Tricin, 4',5,7-trihydroxy-3',5'-dimethoxyflavone, exhibits potent antiangiogenic activity in vitro

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Abstract. Tumor growth and metastasis depend on angiogenesis triggered by chemical signals, such as vascular endothelial growth factor (VEGF), released from tumor cells. Therefore, the specific perturbation of angiogenesis has been considered a powerful strategy for the treatment of cancer. Herein, we report that tricin, 4',5,7-trihydroxy-3',5'-dimethoxyflavone, exhibits potent antiangiogenic activity in vitro. Tricin effectively suppressed the proliferation as well as VEGF-induced invasion and tube formation of human umbilical vein endothelial cells (HUVECs) at subtoxic doses. Furthermore, tricin significantly inhibited the angiogenesis of the chorioallantoic membrane from growing chick embryos without showing cytotoxicity. We also found that tricin blocked tumor cell-induced angiogenesis. Notably, tricin downregulated not only the VEGFR2 signal transduction by reducing reactive oxygen species (ROS) generation in endothelial cells, but also the expression of VEGF by inhibiting hypoxia inducible factor-1α (HIF-1α) accumulation in tumor cells. Moreover, combined treatment with tricin and bevacizumab, an anti-VEGF drug, ameliorated the antiangiogenic effect of bevacizumab. Taken together, our findings demonstrate for the first time that tricin possesses promising antiangiogenic potential and thus may be applied to anticancer therapy by targeting tumor angiogenesis.

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is required for a variety of normal physiological functions such as embryonic development, wound healing and tissue or organ regeneration (1,2). However, angiogenesis is also involved in the pathogenesis of several diseases, including age-related macular degeneration, diabetic retinopathy, psoriasis, rheumatoid arthritis and cancer (3). Particularly, upregulation of angiogenesis is a central step in sustained tumor growth and metastasis. The new blood vessels grow and infiltrate into the tumor, providing it with essential nutrients and oxygen, and a route for tumor metastasis (4,5). Therefore, antiangiogenesis has become an important strategy for the treatment of cancer.

Chemical stimulation of angiogenesis is performed by various angiogenic proteins, including vascular endothelial growth factor (VEGF) (6). VEGF stimulates cellular responses by binding to VEGF receptor 2 (VEGFR2) on the cell surface, causing them to dimerize and become activated through transphosphorylation. Activation of VEGFR2 leads to phosphorylation of specific downstream signal transduction mediators, including extracellular signal-regulated kinases (ERK) and AKT. Signaling from VEGFR2 consequently promotes the proliferation, migration and differentiation of endothelial cells (7,8). Therefore, VEGF2 has been recognized as the most important target for the antiangiogenesis therapy of cancer. Bevacizumab (Avastin®), sunitinib malate (Sutent®, SU11248), and sorafenib (Nexavar®, BAY 43-9006) that were developed for antiangiogenic actions have been approved by the United States Food and Drug Administration (FDA) for treatment of patients with specific types of cancer. All three agents inhibit VEGF signaling by blocking VEGF ligand or VEGF receptor function (9,10). But most of angiogenesis inhibitors have some adverse effects, including hypertension and proteinuria, which emphasizes that discovery of novel VEGFR2 inhibitors with better safety and efficacy in treating human cancer is still needed (11,12).

In general, rapid growth of tumor cells causes hypoxia in tumor tissues, which drives angiogenesis to improve the influx of oxygen. Thus, the hypoxic microenvironment can stimulate the expression of VEGF via hypoxia inducible factor-1 (HIF-1), the transcription factor which binds the regulatory region of VEGF gene and induces its transcription during hypoxia (13,14). HIF-1 is a heterodimeric transcription factor composed of an oxygen-regulated α-subunit (HIF-1α) and a constitutively expressed β-subunit (HIF-1β). HIF-1α plays a key role in the regulation of the expression of many genes involved in metabolic adaptation to low oxygen, survival and angiogenesis. Under normoxic condition, HIF-1α is rapidly degraded by proteasome after post-translational modification, whereas, under hypoxic condition, it remains stable.

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and binds with HIF-1α to activate the transcription of a large number of genes (15). It has been reported that overexpression of HIF-1α was associated with poor prognosis of many human cancers (16). Given the crucial role of VEGF-mediated signaling in promoting tumor angiogenesis, dual inhibition of VEGFR2 and HIF-1α activities could potentiate antiangiogenic therapy in cancer treatment.

