between M13 ssDNA and pol α (Supplemental Fig. 2). These results suggested that GEM-TP and MGDG did not compete with M13 ssDNA and had no interfered with the binding of DNA template-primer substrate to the largest subunit of pol α.

3.4. Inhibition by GEM-TP of mammalian pol activities with or without MGDG

As the pol α inhibitory mode of GEM-TP against nucleotide substrate was different from that of MGDG in kinetic analyses, the question of whether MGDG could enhance pol inhibition by GEM-TP was investigated. As described in Materials and methods, GEM-TP and MGDG were preincubated with the standard pol reaction mixtures containing DNA template-primer/nucleotide substrates and pol enzymes. The concentrations of GEM-TP and MGDG added were one half of the IC_{50} for each pol and 20 μM, respectively. In this study, calf pol α, rat pol β, human pol γ and human pol κ were used as representative of pol families of B, X, A and Y, respectively.

The rate of pol relative activity by GEM-TP at one-half of the IC_{50} value for each mammalian pol was set at 1 (pol relative activity of ~75%), and the ratios of the activities by MGDG alone and in combination with GEM-TP are shown in Fig. 3. As 20 μM MGDG alone did not inhibit the activities of pols β and κ, the rate of pol relative activity was 1.33 (this pol relative activity at 100%), and pols α and γ were slightly inhibited. The combination of GEM-TP with MGDG significantly enhanced the inhibition of pols α and γ, with the inhibitory effect for pol α 2-fold stronger than that for pol γ. The effect of the GEM-TP/MGDG combinations on pols β and κ activity was the almost same as with GEM-TP alone. These results suggested that mixtures of GEM-TP and MGDG showed synergistic effects on the inhibitory activity of DNA replicative pols, such as with pols α and γ according to the differences in the mode of pol inhibition.

3.5. Effects of GEM, phosphorylated GEMs, and MGDG on cultured human cancer cells

As pols conduct cellular DNA synthesis [4–6] and are essential for DNA replication, repair, and subsequent cell division, inhibition of these enzymes will lead to cell death, particularly under proliferative conditions, such that pol inhibitors can be considered potential agents for cancer chemotherapy. GEM is clinically used in the treatment of various

carcinomas and, in particular, possesses greater clinical benefit to pancreatic cancer patients compared with conventional medications [35]. Thus, the effect of GEM, phosphorylated GEMs and MGDG on the cultured cell growth was investigated in human various cancer cells, such as pancreatic carcinoma cell lines.

Here, GEM dose-dependently suppressed cell proliferation of three human pancreatic cancer cell lines, BXPC-3, MIAPaCa2 and PANC-1, with a 50% lethal dose (LD_{50}) of 3.0, 15.1 and 17.6 nM, respectively (Fig. 3A–3C), suggesting that GEM's effect on BXPC-3 cell growth inhibition was the strongest among the three lines. GEM might have been able to penetrate these human cancer cells, undergo phosphorylation to GEM-TP, which then reached the nucleus, and inhibited pol activities, with the specific inhibition of DNA replicative pol activity leading to cell growth suppression. On the other hand, phosphorylated GEM, such as GEM-MP, GEM-DP and GEM-TP, had no effect on cancer cell growth, which suggested that these compounds did not penetrate the cells.

MGDG also suppressed the growth of these human pancreatic cancer cell lines (Fig. 4D), with the growth suppression involving concentrations similar to those for inhibition of mammalian pols α, γ, δ and ε by MGDG, suggesting that the cause of MGDG's intracellular influence in cancer cells may be its effect on pol activities, particularly on the replicative pols α, δ and ε. The cytotoxic dose was almost the same range of enzyme inhibitory concentrations (LD_{50} and IC_{50} for MGDG, 15.1–22.0 and 10.7–35.1 μM, respectively, Fig. 4D and Table 2), suggesting that MGDG penetrated human cancer cells and effectively inhibited nuclear and mitochondrial replicative pol activities. It was considered here that GEM penetrated into cells more effectively than MGDG, as GEM exhibited a ~1000-fold stronger toxicity than MGDG.

Next, additional experiments were carried out on GEM and MGDG in the latter part of this study. As shown in Table 3, the influence of GEM on the growth of human other cancer cell lines, such as lung (A549), prostate (DU145 and PC3), cervix (HeLa), hepatocellular liver

(HepG2) and breast (MCF-7), and a human normal cell line, PHDFCs, was similar to that obtained for the pancreatic cancer cell lines. The LD_{50} values of MGDG for the three pancreatic cancer cell lines were >1.6-fold higher than those for other cancer cell lines. In contrast, MGDG exhibited no effect toward PHDFCs; therefore, MGDG form spinach, which is a food component, could selectively inhibit human cancer cell growth.

3.6. Cell growth inhibition by combinations of GEM with MGDG of cultured human cancer cells

The cell growth suppression effects of combined GEM and MGDG treatments were determined using various timed treatments in which three human pancreatic cancer cell lines were treated with mixtures of GEM & MGDG, GEM→MGDG, or MGDG→GEM [(a), (b), or (c) in Fig. 5A, respectively] for 48 h, the cells were cultured 48 h, and then the cytotoxicity assessed by MTT assay. The GEM and MGDG concentrations added were one half of the LD_{50} for each cell line and 20 μM, respectively.

