Anti-angiogenic effect of α-mangostin

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NOTE

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Abstract Eleven known prenyl xanthones, isolated from the pericarp of *Garcinia mangostana*, were tested for their ability to inhibit the phosphorylation of kinase domain receptor (KDR) tyrosine kinase. α-Mangostin was found to inhibit phosphorylation of KDR. α-Mangostin also showed to inhibit phosphorylation of the Y1175 residue of KDR (10 μM). This is the first report that α-mangostin inhibited the phosphorylation of KDR tyrosine kinase and also the Y1175 residue of KDR. α-Mangostin also showed inhibitory effects on proliferation of human umbilical vein endothelial cells (HUVECs) (IC_{50} 1.2 μM) and human umbilical artery endothelial cells (IC_{50} 2.4 μM), as well as the migration (IC_{50} 0.034 μM) and tubule formation (at the concentrations of 0.6 and 1.2 μM) of HUVECs. These results suggest that the inhibition of the phosphorylation of KDR tyrosine kinase is concerned in the anti-angiogenic activity of α-mangostin.

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Introduction

Tumor angiogenesis, the formation of new blood vessels able to deliver oxygen and nutrients to tumors cells, is a major step in the growth of tumors, with the formation of new blood vessels being an enabling factor in the development of metastasis. Therefore, inhibition of tumor angiogenesis is viewed as an effective avenue for cancer treatment. We have previously reported that various natural products act as inhibitors of vascular endothelial growth factor receptor 2 [VEGFR2: kinase domain receptor (KDR)] tyrosine kinase, or human umbilical vein endothelial cells (HUVECs)/human umbilical artery endothelial cells (HUAECs) proliferation [1–4].

Angiogenesis is a complex process that involves VEGF binding-induced phosphorylation of KDR. Among the various signaling events, KDR mediated signaling is central to proliferation, migration and tubule formation of vascular endothelial cells. KDR activity is regulated by tyrosine phosphorylation and has several major phosphorylation sites (Y951, Y1054, Y1059, Y1175, Y1214). Among these, phosphorylation of Y1175 is very important in angiogenesis. Phosphorylation of Y1175 affects cell proliferation and migration, as well as vascular permeability [5].

Apoptosis in various types of human cancer cells and metastasis inhibitory activity in mammary and prostate cancer cells for α-mangostin has been reported [6–13]. It has also been reported that these effects based on inhibition of serine–threonine kinase activation such as ERK, Akt and JNK.

Here, we focused on KDR tyrosine kinase and found that α-mangostin is a compound with inhibitory activity