There has been increasing interest in the research on flavonoids, a large class of plant metabolites, because of their multifaceted health benefits (17,18). The flavonoid, tricin (4',5,7-trihydroxy-3',5'-dimethoxyflavone) was reported as a valuable anticancer agent having a pharmacokinetic advantage (Fig. 1) (19-21). However, the antiangiogenic potential of tricin has not been explored. In the present study, we investigated the in vitro antiangiogenic effect and the molecular mechanisms of tricin. Our results demonstrated that tricin could efficiently suppress tumor angiogenesis by downregulating both VEGFR2 signaling and HIF-1α activity.

Materials and methods

Materials. Tricin was purchased from ChemFaces (Wuhan, China). Endothelial growth medium-2 (EGM-2) was obtained from Lonza (Walkersville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), RPMI-1640 medium, and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). Recombinant human vascular endothelial growth factor (VEGF), Matrigel and Transwell chamber systems were obtained from BD Biosciences (San Jose, CA, USA) and Corning Costar (Acton, MA, USA), respectively. Anti-hypoxia inducible factor-1α (HIF-1α) antibody was purchased from BD Biosciences. Anti-phospho-VEGFR2, anti-VEGFR2, anti-phospho-AKT, anti-AKT, anti-phospho-ERK1/2, anti-ERK1/2 and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and hypoxic conditions. Human umbilical vein endothelial cells (HUVECs) and U87MG (human glioblastoma) cells were grown in EGM-2 and MEM supplemented with 10% FBS, respectively. AGS (human gastric carcinoma) and HCT116 (human colon carcinoma) cells were maintained in RPMI-1640 medium containing 10% FBS. HeLa (human cervical carcinoma) and HepG2 (human hepatocarcinoma) cells were grown in DMEM supplemented with 10% FBS. All cells were maintained at 37°C in a humidified 5% CO₂ incubator. For hypoxic conditions, cells were incubated in a hypoxic chamber (Forma Scientific, Marietta, OH, USA) under 5% CO₂ and 1% O₂ balanced with N₂.

Cell proliferation assay. HUVECs (3x10³ cells/well) and various cancer cells (2x10³ cells/well) were seeded in 96-well culture plates and then treated with various concentrations of tricin for 72 h. Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

Cell viability assay. HUVECs were seeded at a density of 1x10⁵ cells/well in 12-well culture plates. Tricin (1-20 µM) was added to each well and the cells were incubated for up to 72 h. After 72 h, the cells were stained with Trypan blue and counted using a hemocytometer.

Chemoinvasion assay. The invasiveness of HUVECs was investigated using a Transwell chamber system with polycarbonate filter inserts with a pore size of 8.0 µm. Briefly, the lower side of the filter was coated with 10 µl gelatin (1 mg/ml) and the upper side was coated with 10 µl Matrigel (3 mg/ml). Serum-starved HUVECs (8x10⁴ cells) were placed in the upper chamber of the filter and tricin (2.5-10 µM) was added to the lower chamber in the presence of VEGF (30 ng/ml). The chamber was incubated at 37°C for 18 h, and then the cells were fixed with methanol and stained with hematoxylin and eosin (H&E). The total number of cells that invaded the lower chamber of the filter was counted using an optical microscope (Olympus, Center Valley, PA, USA) at a x10 magnification.

Capillary tube formation assay. Serum-starved HUVECs (8x10⁴ cells) were inoculated on a surface containing Matrigel (10 mg/ml) and were incubated with tricin (2.5-10 µM) for 6 h in the presence of VEGF (30 ng/ml). Morphological changes of the cells and tube formation were visualized under a microscope and photographed at a x10 magnification (Olympus). Tube formation was quantified by counting the total number of branched tubes in randomly selected fields at a x10 magnification.

Chorioallantoic membrane (CAM) assay. Fertilized chick eggs were maintained in a humidified incubator at 37°C for 3 days. Approximately 6 ml egg albumin was removed with a hypodermic needle, allowing the CAM and yolk sac to drop away from the shell membrane. After 2 days, the shell was punched out and peeled away. Thermapan coverslips (Nalge Nunc International, Rochester, NY, USA) with or without tricin were air-dried and applied to the CAM surface. Two days later, 2 ml of 10% fat emulsion (Greencross Co., Yongin, Republic of Korea) were injected into the chorioallantois and the CAM was observed under a microscope.

