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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

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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

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

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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 Sigma-Aldrich Inc. (St. Louis, MO, USA). Dried spinach (*Spinacia oleracea* L.) was purchased from Kodama Foods Co., Ltd. (Hiroshima, Japan). Calf thymus DNA was purchased from Sigma-Aldrich, Inc., 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-benzoyl-gemcitabine 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

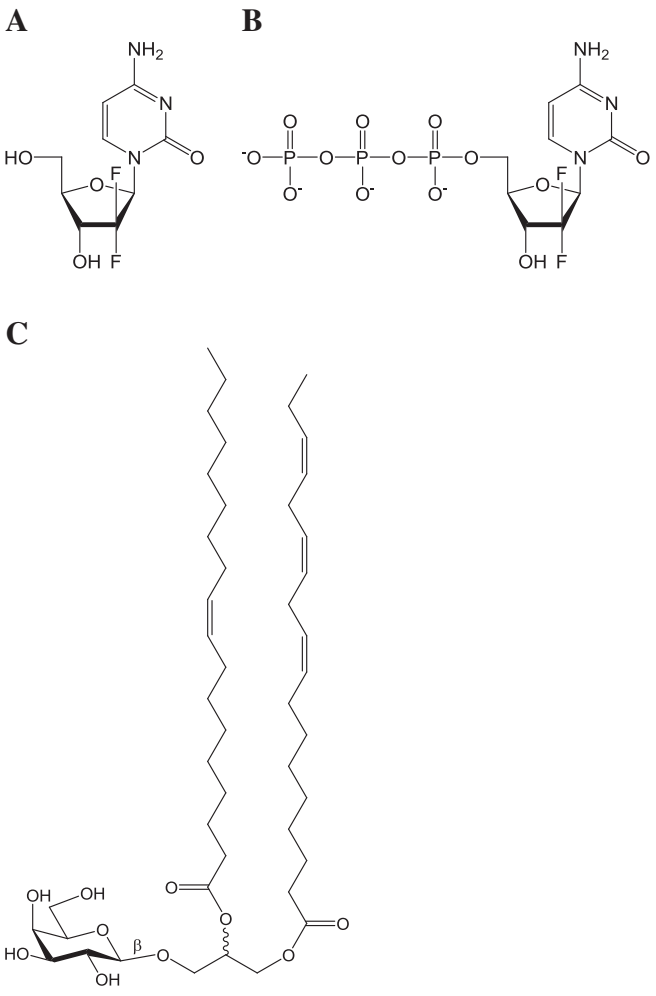

Fig. 1. Chemical structure of GEM (A), GEM-TP (B) and MGDG (C).

treatment of the corresponding N^4-benzoates with 1 M NH_4OH at room temperature for 1 day. 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).