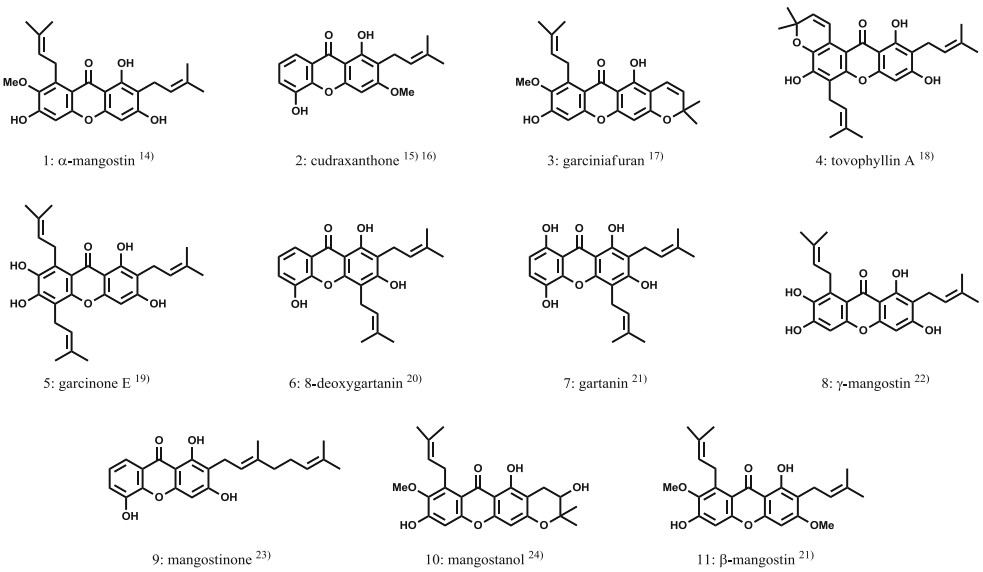

Fig. 1 Chemical structures of 11 known prenyl xanthones

that is present in *Garcinia mangostana* pericarp. α-Mangostin also inhibited phosphorylation of the Y1175 residue of KDR. Furthermore, we tested α-mangostin using bioassays for inhibitory effects on proliferation, migration and tubule formation with HUVECs.

Results and discussion

In the present study, we searched for KDR inhibitors obtained from fruit pericarps. Since pericarps are a non-edible waste material, making use of fruit pericarp is meaningful from an ecological viewpoint.

Initially, dried pericarps from several kinds of fruit were extracted with $CHCl_3$ followed by MeOH. The extracts were tested for KDR kinase inhibitory activity and the $CHCl_3$ extract of pericarp of *Garcinia mangostana* L. exhibited 37.0% inhibition at 100 μg/mL. The $CHCl_3$ extract was subjected to silica gel column chromatography (CC), ODS CC, Sephadex LH-20 CC and HPLC, resulting in the identification of 11 known xanthones **1**–**11** (Fig. 1). The compounds were identified as α-mangostin (**1**) [14], cudraxanthone (**2**) [15, 16], garciniafuran (**3**) [17], tovophyllin A (**4**) [18], garcinone E (**5**) [19], 8-deoxygartanin (**6**) [20], gartanin (**7**) [21], γ-mangostin (**8**) [22], mangostinone (**9**) [23], mangostanol (**10**) [24], and *β*-mangostin (**11**) [21] by comparing their NMR data with previously reported data.

These xanthones were tested for KDR kinase inhibitory activity, and only α-mangostin (**1**) showed week inhibitory activity with an IC_{50} value of 203.5 μM. However, inhibition of KDR tyrosine kinase is effective in eliciting anti-angiogenesis, since tyrosine phosphorylation of KDR is a relatively upstream event in angiogenic signaling in vascular endothelial cells. We examined site-specific

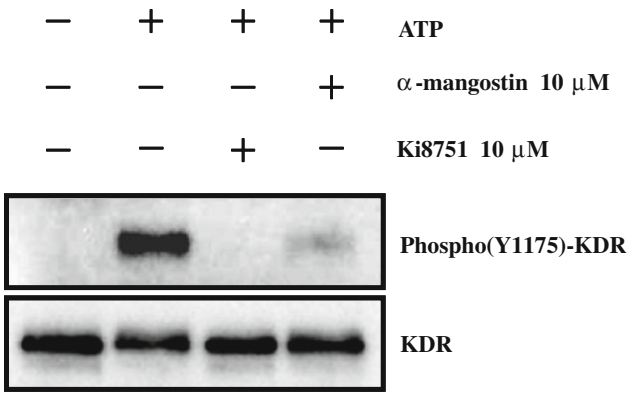

Fig. 2 Inhibitory effect of α-mangostin on phosphorylation of KDR at position Tyr1175

phosphorylation of KDR using Western blotting and found that 10 μM α-mangostin inhibited phosphorylation of Y1175 (Fig. 2). We subsequently tested the inhibition of proliferation in HUVECs and HUAECs using the MTT assay. α-Mangostin showed the activity with IC_{50} value of 1.2 and 2.4 μM, respectively. The effect of α-mangostin on VEGF-induced HUVEC migration was also examined using cell culture inserts. Cell migration was assessed by staining the membranes, containing migrated cells, after culturing for 22 h in the presence of α-mangostin. Enumeration of migrated cells was performed using a microscope. α-Mangostin was found to inhibit on HUVEC migration with an IC_{50} 0.034 μM (Fig. 3). Although α-mangostin inhibited proliferation of HUVECs with an IC_{50} of 1.2 μM, this compound was capable of inhibiting tubule formation at 0.6 and 1.2 μM (Fig. 4).