Tumor cell-induced angiogenesis assay. Tumor-induced angiogenesis was assessed using an in vitro co-culture system based on the chemoinvasion assay (22). U87MG cells were plated in the lower chamber and treated with tricin (2.5-10 µM) for 24 h. And then the medium in each lower chamber was replaced with fresh medium without tricin, and serum-starved HUVECs (8x10⁴ cells) were placed in the upper chamber. The chamber was incubated at 37°C for 18 h, and HUVECs that invaded the lower chamber of the filter were analyzed using the same procedure as described in the chemoinvasion assay. To further verify the activity of tricin to tumor cell-induced

Figure 1. The structure of tricin.
angiogenesis, a conditioned medium was collected from U87MG cells and used as the angiogenic stimuli for the tube formation of HUVECs (22). Briefly, U87MG cells were treated with tricin (2.5-10 µM) for 24 h, and then the medium was replaced with fresh medium without tricin. The conditioned medium was used in the in vitro tube formation assay.

Western blot analysis. Cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using standard electroblotting procedures. The blots were blocked and immunolabeled with primary antibodies against phospho-VEGFR2, VEGFR2, phospho-AKT, AKT, phospho-ERK1/2, ERK1/2, HIF-1α and β-actin overnight at 4˚C. Immunolabeling was detected with an enhanced chemiluminescence (ECL) kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions.

Reactive oxygen species (ROS) measurement. ROS levels were detected with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes, Eugene, OR, USA). For the assay, serum-starved HUVECs seeded at a density of 1x10^5 cells/well in 96-black well culture plates were pretreated with tricin (2.5-10 µM) for 3 h. After incubation with H2DCFDA (10 µM) for 5 min, the cells were stimulated to VEGF (30 ng/ml) for 5 min. The fluorescence intensity of DCF was detected using a multimode microplate reader (Thermo Fisher Scientific, Vantaa, Finland) at the excitation and emission wavelengths of 495 and 529 nm, respectively.

Measurement of VEGF by enzyme-linked immunosorbent assay (ELISA). VEGF concentration in the media from the tricin-treated cells was determined using a VEGF immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The results were expressed as the concentration of VEGF relative to the total amount of protein from each well.

Statistical analysis. The results are expressed as the mean ± standard error (SE). Student's t-test was used to determine statistical significance between the control and the test groups. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

The effect of tricin on the proliferation of human umbilical vein endothelial cells. We first investigated the effect of tricin on the growth of various cell lines, including several cancer cells and human umbilical vein endothelial cells (HUVECs), using the MTT colorimetric assay. As shown in Fig. 2A, tricin inhibited the proliferation of each cell line with a different sensitivity to growth inhibition. Notably, tricin showed comparatively better inhibition effect on the growth of HUVECs with an IC50 of 27.32 µM among the tested cell lines. To further evaluate whether the endothelial cell growth inhibition by tricin was due to cytotoxic or cytostatic activity, a viability assay was performed using the Trypan blue exclusion method. As shown in Fig. 2B, the viability of HUVECs was not affected up to 10 µM of tricin treatment, indicating that the antiproliferative activity of tricin shown at range of <10 µM is not due to mere cytotoxicity of the compound.

The in vitro antiangiogenic activity of tricin. We next examined the effect of tricin on key angiogenic phenotypes such as endothelial cell invasion and tube formation. The in vitro angiogenesis assays were conducted in a non-cytotoxic concentration range of tricin (2.5-10 µM). To elucidate the inhibitory activity of tricin on VEGF-induced angiogenesis, serum starved HUVECs were stimulated by VEGF with or without tricin. As shown in Fig. 3A and B, tricin significantly decreased the VEGF-induced invasiveness and tube forming ability of HUVECs in a dose-dependent manner.

Furthermore, the antiangiogenic activity of tricin was validated using a chorioallantoic membrane (CAM) assay. Coverslips containing tricin were placed on the CAM surface, and angiogenesis zones were observed under a microscope.
As shown in Fig. 3C, the inhibition of neovascularization on control coverslips was 13% (n=15), whereas tricin much more potently inhibited the angiogenesis of the CAM (53% at 2 µg/egg, n=19; 64% at 5 µg/egg, n=22) without toxicity against pre-existing vessels. These results demonstrate that tricin significantly inhibited angiogenesis without exhibiting cytotoxicity on endothelial cells in vitro.

