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Inhibition of cyclooxygenase and prostaglandin E_2 synthesis by γ-mangostin, a xanthone derivative in mangosteen, in C6 rat glioma cells☆

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Abstract

The fruit hull of mangosteen, *Garcinia mangostana* L., has been used for many years as a medicine for treatment of skin infection, wounds, and diarrhea in Southeast Asia. In the present study, we examined the effect of γ-mangostin, a tetraoxygenated diprenylated xanthone contained in mangosteen, on arachidonic acid (AA) cascade in C6 rat glioma cells. γ-Mangostin had a potent inhibitory activity of prostaglandin E_2 (PGE_2) release induced by A23187, a Ca^{2+} ionophore. The inhibition was concentration-dependent, with the IC_{50} value of about 5 μM. γ-Mangostin had no inhibitory effect on A23187-induced phosphorylation of p42/p44 extracellular signal regulated kinase/mitogen-activated protein kinase or on the liberation of [^{14}C]-AA from the cells labeled with [^{14}C]-AA. However, γ-mangostin concentration-dependently inhibited the conversion of AA to PGE_2 in microsomal preparations, showing its possible inhibition of cyclooxygenase (COX). In enzyme assay *in vitro*, γ-mangostin inhibited the activities of both constitutive COX (COX-1) and inducible COX (COX-2) in a concentration-dependent manner, with the IC_{50} values of about 0.8 and 2 μM, respectively. Lineweaver–Burk plot analysis indicated that γ-mangostin competitively inhibited the activities of both COX-1 and -2. This study is a first demonstration that γ-mangostin, a xanthone derivative, directly inhibits COX activity.

Keywords: γ-Mangostin; Cyclooxygenase; PGE_2; C6 rat glioma cells; Competitive inhibition

1. Introduction

Mangosteen, *Garcinia mangostana* L. (Guttifereae), is a tree that is fairly widespread in Thai, India, Srilanka, and Myanmar and is known for its medicinal properties. The fruit hull of this plant has been in use in Thai indigenous medicine for the treatment of skin infections, wounds, and diarrhea for many years [1]. Thus, mangosteen may be expected to contain an anti-inflammatory drug. γ-Mangostin, a constituent of the fruit hull, is a tetraoxygenated diprenylated xanthone derivative. Xanthone derivative has been reported to possess several pharmacological activities, such as antimalarial activity [2], antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) [3], and inhibitory activity for monoamine oxidase [4].

In the brain, prostaglandin E_2 (PGE_2) levels are very low or undetectable in normal conditions, but can rise during inflammatory processes, multiple sclerosis, and AIDS-associated dementia [5,6]. High levels of PGE_2 can affect the activities of several cell types, including neurons, glial, and endothelial cells, and can regulate microglia/macrophage and lymphocyte functions during inflammatory and immune processes [7]. Therefore, the interplay between PGE_2 and

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[1] Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; COX, cyclooxygenase; COX-1, constitutive COX; COX-2, inducible COX; $cPLA_2$, cytosolic PLA_2; EMEM, Eagle's minimum essential medium; ERK, extracellular signal regulated kinase; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MAPK, mitogen-activated protein kinase;PGE_2, prostaglandin E_2; $sPLA_2$, secretory phospholipase A_2; TBST, Tris-buffered saline containing 0.05% Tween 20.

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other local factors, including pro- and anti-inflammatory cytokines, is likely to influence the outcome of inflammatory and immune responses in the central nervous system (CNS). There is considerable evidence linking the generation of prostaglandins (PGs) with inflammation, pain, and fever. Glial cells, which outnumber neurons by about 10 to 1 in the brain, provide both mechanical and metabolic support for neurons. Glial cells are assumed to be an important source of PGs in the CNS [8]. It is suggested that regulation of arachidonic acid (AA) metabolism, particularly PGE_2 production, appears beneficial in patients with inflammatory conditions [9].