With the rate of cell viability by GEM at one-half of the LD_{50} set at 1, as described above, the ratios of the viability by MGDG alone and the combination of GEM with MGDG were determined (Fig. 5B). The cell viability by 20 μM MGDG alone was 32.5–43.3% in the three human pancreatic cancer cell lines, and the ratio of cell viability of MGDG and GEM at one-half of the LD_{50} value was 0.43–0.58. In combining GEM and MGDG in treatments, the effects of schedules (a) (i.e., GEM & MGDG) and (b) (i.e., GEM→MGDG) were almost the same strength as MGDG alone, whereas schedule (c) (i.e., MGDG→GEM) showed synergistic effects on overall cytotoxicity, showing clearly that the latter combination was superior. The synergistic effect of schedule (c) on BxPC-3 cells, MIAPaCa2 cells and PANC-1 cells was 6.60, 28.7 and 13.0-fold stronger, respectively, than MGDG treatment alone; therefore, these effects showed the same tendency in all three cell lines. In contrast, the

enhancement of cell growth suppression by combination of GEM and MGDG was not shown against the other human cancer cell lines tested, because the cytotoxic rations of MGDG / schedule (c) (i.e., MGDG→GEM) were 0.94–1.47 (Table 4). These results suggested that the timing of the application of GEM and MGDG might be very important for the suppression of human pancreatic cancer cell proliferation in the use of clinical GEM-MGDG combination treatments.

3.7. Apoptosis induction in cultured human pancreatic cancer cells by combinations of GEM with MGDG

As MIAPaCa2 cells showed the highest synergistic effect from schedule (c) on cell growth suppression among the human pancreatic cancer cell lines tested (Fig. 4A–4C), the effects on this cell line were investigated further regarding the induction of apoptosis by a treatment combining 50 nM GEM with 70 μM MGDG as half time of schedule (c) (i.e., MGDG→GEM [GEM for 12 h after treated with MGDG for 12 h]) in Fig. 5A. In the DNA fragmentation assay using flow cytometry, the apoptotic cell rate of untreated MIAPaCa2 cells was normalized to 1, and treatment of both GEM alone and MGDG alone was found to slightly induce apoptosis (Fig. 6). On the other hand, the present combination of GEM with MGDG significantly enhanced the appearance of apoptotic cells, suggesting that the synergistic effect on cell proliferation suppression may also induce apoptosis (Fig. 5B(c)). The direct DNA fragmentation of MIAPaCa2 cells was analyzed by electrophoresis, and these cells clearly underwent DNA fragmentation by GEM, MGDG and the combination of MGDG→GEM (Supplemental Fig. 3). It is thus considered that DNA damage in the cells was caused immediately with GEM treatment and that pol inhibition by phosphorylated GEM, such as GEM-TP and MGDG might have promoted apoptosis.

Discussion

Pancreatic cancer remains one of the most deadly and chemoresistant cancers. Multiple studies have evaluated various chemotherapeutic agents, but few have produced significant improvement in patient survival [36]. GEM remains the first-line drug for the treatment of advanced pancreatic cancer, either alone or in combination with other chemotherapeutic agents, but the inherent resistance of pancreatic cancer to currently available chemotherapeutic agents presents a major challenge [36–38]. Identification of robust new molecular targets and relevant pathways to produce greater sensitivity to chemotherapeutic agents is a top priority [36]. Several mechanisms, involving factors such as STAT3 and NF-κB, have been reported to induce GEM resistance in preclinical models [39,40]. However, clinically, only one study has reported a survival improvement, of <2 weeks, with a combination of erlotinib and GEM [41], which highlights the urgent need to find novel agents against relevant molecular targets to produce new, more effective treatments. In this study, the focus was on DNA replicative pol species, especially the B-family pol α among 15 mammalian pols, as a new, metabolically critical molecular target to GEM treatment for human pancreatic cancer.

GEM is a nucleoside analog of dCTP, in which the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms (Fig. 1A), and can replace dCTP, one of the building blocks of nucleic acids during DNA replication by pols. Thus, the inhibitory mode of GEM-TP for pol α, a DNA replicative pol, was predictably competitive with the nucleotide substrate (Table 2). In the cells tested, GEM was enzymatically phosphorylated, such as to GEM-TP, and then arrested cell growth (Fig. 4A–4C), as only one additional nucleoside could be attached to the "faulty" nucleoside, resulting in cytotoxicity of both normal and cancer cells (Table 3) and induction of apoptosis (Fig .6).

MGDG is a glycoglycerolipid non-nutrient compound found in vegetables, grains and fruits and, although its content varies among these plants [42], it is ingested daily in food. MGDG's chemical structure includes two acyl groups derived from two fatty acid molecules (Fig. 1). Wheat flour MGDG includes non-*n*-3 fatty acids, such as *n*-6 linoleic acid and saturated fatty acids [43], and the fatty acyl component appears to influence MGDG's antitumor effects. Therefore, the present findings suggested that researchers should observe and pay close attention to the lipid content and inherent fatty acid composition in MGDG studies.