The inhibitory effect of tricin on tumor cell-induced angiogenesis. Angiogenesis is recognized as a crucial step in the transition of tumors from a dormant condition to a malignant state by inducing tumor growth and metastasis (4,5). To evaluate whether tricin inhibits tumor cell-induced angiogenesis, we investigated the effect of tricin on HUVEC invasion induced by U87MG glioblastoma cells using a coculture assay.

As shown in Fig. 4A, the inhibition of tumor cell invasion on control coverslips was 13% (n=15), whereas tricin much more potently inhibited tumor cell invasion (53% at 2 µg/egg, n=19; 64% at 5 µg/egg, n=22) without toxicity against pre-existing vessels. These results demonstrate that tricin significantly inhibited tumor cell invasion without exhibiting cytotoxicity on endothelial cells in vitro.
As shown in Fig. 4A, HUVECs cocultured with U87MG cells invaded 2-fold faster compared to HUVECs alone. The increased invasion of HUVECs was effectively prevented when U87MG cells were treated with tricin. To further verify its activity to tumor cell-induced angiogenesis, we also assessed the effect of tricin on the tube formation of HUVECs induced by U87MG cells. As shown in Fig. 4B, the conditioned medium from U87MG cells induced tube formation of HUVECs by 1.6-fold compared to control (medium only). However, tricin-treated conditioned medium from U87MG cells blocked the stimulated tube formation of HUVECs in a dose-dependent manner, implying that tricin could inhibit tumor cell-induced angiogenesis.

Downregulation of VEGFR2 signal transduction by tricin. VEGFR2 signal transduction leads to the activation of various downstream signaling substrates that are involved in proliferation, migration and capillary tube formation of endothelial cells (7,8). We, thus, evaluated the effect of tricin on VEGF-mediated VEGFR2 signaling pathways in HUVECs. As shown in Fig. 5A, tricin efficiently suppressed the phosphorylation of VEGFR2, AKT and ERK1/2 induced by VEGF, without affecting the total protein levels, suggesting that tricin exhibits the antiangiogenic activity by inhibiting VEGFR2-mediated downstream signaling cascades. In addition, we found that tricin dose-dependently reduced the generation of ROS induced by VEGF in HUVECs (Fig. 5B). It has been previously reported that VEGF stimulates ROS production and in turn promotes VEGFR2 autophosphorylation by reversibly oxidizing and inactivating protein tyrosine phosphatases (PTPs) (23,24). Taken together, these results suggest that tricin may block the VEGFR2 signaling in HUVECs via the downregulation of ROS generation.

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**The effect of tricin on hypoxia-induced accumulation of HIF-1α protein.** HIF-1α pathway activation in hypoxic tumor cells is an important stimulus for tumor angiogenesis through the regulation of the expression of proangiogenic genes such as VEGF (13,14). To determine the role of HIF-1α in mediating the antiangiogenic effect of tricin, we evaluated the HIF-1α inhibitory activity of tricin in the human hepatoma cell line HepG2, a hypervascularized tumor. As shown in Fig. 6A, tricin-treated HepG2 cells reduced the hypoxia-induced accumulation of HIF-1α protein in a dose-dependent manner. We further assessed the effect of tricin on the expression of VEGF induced by hypoxia. Tricin dose-dependently decreased VEGF production in HepG2 cells under hypoxic condition (Fig. 6B). These data indicate that tricin could inhibit tumor angiogenesis by downregulating HIF-1α and its target gene, VEGF.
The enhanced antiangiogenic effect of combined treatment with tricin and bevacizumab. Bevacizumab (Avastin) is a monoclonal antibody that blocks angiogenesis by inhibiting vascular endothelial growth factor (9). Although the drug has been used in treatment of various solid tumors in combination with several anticancer agents, the efficacy of this treatment is limited due to the development of resistance (25). We, thus, evaluated whether tricin elevates the antiangiogenic function of the VEGF blocker using a chemoinvasion assay. As shown in Fig. 7, treatment with both 2.5 µM of tricin and 100 ng/ml of bevacizumab resulted in additive inhibition of VEGF-induced endothelial cell invasion (inhibition of 45, 53 and 94% with tricin, bevacizumab, and tricin/bevacizumab combination, respectively), suggesting that tricin potentiates the antiangiogenic activity of bevacizumab. Thus, tricin administered alone or in combination with bevacizumab may overcome the resistance to bevacizumab for antiangiogenesis.