Phospholipase A_2 (PLA_2) is subdivided into several groups based on their structures and enzymatic characteristics [10–12]. Secretory (s)PLA_2 is a family of low-molecular-mass (~14 kDa) enzymes that require millimolar concentration of Ca^{2+} for enzymatic activity. Cytosolic ($cPLA_2$), or group IV PLA_2, is a ubiquitously distributed 85 kDa enzyme, the activation of which is tightly regulated by postreceptor transmembrane signaling. $cPLA_2$ is activated by p42/p44 extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) under cytosolic Ca^{2+} concentration of the submicromolar or micromolar range, and p42/p44 ERK/MAPK was activated by dual phosphorylation of tyrosine/threonine residues [11,13].

Cyclooxygenase (COX) is the rate-limiting enzyme in PG synthesis and exists as two isoforms, constitutive (COX-1) and inducible (COX-2). These isoforms originate from distinct genes, but are structurally conserved [14,15]. COX-1 is regarded as a constitutive enzyme whose expression is developmentally regulated. PGs produced by COX-1 primarily function in fluid and electrolyte homeostasis, gastric acid secretion, and platelet aggregation. In contrast, COX-2 is expressed in response to inflammatory stimuli and is active in physiological responses to growth factors and glucocorticoids [16].

In this study, we examined the effect of γ-mangostin (Fig. 1), a tetraoxygenated diprenylated xanthone contained in mangosteen, on AA cascade in C6 rat glioma cells. The results suggest that γ-mangostin, a xanthone derivative, directly inhibits COX activity.

2. Materials and methods

2.1. Materials

Fetal bovine serum was obtained from Cell Culture Laboratory (Cleveland, OH, USA); horse serum was purchased from Dainippon Pharmaceutical Co. Ltd.; F-10 (Nutrient Mixture: Ham) was obtained from GIBCO BRL; γ-Mangostin was purified from the fruit hull of *G. mangostana* L., with its purity of more than 90% determined by an high-performance liquid chromatography analysis. It was dissolved in dimethyl sufoxide to make a concentration of 20 mM and used after its dilution. Eagle's minimum essential medium (EMEM) was purchased from Nissui Pharmaceutical Co. Ltd. PGE_2 was a generous from Ono Pharmaceuticals. Anti-PGE_2 antibody was obtained from Chemicon International Inc. [^{3}H]-PGE_2 (200 Ci/mmol) and [^{14}C]-AA (51 mCi/mmol) were from NEN/DuPont. Anti-phospho-p44/p42 ERK/MAPK antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG were obtained from New England Biolabs. COX-1 and -2 were purchased from Cayman Chemical. AA was obtained from Sigma Chemical Co., Ltd. AA and [^{14}C]-AA were diluted by dimethyl sulfoxide. A final concentration of less than 0.5% dimethyl sulfoxide was kept. Other chemicals and drugs were of reagent grade or of the highest quality available.

2.2. Cell culture

C6 rat glioma cells were grown in F-10 medium containing 15% horse serum and 2.5% fetal bovine serum in a 37° humidified incubator in an atmosphere of 5% CO_2 in air.

2.3. Assay of PGE_2

C6 cells were seeded into 12-well plates at the density of 1.0×10^5 cells per well. The experiment was performed 2 days after seeding of cells. The cells were washed twice with EMEM buffered with 20 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; EMEM-HEPES), pH 7.35, and were pre-incubated with or without γ-mangostin for 10 min. The cells were further incubated with or without 10 μM A23187 for an additional 10 min. The medium was acidified to pH 4.0 by addition of 1 N HCl, and PGE_2 was extracted twice with ethyl acetate. After ethyl acetate was evaporated under a stream of N_2 gas, the sample was dissolved in 10 mM Tris-HCl (pH 7.6). PGE_2 was determined by radioimmunoassay, as described previously [17,18].