Garcinia mangostana pericarp-derived α-mangostin inhibited phosphorylation of KDR tyrosine kinase and also

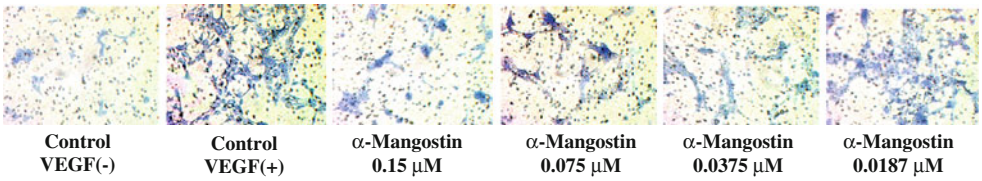

Fig. 3 Inhibitory effect of α-mangostin on migration of HUVECs

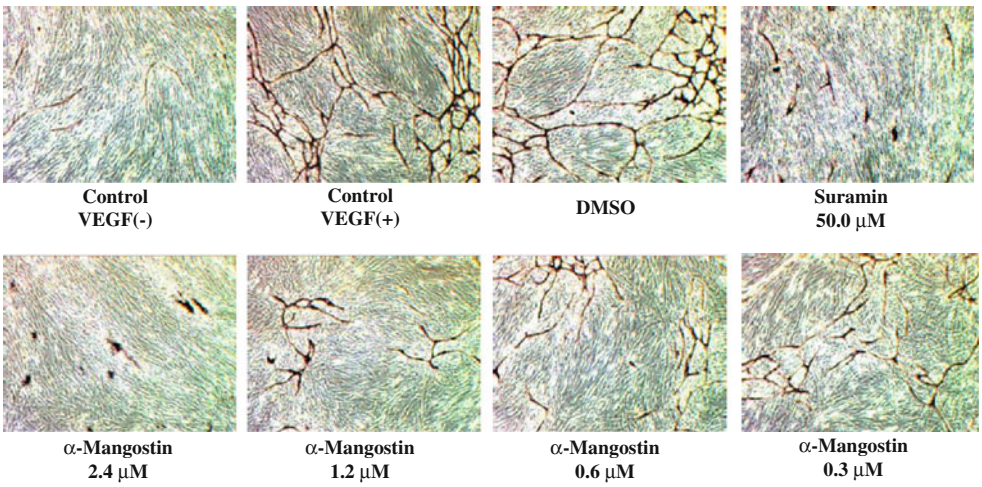

Fig. 4 Inhibitory effect of α-mangostin on tubule formation of HUVECs

the Y1175 residue of KDR, as well as proliferation, migration, and tubule formation in HUVECs.

α-Mangostin has been reported the inhibition of ERK, Akt, JNK activation [6–13]. When the results of our experiment and the already reported are considered, it is suggested that α-mangostin showed anti-angiogenic activity by inhibiting not only KDR tyrosine kinase but serine–threonine kinase such as ERK, Akt, and JNK.

Experimental

General experimental procedures

Melting points were determined on a Yanaco MP apparatus. UV spectra were recorded with a Shimadzu UV-240 spectrophotometer. IR spectra were recorded with a Jasco IR Report-100 spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were measured with JEOL JNM-AL-400 (^{1}H 400 and ^{13}C 100 MHz) spectrometer using tetramethylsilane as the internal standard. Low- and high-resolution EIMS and FABMS spectra were measured with a JEOL JMS-700 spectrometer. CC was performed using silica gel 60N (63–210 μm) from Kanto Chemical (Tokyo, Japan) and Sephadex LH-20 from GE Healthcare. HPLC (columns, Senshu Pak Silica-4251-N, 10 mm i.d. × 250 mm and Senshu Pak ODS-4251-SS, 10 mm i.d. × 250 mm) was performed using a SSC-3461 and JASCO PU2080 PLUS pump and measured with a JASCO UV-970 and JASCO PU 2075 PLUS detector. CKX 31 Microscope (Olympus) which connects object glass (10×) and C-mount relay lens (Wraymer, Inc., Japan) as eye glass was used. IC_{50} value was determined by Graphpad Prism 5.0 (Graphpad Software, Inc.).