In this study, MGDG, isolated from 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 1). The mechanism of selective inhibition between A and B-families pols, which are DNA replicative pols, and X and Y-families of pols, which are DNA repair-related pols, by MGDG remains unclear and will require further study. This spinach MGDG prevented cell growth in three cancer cell lines (Fig. 4D) and, as the MGDG LD_{50}'s on cell growth were almost the same of MGDG's IC_{50}'s on pol activities, this inhibition was concluded to be mostly caused by direct effects on pol functions. MGDG appeared to be able to penetrate cancer cells and reach the nucleus and mitochondria, thus inhibiting mammalian pol α, γ, δ and ε activities and leading to cell growth suppression. It may be considered that the expression amounts and activities of pols α, δ and ε, all nuclear DNA replicative pols, as well as pol γ, a mitochondrial DNA replicative pol, in cancer cells are high and, thus, MGDG could inhibit various cancer cell proliferation, but did not influence normal cell growth (Table 3).

The present study is the initial investigation into the effects of combinations of GEM with MGDG, the latter a selective mammalian pol inhibitor. The results of this study indicated that GEM/MGDG enhanced the growth suppression of cells based on the inhibition

of pol activities (Table 1, Figs. 2 and 3). Furthermore, treatment of cells by MGDG→GEM addition [schedule (c) of Fig. 5A] showed significantly greater anticancer effects, such as inductions of cell death and apoptosis, than other combined GEM and MGDG treatments in human pancreatic cancer cells (Fig. 5 and Table 4). It is not clear at this moment why MGDG-treated cells followed by GEM addition (i.e., MGDG→GEM) significantly enhanced cell proliferation suppression in human pancreatic cancer cell lines, but had no affected on the enhancement in the other human cancer cell lines; therefore, we will consider them more stringently in future studies. These results indicated that combining selective inhibitors of mammalian pols, such as MGDG and GEM, might have clinical potential in a pancreatic cancer treatment strategy.

In conclusion, the present findings suggest that an anticancer mechanism in which GEM easily enters and is quickly phosphorylated to GEM-TP within human pancreatic cancer cells and subsequently suppresses cell proliferation based on pol inhibition. Spinach MGDG was found to selectively inhibit the activities of mammalian DNA replicative pols. In particular, MGDG's inhibitory activity against pol α was different from that of GEM-TP and, therefore, might further enhance the chemotherapeutic potential of GEM against pancreatic cancer. Spinach MGDG could be a functional food for anticancer regimes without side effects.

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Fig. 1. Chemical structure of GEM (A), GEM-TP (B) and MGDG (C).

Fig. 2. Effect of GEM and phosphorylated derivatives (GEM-MP, GEM-DP and GEM-TP) on mammalian pol activities. Compounds at 10 μM incubated with each enzyme at 0.05 units; % of relative activity; activities in absence of compounds, 100%; data, means ±SD of three independent experiments.

Fig. 3. Inhibitory effect of the combination of GEM-TP with MGDG on mammalian pol activities. GEM-TP and MGDG concentrations at one-half of IC_{50} of each pol (1.9, 9.3, 4.0 and 8.0 μM for pols α, β, γ and κ, respectively) and 20 μM, respectively; GEM-TP and MGDG preincubated with standard pol reaction mixtures, one containing DNA template-primer/nucleotide substrate and the other 0.05 units of pol enzyme, for 10 min at room temperature and these solutions were then mixed and incubated for 60 min at 37 °C; pol relative activity by GEM-TP at one-half of IC_{50} of each pol normalized to 1, and the ratios of the activity shown; data, means ±SD of three independent experiments.

Fig. 4. Dose-response curves of growth inhibition on cultured human pancreatic cancer cell lines by GEM, phosphorylated GEM and MGDG. (A–C) GEM and phosphorylated derivatives, GEM-MP, GEM-DP and GEM-TP, added to cultures of PXPC-3 cells, MIAPaCa2 cells and PANC-1 cells (A–C, respectively); (D) MGDG added to cell cultures, each incubated with various concentration of each compound for 96 h; cell proliferation determined by MTT assay [31]; % of relative activity; activities in absence of compounds, 100%; data, means ±SD of three independent experiments.

Fig. 5. Inhibitory effect of the combinations of GEM and MGDG on cultured human pancreatic cancer cell proliferation. (A) Treatments combining GEM and MGDG for three cell lines; GEM and MGDG concentrations added at one-half of LD_{50} of each cell line (1.5, 7.6 and 8.8 nM for BxPC-3 cells, MIAPaCa2 cells and PANC-1 cells, respectively) and 20 μM, respectively; (a), mixture of GEM and MGDG exposure for 48 h (i.e., GEM & MGDG); (b), GEM exposure for 24 h and then MGDG exposure for 24 h (i.e., GEM→MGDG); and (c), MGDG exposure for 24 h and then GEM exposure for 24 h (i.e., MGDG→GEM). (B) Cytotoxicity of cancer cells by MTT assay following treatments; cell viability by GEM at one-half of LD_{50} of each cell line normalized at 1, ratios of viability shown; data, means ±SD of three independent experiments.