2.4. Immunoblotting

C6 cells were seeded into 6-well plates at the density of 2.0×10^5 cells per well. Two days after seeding, the cells were washed twice with EMEM-HEPES and pre-incubated with or without γ-mangostin for 10 min at 37°. After the cells were incubated with or without 10 μM A23187 for an additional 2 min, the medium was aspirated. The cells were solubilized by the addition of Laemmli sample buffer [19], the composition of which was Tris-HCl 187.5 mM, sodium dodecyl sulfate 6%, glycerol 30%, 2-mercaptoethanol 15%, pH 6.8. The sample was boiled at 95° for 5 min. Electrophoresis was performed on 11% acrylamide gels. Proteins were transferred electrically from the gel onto Immobilon polyvinylidene difluoride membranes (Millipore) by the semi-dry blotting method [20]. The immunoblots were blocked for 2 h with 2% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween 20 (TBST) at

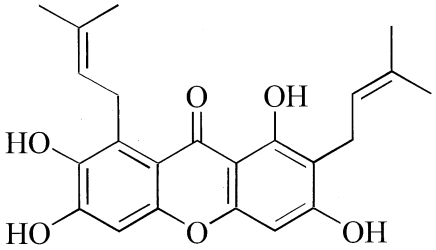

Fig. 1. Chemical structure of γ-mangostin.

25° and incubated with anti-phospho-p42/p44 ERK/MAPK antibody (rabbit) at 1 μg/mL for 2 h at 25°. The immunoblots were washed several times and incubated with a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG in TBST containing 2% BSA overnight at 4°. Blots were developed by using a chemiluminescence assay kit (Bio Rad) and visualized by exposing the membrane to Hyper-film ECL (Amersham).

2.5. Analysis of AA liberation

C6 cells were seeded into 12-well plates at the density of 1.0×10^5 cells per well. Two days after seeding, the medium was changed to Dulbecco's modified Eagle's medium containing 0.3 μCi/ml of [^{14}C]-AA, and the cell were incubated for 18 h. The cells were washed twice with EMEM-HEPES-3 mg/mL albumin solution (pH 7.35) and pre-incubated with or without γ-mangostin for 10 min at 37°C. After the cells were incubated with or without 10 μM A23187 for 5 min, the reaction was terminated by removing the medium to tubes [21]. After acidifying the medium (0.6 mL) to pH 4.0 by 1N HCl, it was mixed with chloroform (1.2 mL) and water (0.6 mL). The lower phase was dried by the stream of nitrogen gas and applied to a thin layer chromatography (TLC) plate (LK5D, Whatman). The developer used was the upper phase of benzene-isooctane-acetic acid (60:30:3, v/v) [21]. [^{14}C]-AA metabolites were visualized as radioluminogram with a Molecular Imager (GS363, Bio-Rad).

2.6. Assay of AA conversion by microsomes of C6 cells

C6 cells were seeded into 150-mm dishes at the density of 5.0×10^5 cells per dish. Three days after seeding, C6 cells were homogenized in 50 mM Tris-buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid. Microsomes were prepared by centrifugation at $3{,}000 \times g$ for 5 min at 4° followed by centrifugation of the supernatant at $100{,}000 \times g$ for 60 min at 4°. Microsomes (90 μg of protein/tube) were pre-incubated in 50 mM Tris-buffer containing 2 μM hematin and 5 mM tryptophan with or without γ-mangostin for 10 min at 37°, and they were incubated with 0.1 μCi/mL of [^{14}C]-AA for 40 min at 37°. After the cells were incubated, the reaction was terminated by the addition of ice-cold diethyl ether/methanol/1 M citric acid (30:4:1, v/v). The ether layer was dried by a stream of nitrogen gas and applied to a TLC plate (LK5D, Whatman). The developer used was the upper phase of ethyl acetate/water/isooctane/acetic acid (110:100:50:20, v/v). [^{14}C]-AA metabolites were visualized as radioluminogram with a Molecular Imager (GS363, Bio-Rad).