Plant material

The pericarp of *Garcinia mangostana* was purchased from a market in Thailand in June 2010. A voucher specimen (THAI 20106) was deposited at our laboratory.

Extraction and isolation

Dried pericarp of *Garcinia mangostana* (271.1 g) were cut into small slices and extracted with $CHCl_3$ (2 L × 3) at room temperature. The $CHCl_3$ extract was concentrated under reduced pressure and 22.8 g of the concentrated extract was subjected to silica gel CC with a solvent system consisting of *n*-hexane–$CHCl_3$ (1:1), $CHCl_3$, $CHCl_3$–MeOH (50:1, 10:1, 1:1) and MeOH to yield seven fractions (Fr. A1 to Fr. A7). Fr. A3 (8.2 g) was separated by silica gel CC with a solvent system consisting of *n*-hexane–EtOAc (5:1, 3:1, 2:1) and MeOH to yield six fractions (Fr. B1 to Fr. B6). Compound **1** (111.5 mg) was isolated from Fr. B3 (198.1 mg) as a $CHCl_3$-insoluble substance. Fr. A2 (9.5 g) was separated by silica gel CC with a solvent system consisting of *n*-hexane–$CHCl_3$–EtOAc (6:1:1, 5:1:2, 5:1:4, 5:1:6), $CHCl_3$–EtOAc (1:2) and MeOH to

yield seven fractions (Fr. C1 to Fr. C7). Compound **2** (9.9 mg) was isolated from Fr. C5 (3.3 g) as a $CHCl_3$-insoluble substance. Fr. C4 (2.3 g) was separated by silica gel CC with a solvent system consisting of *n*-hexane–$CHCl_3$–EtOAc (6:1:1, 5:1:1, 5:1:2), EtOAc and MeOH to yield eight fractions (Fr. D1 to Fr. D8). Compounds **3** (3.6 mg) and **4** (1.3 mg) were isolated from Fr. D1 (24.4 mg) by HPLC (eluent *n*-hexane–acetone = 5:1, R_t = 10.0 and 13.2 min). Fr. D4 (1.0 g) was separated by silica gel CC with a solvent system consisting of *n*-hexane–$CHCl_3$ (1:1, 1:2), $CHCl_3$ and $CHCl_3$–MeOH (2:1) to yield seven fractions (Fr. G1 to Fr. G7). Compound **5** (1.9 mg) was isolated from Fr. G2 (12.7 mg) as a $CHCl_3$-insoluble substance. Fr. C3 (640.0 mg) was separated by silica gel CC with a solvent system consisting of *n*-hexane–$CHCl_3$ (2:1, 1:2), $CHCl_3$, $CHCl_3$–MeOH (2:1, 1:2) and MeOH to yield seven fractions (Fr. E1 to Fr. E7). Compounds **6** (12.0 mg), **7** (23.2 mg) and **11** (2.3 mg) were isolated from Fr. E3 (228.5 mg) by the combination of Sephadex LH-20 CC (eluent MeOH) and ODS-HPLC (eluent MeOH, R_t = 16.5, 18.0 and 19.0 min). Fr. A4 (2.2 g) was separated by silica gel CC with a solvent system consisting of $CHCl_3$ and $CHCl_3$–MeOH (100:1, 80:1, 50:1) to yield six fractions (Fr. F1 to Fr. F6). Compound **8** (131.3 mg) was isolated from Fr. F5 (257.7 mg) as a $CHCl_3$-insoluble substance. The $CHCl_3$-soluble (126.4 mg) component of Fr. F5 of was separated by HPLC (eluent $CHCl_3$–MeOH 40:1, R_t = 24.0 min) to yield compound **9** (16.7 mg). Fr. A5 (0.2 g) was separated by Sephadex LH-20 CC (eluent MeOH) to yield six fractions (Fr. N1 to Fr. N6). Compound **10** (9.9 mg) was isolated from Fr. N3 (48.0 mg) as a $CHCl_3$-insoluble substance.