Fig. 6. Detection of apoptosis in cultured human pancreatic cancer cells by combination of GEM with MGDG. Schedule (c) treatment (i.e., MGDG→GEM) using 50 nM GEM and 70 μM MGDG on MIAPaCa2 cells (Fig. 5A); treated cells cultured, harvested, stained with APO-Direct™ Assay Staining kit, analyzed using flow cytometry; nontreated apoptotic cell rate normalized to 1; ratios of apoptotic cell by GEM alone, MGDG alone, GEM-MGDG combination shown; data, means ±SD of three independent experiments.

Table 1

IC_{50} of GEM-TP and MGDG on mammalian pol activities

Mammalian pols	IC_{50} (μM)	
	GEM-TP	MGDG
[A-Family]		
Human pol γ	7.9 ±0.5	35.1 ±2.1
[B-Family]		
Calf pol α	3.7 ±0.3	22.0 ±1.4
Human pol δ	3.8 ±0.3	19.9 ±1.2
Human pol ε	3.4 ±0.2	10.7 ±0.7
[X-Family]		
Rat pol β	18.5 ±1.1	>200
Human pol λ	18.9 ±1.2	>200
Human pol μ	17.2 ±1.0	>200
Calf TdT	20.1 ±1.2	>200
[Y-Family]		
Human pol η	15.7 ±0.9	>200
Mouse pol ι	17.1 ±1.0	>200
Human pol κ	16.0 ±1.6	>200

Compounds incubated with each pol (0.05 units); 1 unit of pol activity, amount of enzyme catalyzing 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 taken as 100%; data, mean ±SD (n=3).

Table 2

Kinetic analysis of the inhibitory effects of GEM-TP and MGDG on calf pol α activity as a function of the DNA template-primer substrate dose and the nucleotide substrate concentration

Compound	Conc. (μM)	Substrate	K_m [a] (μM)	V_{max} [a] (pmol/h)	K_i [b] (μM)	Inhibitory mode
GEM-TP	0	DNA [c] template-primer	7.80	55.6	1.35	Noncompetitive
	1			27.8		
	2			18.5		
	3			13.9		
	0	Nucleotide [d]	1.65	29.2	0.67	Competitive
	1		3.23			
	2		6.25			
	3		11.1			
MGDG	0	DNA [c] template-primer	7.80	55.6	5.08	Noncompetitive
	5			29.4		
	10			20.0		
	15			15.2		
	0	Nucleotide [d]	1.65	29.2	6.44	Noncompetitive
	5			12.5		
	10			8.00		
	15			5.88		

[a] Data obtained from Lineweaver–Burk plot.

[b] Data obtained from Dixon plot.

[c] That is, activated DNA.

[d] That is, four dNTPs.

Table 3

LD_{50} of GEM and MGDG on the proliferation of human cancer and normal cells

	Cell line	LD_{50}	
		GEN (nM)	MGDG (μM)
Cancer cells	BxPC-3 (Pancreatic cancer)	3.0 ± 0.4	15.1 ± 1.7
	MIAPaCa2 (Pancreatic cancer)	15.1 ± 1.6	18.8 ± 2.0
	PANC-1 (Pancreatic cancer)	17.6 ± 1.8	22.0 ± 2.1
	A549 (Lung cancer)	17.1 ± 1.7	8.0 ± 0.9
	DU145 (Prostate cancer)	4.4 ± 0.5	9.4 ± 1.0
	HeLa (Cervix cancer)	23.4 ± 2.2	8.8 ± 1.0
	HepG2 (Hepatocellular liver cancer)	2.9 ± 0.3	0.33 ± 0.05
	MCF-7 (Breast cancer)	3.6 ± 0.4	6.3 ± 0.7
	PC3 (Prostate cancer)	7.7 ± 0.9	8.3 ± 1.0
Normal cells	PHDFCs (Primary human dermal fibroblast cells)	5.0 ± 0.6	> 100

The 9 human cancer cell lines and a normal human cell line, PHDFCs, were incubated with each compound for 48 h. Cell viability was determined using the MTT assay; data, mean ±SD (n=5).

Table 4

Effect of the combinations of GEM and MGDG on cell growth suppression against cultured human cancer cell lines

Cell line	Rate of cell viability (%)				
	GEM [a)]	MGDG [b)]	GEM & MGDG [c)]	GEM→MGDG [d)]	MGDG→GEM [e)]
BxPC-3	75 ± 7	33 ± 3	33 ± 4	38 ± 5	5.6 ± 0.7
MIAPaCa2	75 ± 7	43 ± 5	34 ± 4	30 ± 3	1.5 ± 0.2
PANC-1	75 ± 7	39 ± 4	36 ± 4	32 ± 4	3.0 ± 0.4
A549	75 ± 6	47 ± 8	46 ± 6	46 ± 9	43 ± 8
DU145	75 ± 6	48 ± 7	59 ± 3	67 ± 10	51 ± 11
HeLa	75 ± 9	48 ± 9	40 ± 8	47 ± 9	33 ± 6
HepG2	75 ± 6	46 ± 9	44 ± 8	42 ± 9	34 ± 4
MCF-7	75 ± 7	49 ± 7	46 ± 9	45 ± 8	39 ± 9
PC3	75 ± 8	47 ± 6	33 ± 3	35 ± 2	32 ± 4

Cell line	Ratio of cytotoxicity		
	MGDG / GEM & MGDG	MGDG / GEM→MGDG	MGDG / MGDG→GEM
BxPC-3	1.00	0.88	6.60
MIAPaCa2	1.26	1.43	28.7
PANC-1	1.08	1.22	13.0
A549	1.09	1.09	1.16
DU145	0.81	0.72	0.94
HeLa	1.20	1.02	1.45
HepG2	1.05	1.10	1.35
MCF-7	1.07	1.09	1.26
PC3	1.42	1.34	1.47

[a)] One-half of LD_{50} values of GEM were used.