2.7. Assay of the enzyme activities of COX-1 and -2

Activities of COX-1 and -2 were determined according to the procedure described [22]. COX-1 or -2 enzyme protein (each 0.5–1.0 unit) was dissolved in 150 μL of 50 mM Tris-buffer containing 2 μM hematin and 5 mM tryptophan (reaction mixture). The reaction mixture was pre-incubated with or without drugs for 10 min at 37° and further incubated with 50 μL AA for 10 min at 37°. Then, the reaction was terminated by the addition of 20 μL of 1N HCl. AA metabolites were extracted twice with ethyl acetate. After ethyl acetate was evaporated under a stream of nitrogen gas, the sample was dissolved in 10 mM Tris-HCl (pH 7.6). PGE_2 was determined by radioimmunoassay, as described previously [17].

2.8. Data analysis

IC_{50} values were calculated from non-linear regression analysis of the data. The statistical differences ($P < 0.05$) of values was determined with ANOVA.

3. Results

3.1. Effect of γ-mangostin on PGE_2 synthesis stimulated by A23187 in C6 cells

A23187, a Ca^{2+} ionophore, is known to stimulate PG synthesis mediated through an activation of $cPLA_2$ following by AA liberation in glial cells [23]. In C6 rat glioma cells, A23187 potently stimulated PGE_2 release (2.3 ng/well from 0.05 ng/well), and γ-mangostin inhibited the release in a concentration-dependent manner, with the IC_{50} value of about 5 μM (Fig. 2).

3.2. Effect of γ-mangostin on p42/p44 ERK/MAPK phosphorylation and AA liberation

To determine the site of action of γ-mangostin, we first examined upstream of AA liberation. The p42/p44 ERK/MAPK is known to be involved in a wide range of cellular functions, including $cPLA_2$ activation [24,25]. γ-Mangostin (3–30 μM) slightly augmented, but do not inhibited A23187-induced phosphorylation of p42/p44 ERK/MAPK (data not shown), indicating that the acting site of γ-mangostin for inhibition of PGE_2 release is downstream of p42/p44 ERK/MAPK.

Because γ-mangostin did not cause the inhibition of

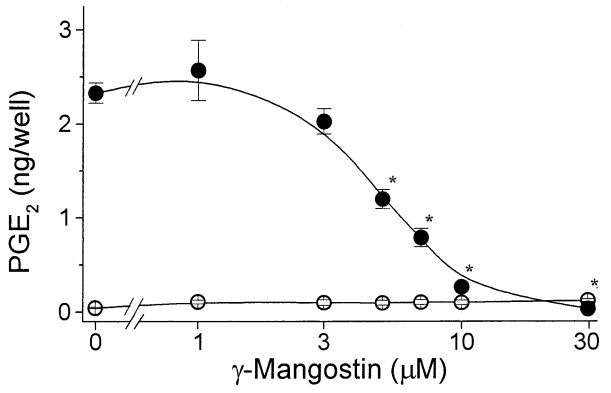

Fig. 2. Effect of γ-mangostin on PGE_2 release from C6 cells. The cells were prei-ncubated with indicated concentrations of γ-mangostin for 10 min, and they were stimulated by 10 μM A23187 (●) or vehicle (○) for 10 min. The released PGE_2 in the medium was determined by radioimmunoassay. Each point represents the mean with S.E. of three determinations. *Significant difference from A23187 alone.

phospholyration of p42/p44 ERK/MAPK, we examined the effect of γ-mangostin on AA liberation from [^{14}C]-AA-labeled cells (Fig. 3). γ-Mangostin slightly augmented, but did not inhibited A23187-induced AA liberation, suggesting that γ-mangostin inhibits PGE_2 synthesis at the downstream of AA liberation. Interestingly, γ-mangostin alone at a concentration of 30 μM did activate rather than inhibit the liberation of AA.