KDR kinase inhibition assay

Analysis of inhibition of recombinant KDR protein (Millipore) was performed on 96-well plates using a universal tyrosine kinase assay kit (Takara Bio, Inc.) according to a previous report [2].

Western blot analysis

Recombinant KDR protein (Invitrogen) was incubated with 10 μM of each test compound and 4 mM ATP in kinase reaction solution (Takara Bio, Inc.) for 10 min at 37°C. The samples were separated on 5–20% SDS-PAGE and transferred to polyvinylidene fluoride membrane (Invitrogen). The membrane was blocked with block Ace (DS Pharma Biomedical) in Tween-PBS for 1 h at room temperature, and then incubated with anti-KDR (Santa Cruz Biotechnology) and anti-phospho (Tyr1175)-VEGF Receptor 2 (Cell Signaling Technology) for overnight at 4°C. Immunoreactive bands were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector Laboratories) and visualized with a chemiluminescent detection substrate (Millipore). Ki8751 (Calbiochem), which is a VEGFR2 kinase inhibitor, was used as a positive control.

Cell culture

HUVECs and HUAECs were purchased from Lonza Walkersville, Inc. HUVECs were cultured using an EGM-2 Bulletkit (Lonza Walkersville, Inc.) at 37°C in 5% CO_2. HUAECs were cultured using an EGM-2MV Bulletkit (Lonza Walkersville, Inc.) at 37°C in 5% CO_2.

Proliferation inhibitory assay

Cells (3×10^3 cells/well) were seeded in 96-well plates with EBM-2 Bulletkit for 3 h at 37°C in 5% CO_2. The medium was removed, replaced with 1% FBS–EBM-2, and incubated for 21 h at 37°C in 5% CO_2. VEGF (1 nM) and the test compounds were added to each well and incubated for 72 h at 37°C in 5% CO_2. Cell proliferation was detected using the WST-8 reagent, and the inhibition of proliferation was measured at an absorbance wavelength of 450 nm using a plate reader.

Migration inhibitory assay

Inhibition of cell migration was determined using PET membrane filters from the inner chambers of 8 μm Falcon cell culture inserts (Becton-Dickinson). HUVECs (5×10^4 cell/well) suspended in EBM-2 medium containing 0.2% FBS with various concentrations of test compounds dissolved in DMSO were seeded on the inner chamber. Next, the inner chamber was placed into 24-well plate (outer chamber), which was filled with the same medium containing VEGF (20 ng/mL). After a 22 h incubation at 37°C in 5% CO_2, the non-migrated cells on the upper surface of the filter were removed by wiping with cotton swabs. The remaining cells were then fixed and stained using the Diff-Quik system (Sysmex). Cells that had migrated through the filter were manually counted in six different microscopic fields.

Tubule formation inhibitory assay

The tubule formation assay was performed using an Angiogenesis Kit (Kurabo, Japan). HUVECs and NHDF cells were co-cultured in 24-well plates and incubated with Optimized Medium. After a 3 h incubation at 37°C in 5% CO_2, the medium was replaced with fresh medium containing VEGF (10 ng/mL) and various concentrations of the test compounds, and the plates were incubated for

11 days at 37°C in 5% CO_2 (medium was replaced on days 4, 7, 9). After 11 days, the cells were fixed with 70% EtOH and the formed tubes stained with mouse anti-human CD31, goat anti-mouse IgG alkaline phosphatase conjugate, and BCIP/NBT. Tubule network patterns were identified under a microscope and photographed.

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