[b)] The used MGDG concentration for pancreatic cells (BxPC-3, MIAPaCa2 and PANC-1) was 20 μM. MGDG concentrations of A549, DU145, HeLa, HepG2, MCF-7 and PC3 were 10, 10, 10, 0.5, 8 and 10 μM, respectively.

[c)] Mixture of GEM and MGDG exposure for 48 h (i.e., GEM & MGDG), the treatment schedule of Fig. 5A (a).

[d] GEM exposure for 24 h and then MGDG exposure for 24 h (i.e., GEM→MGDG), the treatment schedule of Fig. 5A (b).

[e] MGDG exposure for 24 h and then GEM exposure for 24 h (i.e., MGDG→GEM), the treatment schedule of Fig. 5A (c).

Data, mean ±SD (n=5).

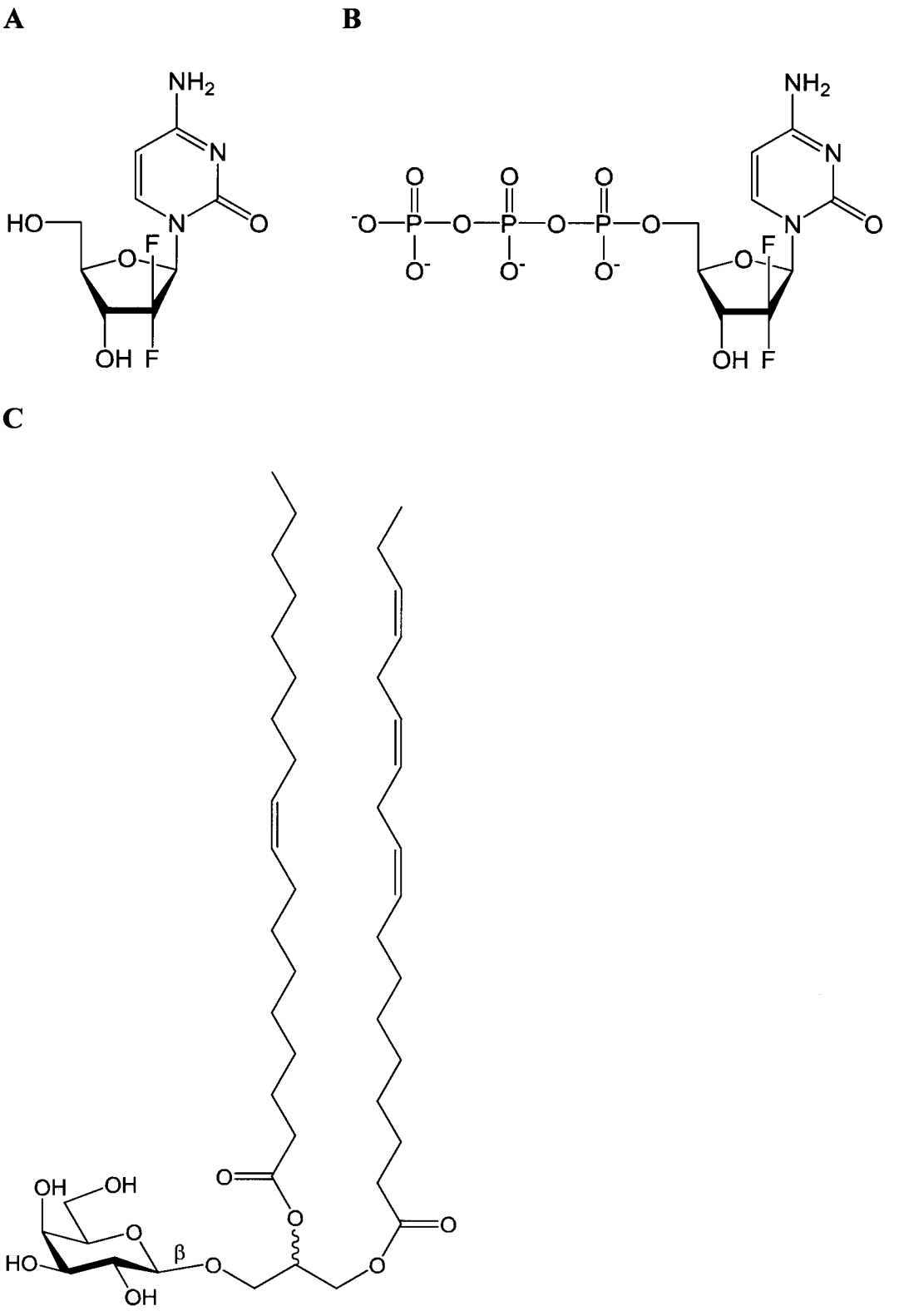


Fig. 1. Akasaka *et al.*

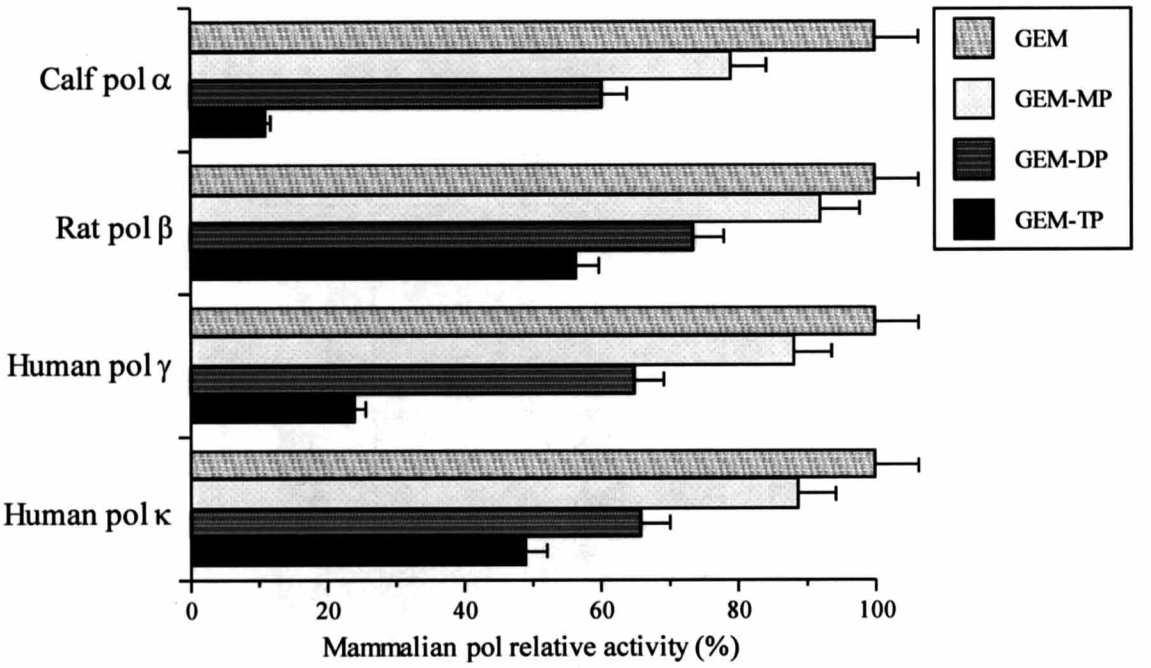


Fig. 2. Akasaka *et al.*

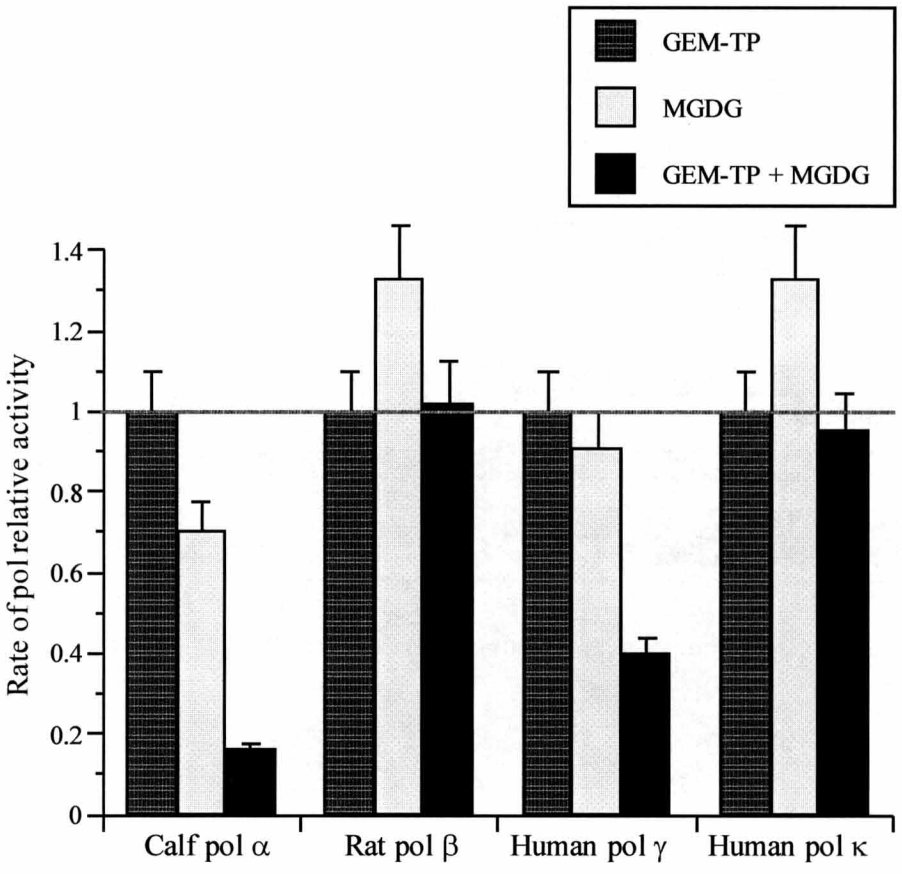


Fig. 3. Akasaka *et al.*

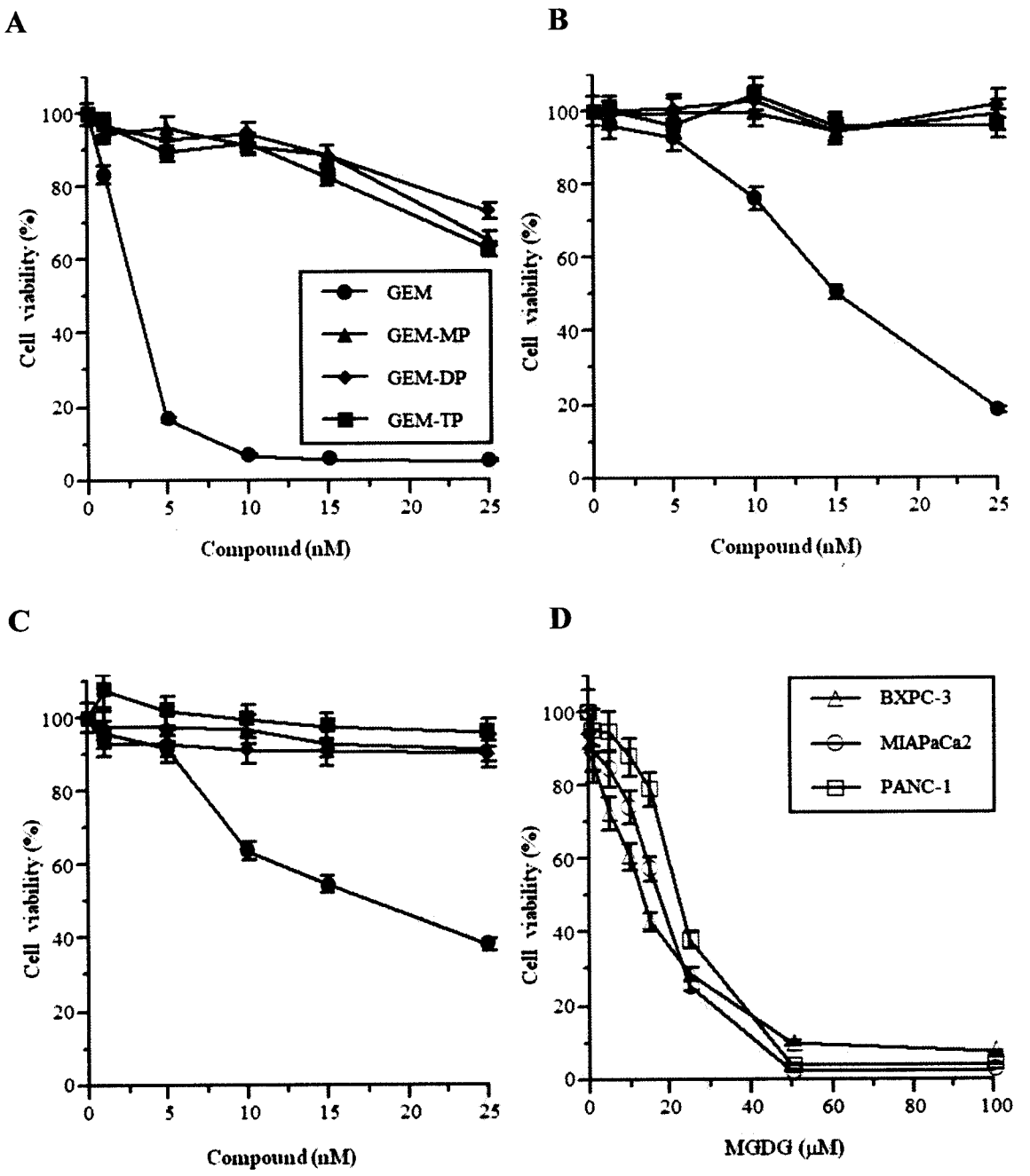
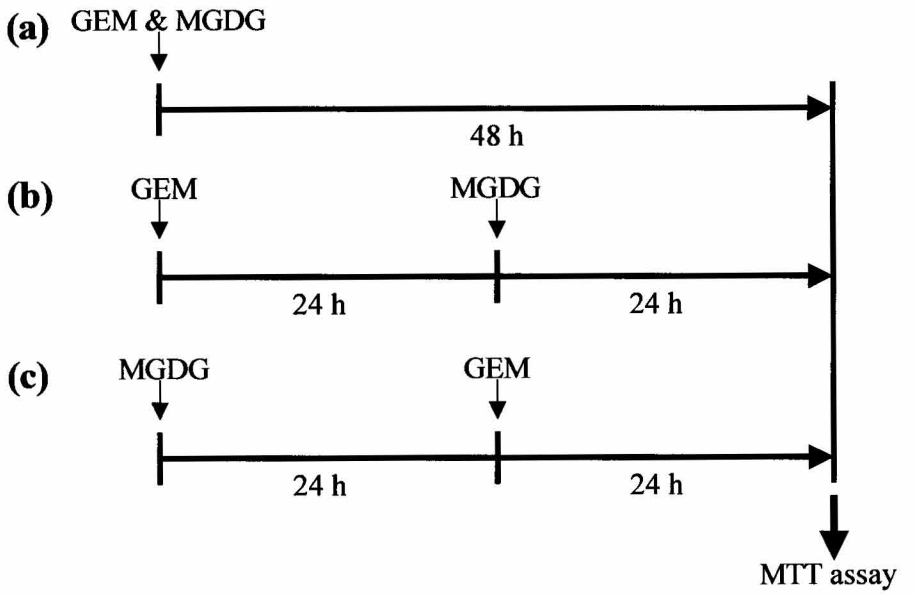