3.4. Effect of γ-mangostin on the conversion of AA to PGE_2 in microsomal fraction

COX, existing in microsomes, is the rate-limiting enzyme in the conversion of AA to prostanoids. Therefore, we

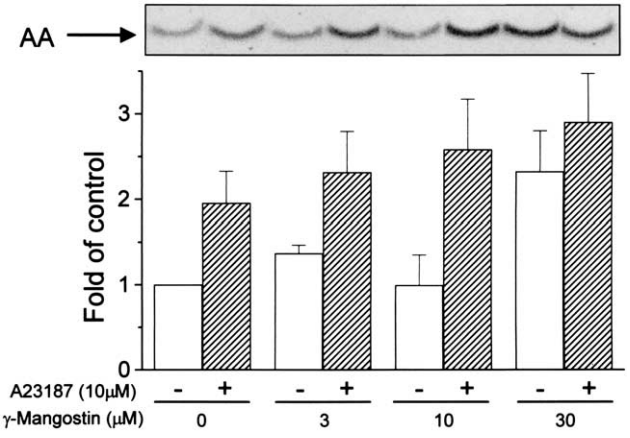

Fig. 3. Effect of γ-mangostin on liberation of AA. The cells were labeled with 0.3 μCi/mL of [^{14}C]-AA for 18 h. After washing out, the cells were pre-incubated with indicated concentrations of γ-mangostin for 10 min, and they were stimulated by 10 μM A23187 for 5 min. [^{14}C]-AA was analyzed in radioluminogram with a molecular imager (GS363, Bio-Rad) after separation by TLC (upper figure). Densitometric analysis of radioluminogram (lower figure). Open column: basal liberation, hatched column: A23187-induced liberation. The results are shown as the -fold increase from control (without A23187). Each column represents the mean with S.E. of three determinations.

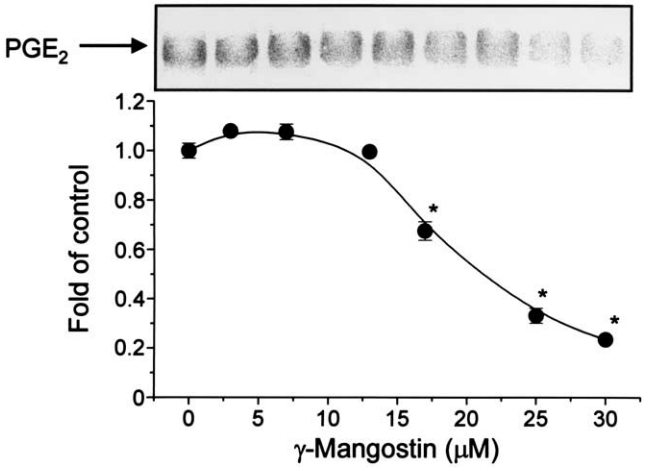

Fig. 4. Effect of γ-mangostin on COX activity of microsomes of C6 cells. Microsomes of C6 cells were pre-incubated with indicated concentrations of γ-mangostin for 10 min and were incubated with [^{14}C]-AA for 40 min. [^{14}C]-AA metabolites were analyzed in radioluminogram with a molecular imager (GS363, Bio-Rad) after separation by TLC (upper figure). Densitometric analysis of radioluminogram (lower figure). The results are shown as the -fold increase as without γ-mangostin of 1. Each point represents the mean with S.E. of three determinations. *Significant difference from without γ-mangostin.

examined the conversion of AA to PGE_2 in the microsomal fraction. Western blotting analysis revealed that COX-1, but not COX-2, was expressed in C6 cells under this experimental condition (data not shown). Incubation of the microsomes with [^{14}C]-AA resulted in the productions of [^{14}C]-eicosanoids. Among them, [^{14}C]-PGE_2 was a main metabolite. γ-Mangostin inhibited [^{14}C]-PGE_2 production from [^{14}C]-AA in a concentration-dependent manner, with the IC_{50} value of about 17 μM (Fig. 4).