Discussion

Owing to the crucial role of angiogenesis in the growth and metastasis of solid tumors, the specific perturbation of angiogenesis has been considered a powerful strategy for anticancer therapy. The inhibition of VEGF pathway has become the focus of antiangiogenesis research since VEGF is a pivotal stimuli of angiogenesis. Strategies to inhibit the VEGF pathway include the blockade of VEGFR2-mediated angiogenic signal transduction in endothelial cells and the prevention of VEGF expression by suppressing its transcription regulators such as HIF-1α in tumor cells (26). Therefore, the novel angiogenesis inhibitors that target both VEGFR2 and HIF-1α activities could provide more effective therapeutic potential for the treatment of hypervascularized tumors.

In recent years, research on dietary flavonoids has become increasingly important with the discovery of their diverse biological activities at non-toxic concentrations. Previous studies have revealed that tricin, a naturally occurring flavone, possesses antiviral, antiinflammatory, antioxidant, antitubercular, antiulcerogenic, antimelanogenic, antihistaminic and anticancer effects (27). In addition, the molecular mechanisms for its biological effects were partly identified. Tricin reduced inflammatory responses in human peripheral blood mononuclear cells (hPBMCs) by regulating the TLR4/NF-κB/STAT, p38MAPK and PI3K/AKT pathways (28,29). It has been also found that tricin exhibits potent anticancer effects in various cancer cells including breast and colon cancers via the inhibition of cyclooxygenase and P-glycoprotein activities (30,31). The antitumor effect of tricin was also demonstrated in the...
present study. Tricin significantly inhibited the growth of human brain, gastric, colon, cervical and liver cancer cell lines (Fig. 2A). Moreover, in recent research, tricin showed pharmaco-kinetic benefit compared with apigenin, a known anticancer flavone. The dietary administration of tricin in mice was more available than apigenin in blood and tissues (32). Moreover, tricin did not show genotoxic properties in mice, suggesting that the safety of tricin may increase its potential clinical usefulness (33). However, to the best of our knowledge, no evaluation of the antiangiogenic activity of tricin has been reported to date.

In the present study, we describe for the first time the antiangiogenic activity and underlying molecular mechanisms of tricin. The flavone exhibited potent antiangiogenic activity in vitro with no obvious cytotoxicity (Fig. 3). We also found that tricin effectively suppressed tumor cell-induced angiogenesis (Fig. 4). Furthermore, our results demonstrated that tricin may inhibit tumor angiogenesis by modulating at least two angiogenic pathways (Fig. 8). In this study, both the VEGFR2 signaling of endothelial cells and the HIF-1α and VEGF expression levels of tumor cells were downregulated by tricin treatment (Figs. 5 and 6). In addition, the blockade of VEGFR2 signal transduction may be associated with the reduction of ROS, generated from NADPH oxidase or mitochondria, by tricin.

An anti-VEGF monoclonal antibody, bevacizumab, has been approved for combination use with standard chemotherapy in certain metastatic cancers (9,10). However, recent clinical results of bevacizumab have revealed its limited therapeutic efficacy in drug-resistant solid tumors as well as several adverse effects such as hypertension, proteinuria and hemorrhage (11,12,25). We, thus, evaluated whether tricin ameliorates the antiangiogenic effect of bevacizumab. Combined treatment with tricin and bevacizumab more effectively inhibited VEGF-induced angiogenesis compared with single agent treatment (Fig. 7). Therefore, these results suggest that tricin may have promising therapeutic potential to overcome the resistance to bevacizumab, alone or in combination with bevacizumab.

Taken together, our results provide new therapeutic aspect for tricin as a potent inhibitor of angiogenesis via the dual blocking of VEGFR2 and HIF-1α pathways. In addition, the known pharmacokinetic advantages of tricin may contribute to its clinical application to antiangiogenic therapy for cancer. Although the inhibitory effect of tricin on the VEGFR2 signaling was partially associated with the decrease of ROS generation, precise mechanisms of how tricin controls bidirectionally angiogenic pathways remain unclear. Further studies to understand the action mechanism of flavonoids will help the discovery of the upstream cellular mediators of tumor angiogenesis regulated by tricin.

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