Fig. 4. Akasaka *et al.*

A



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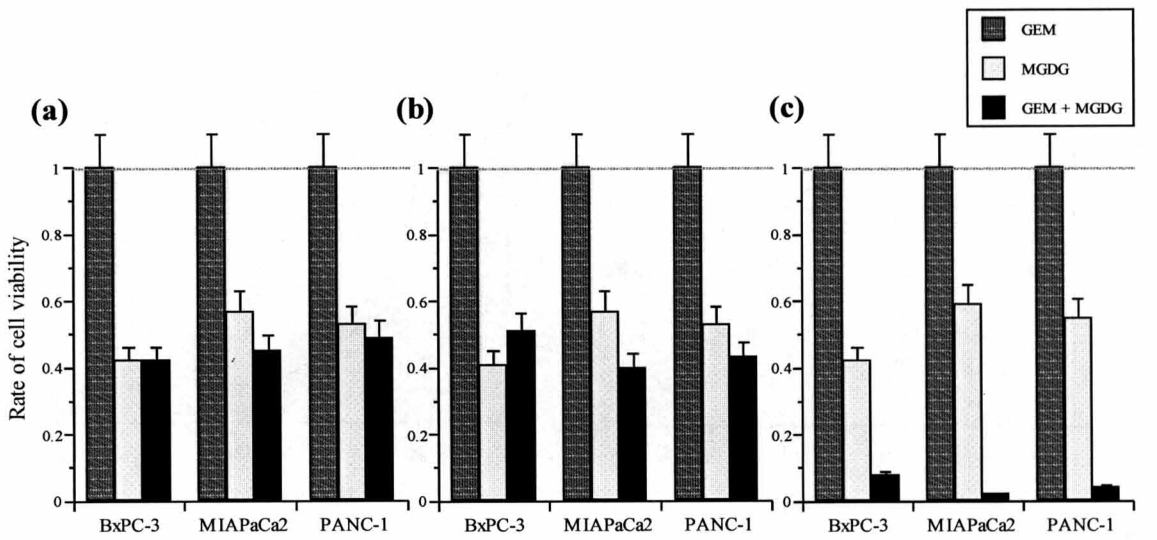

Fig. 5. Akasaka *et al.*

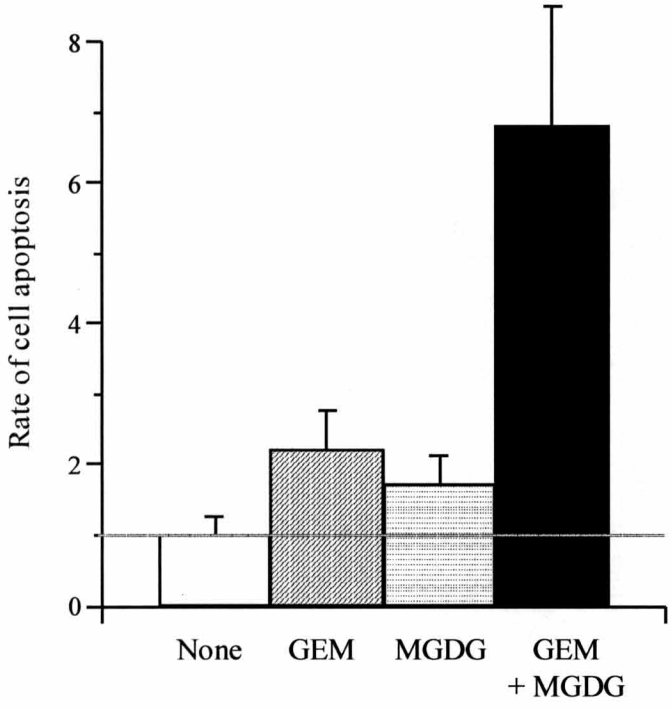

Fig. 6. Akasaka *et al.*

Highlights

Gemcitabine (GEM) suppressed growth in the human pancreatic cancer cell lines.

Spinach monogalactosyl diacylglycerol (MGDG) suppressed cancer cell growth.

GEM-MGDG combination revealed synergistic effects on the cancer cell growth.

These enhancements might cause the inhibition of mammalian DNA polymerase.

MGDG from spinach could be an effective clinical anticancer chemotherapy of GEM.