3.5. Effect of γ-mangostin on enzyme activities of COX-1 and -2 in vitro

Because γ-mangostin inhibited the conversion of AA to PGE_2 in microsomes, we examined the direct effect of γ-mangostin on COX-1 or -2 enzyme *in vitro*, comparing the effects of indomethacin, a non-selective inhibitor of COX, and NS398, a selective inhibitor of COX-2. Indomethacin strongly inhibited both the COX-1 and -2 activities in a concentration-dependent manner (Figs. 5C and D). On the other hand, NS-398 inhibited the COX-2 activity in a concentration-dependent manner, but not COX-1 activity (Figs. 5E and F). γ-Mangostin inhibited both the COX-1 and -2 activities in a concentration-dependent manner, with the IC_{50} values of about 0.8 μM and 2 μM, respectively (Figs. 5A and B).

To clarify the characteristics of the inhibition of COX-1 or -2, we analyzed the kinetics of the inhibitory effect of γ-mangostin. Figs. 6 and 7 show typical saturation curves (Figs. 6A and 7A) and Lineweaver-Burk plots (Figs. 6B and

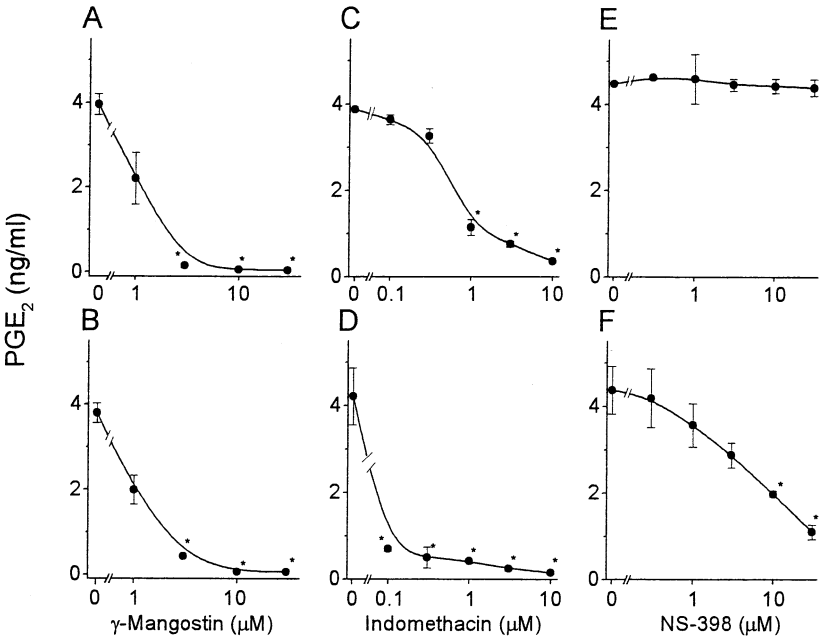

Fig. 5. Effects of γ-mangostin, indomethacin, and NS-398 on enzyme activities of COX-1 and -2. The reaction mixture with COX-1 (A, C, E) or -2 (B, D, F) were pre-incubated with indicated concentrations of γ-mangostin (A, B), indomethacin (C, D), or NS-398 (E, F) for 10 min and were incubated with AA for 10 min. The released PGE_2 in the medium was determined by radioimmunoassay. Each point represents the mean with S.E. of three determinations. *Significant difference from without γ-mangostin.

7B) for COX-1 and -2 activities in the presence or absence of 0.5 μM γ-mangostin. In the COX-1 activity (Fig. 6), the K_m value was increased from 0.75 to 1.7 μM in the presence of γ-mangostin, whereas the V_{max} value was unaffected (6.24 nmol/l·min for control and 6.53 nmol/l·min with γ-mangostin). In the COX-2 activity (Fig. 7), the K_m value was increased from 0.25 to 0.75 μM in the presence of γ-mangostin, whereas the V_{max} value was unaffected (6.24 nmol/l·min for control and 6.53 nmol/l·min with γ-mangostin). Thus, γ-mangostin has an ability to inhibit the COX-1 and -2 activities competitively.

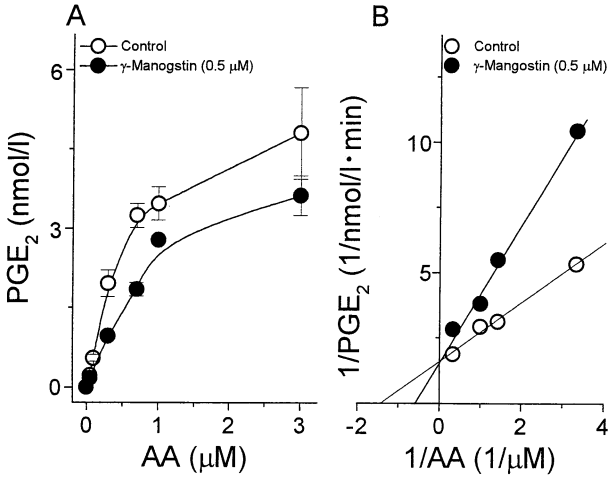

Fig. 6. (A) Saturation curve and (B) Lineweaver-Burk plot of COX-1 activity of γ-mangostin in a cell-free system. The reaction mixture with COX-1 were pre-incubated with γ-mangostin for 10 min and were incubated with indicated concentrations of AA for 10 min. The released PGE_2 in the medium was determined by radioimmunoassay. Each point represents the mean with S.E. of three determinations.

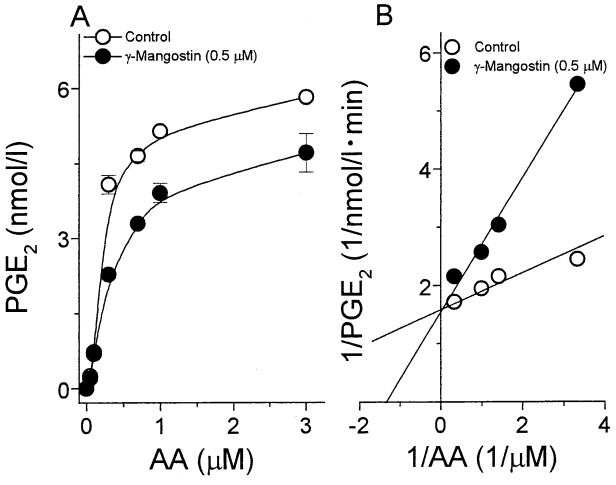

Fig. 7. (A) Saturation curve and (B) Lineweaver-Burk plot of COX-2 activity of γ-mangostin in a cell-free system. The reaction mixture with COX-2 were pre-incubated with γ-mangostin for 10 min and were incubated with indicated concentrations of AA for 10 min. The released PGE_2 in the medium was determined by radioimmunoassay. Each point represents the mean with S.E. of three determinations.

4. Discussion

The fruit hull of *G. mangostana* L., mangosteen, has been widely used as an anti-inflammatory agent for the treatment of skin infections, wounds, and diarrhea for many years in Southeast Asia [1]. The crude extract of the fruit hull has been reported to possess several pharmacological activities, such as inhibitory activity against HIV-1 protease [26] and antimicrobial activity [27]. Several constituents contained in the fruit hull of mangosteen, such as α-mangostin, γ-mangostin, or another substances, have diverse pharmacological activities, such as anti-histamine activity [28], inhibition of the sarcoplasmic reticulum Ca^{2+}-pumping adenosine 5′-triphosphate (ATP)ase [29], and anti-serotonin activity [30]. Among then, we selected γ-mangostin to examine its activity of PGE_2 synthesis. In the analysis using C6 rat glioma cells, we show that γ-mangostin has a potent inhibitory effect on PGE_2 synthesis. A synthetic xanthone derivative, 3-[2-(cyclopropylamino)ethoxy] xanthone inhibits AA-induced platelet aggregation and thromboxane formation [31], showing its possible inhibition of COX activity. In the present study, we showed for the first time that γ-mangostin, a xanthone derivative derived from natural products, directly binds to COX and inhibits its activity. This effect of γ-mangostin may contribute to its anti-inflammatory activity.

C6 cells, derived from rat glial tumors induced by N-nitrosomethyl urea [32], are commonly used as an established cell line for a model of glial cells. Glial cells are known as an important source of PGs in the CNS [33]. Because a high PGE_2 level is observed in some diseases, such as multiple sclerosis and AIDS-associated dimension, drugs reducing PGE_2 synthesis in glial cells have a possibility to improve the diseases with inflammation in the brain. Thus, γ-mangostin is one of the candidates of the drugs for treatment brain diseases accompanied with inflammation, although further detailed analysis is necessary, such as metabolism and distribution of the drug *in vivo.*

Because γ-mangostin has an inhibitory effect of PGE_2 synthesis, we examined its site of action. However, γ-mangostin slightly augmented, but not inhibited, the phosphorylation of p42/p44 ERK/MAPK. Furthermore, the A23187-induced AA liberation was slightly augmented, but not inhibited, by γ-mangostin. Interestingly, γ-mangostin alone at a concentration of 30 μM did activate rather than inhibit the liberation of AA, although it inhibited PGE_2 synthesis. Because the acting site of γ-mangostin may be downstream of AA liberation, we examined the effect of γ-mangostin on the conversion of AA to PGE_2 in microsomal preparations. The conversions of AA to PGE_2 was potently inhibited by γ-mangostin, suggesting that the site of γ-mangostin was COX. In the analysis of enzyme activity *in vitro,* we found that γ-mangostin directly interacted with COX-1 and -2 and inhibited their activities. There is a certain difference in the IC_{50} values of γ-mangostin between intact cells and microsomal preparations. However, the IC_{50} values of γ-mangostin in purified COX enzymes are similar to the IC_{50} values in intact cells. Then, the microsomal preparation was exceptional, and γ-mangostin might be degraded with P450 enzymes in the microsomal preparation. Because γ-mangostin inhibited both the COX-1 and -2, the drug seems to be similar to indomethacin in its selectivity to COX. Indomethacin is known as a competitive, time-dependent, reversible COX inhibitor. It forms an enzyme-inhibitor (EI) complex but then secondarily changes the structure of the protein to produce an intermediate state of enzyme-inhibitor (EI*) complex without covalently modifying the protein [34]. EI* complex formation is relatively slow, occurring in seconds to minutes, and EI* slowly reverts to EI [14]. Aspirin is a competitive, irreversible COX inhibitor. It converts EI to an EI* complex by covalent modification (acylation) of the protein. Once an EI* complex is formed with aspirin, it is not possible for the protein to revert to EI [14]. Like indomethacin, γ-mangostin does not have a site of covalent modification (e.g. acetoxyl group). Then, γ-mangostin may be a reversible COX inhibitor. Furthermore, the kinetic analysis in this study indicated that γ-mangostin inhibited the COX-1 and -2 activities competitively. Thus, γ-mangostin competes with arachidonate for binding to the COX-1 and -2 active site.

In conclusion, we showed for the first time that γ-mangostin, a tetraoxygenated diprenylated xanthone from mangosteen, reduces PG generation through its direct inhibition of COX. γ-Mangostin is an attractive drug because its analogs of tetraoxygenated diprenylated xanthones are contained in many plants, vegetables, and fruits.

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