Phenethyl Isothiocyanate Inhibits Angiogenesis

In vitro and Ex vivo

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Abstract

Previous studies, including those from our laboratory, have revealed that phenethyl isothiocyanate (PEITC), a constituent of many edible cruciferous vegetables, not only affords significant protection against chemically induced cancer in animal models but also inhibits growth of cancer cells in culture and in vivo by causing cell cycle arrest and apoptosis induction. We now report a novel response to PEITC involving inhibition of angiogenesis in vitro and ex vivo at pharmacologically achievable concentrations. The PEITC treatment caused a decrease in survival of human umbilical vein endothelial cells (HUVEC) in a concentration- and time-dependent manner. The capillary-like tube structure formation (in vitro neovascularization) and migration (invasion potential) by HUVEC was also inhibited significantly in the presence of PEITC at pharmacologically relevant concentrations (<1 μmol/L). The PEITC-mediated inhibition of angiogenic features of HUVEC in vitro was associated with suppression of vascular endothelial growth factor (VEGF) secretion, down-regulation of VEGF receptor 2 protein levels, and inactivation of prosurvival serine-threonine kinase Akt. The PEITC treatment reduced migration by PC-3 human prostate cancer cells, which correlated with inactivation of Akt and suppression of VEGF, epidermal growth factor (EGF), and granulocyte colony-stimulating factor (G-CSF) secretion. The PEITC-mediated inhibition of PC-3 cell migration was statistically significantly attenuated by ectopic expression of constitutively active Akt. Most importantly, PEITC treatment inhibited ex vivo angiogenesis as revealed by chicken egg chorioallantoic membrane assay. In conclusion, the present study suggests that inhibition of angiogenesis may be an important mechanism in cancer chemoprevention by PEITC.

Introduction

Angiogenesis (formation of new blood vessels) is a highly complex and tightly regulated physiologic process that is implicated in pathogenesis of many chronic diseases, including cancer, rheumatoid arthritis, endometriosis, and diabetic retinopathy (1, 2). Angiogenesis in tumors permits the growth and invasiveness of cancer cells leading to metastasis to distant organs (3, 4). Evidence is accumulating to indicate that angiogenesis is especially critical for growth and progression of solid tumors because growth in tumor mass beyond 2 to 3 mm is often preceded by an increase in formation of new blood vessels presumably essential for delivery of nutrients and oxygen to the tumor microenvironment (2–5). Therefore, antiangiogenic therapy represents one of the most promising approaches to control tumor growth and invasiveness (5–7). Interestingly, many of the antiangiogenic agents currently in clinical trials are natural products (6). Drug development from natural products is a rapidly emerging and highly promising strategy to identify novel antiangiogenic and anticancer agents.

Epidemiologic studies continue to support the premise that dietary intake of cruciferous vegetables may be protective against the risk of various types of malignancies (8). Anticarcinogenic effect of cruciferous vegetables is attributed to organic isothiocyanates (ITC) that occur naturally as thioglucoside conjugates (glucosinolates) in a variety of edible cruciferous vegetables, such as broccoli, watercress, and cabbage (9). Organic ITCs are generated due to hydrolysis of corresponding glucosinolates through catalytic mediation of myrosinase, which is released on damage of the plant cells during processing (cutting or chewing) of cruciferous vegetables. Phenethyl-ITC (PEITC) is one of the best-studied members of the ITC family of compounds that has generated a great deal of research interest due to its cancer chemopreventive activity (10–14). For example, PEITC administration was shown to significantly inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane none–induced pulmonary neoplasia in mice (10), N-nitrosobenzyl methylamine–induced esophageal cancer in rats (11), and benzo(a)pyrene–induced carcinogenesis in mice (14). Impaired carcinogen activation due to inhibition of cytochrome P450–dependent monoxygenases and/or increased detoxification of the carcinogenic intermediates due to induction of phase 2 enzymes (e.g., glutathione transferases) are considered important mechanisms for PEITC-mediated inhibition of chemically induced cancers in animal models (reviewed in ref. 9).

Studies, including those from our laboratory, have revealed that PEITC and other naturally occurring ITC compounds have the ability to suppress growth of human cancer cells in culture as well as in vivo in xenograft assays by causing cell cycle arrest and apoptosis induction (15–22). For instance, p.o. gavage of PEITC significantly retards growth of PC-3 and TRAMP-C1 prostate cancer xenografts in nude mice in association with induction of proapoptotic Bcl-2 family members (19, 21). It was also shown recently that PEITC inhibits transcriptional activity of nuclear factor-κB (NF-κB) and suppresses expression of NF-κB–regulated genes, including vascular endothelial growth factor (VEGF) in PC-3 human prostate cancer cells (23). Because VEGF plays an important role in angiogenesis by promoting endothelial cell proliferation, migration, and differentiation (24), we hypothesized that PEITC may inhibit angiogenesis.

We now show that PEITC effectively inhibits in vitro angiogenic features (capillary-like tube structure formation and migration potential) of human umbilical vein endothelial cells (HUVEC) and PC-3 cells by causing suppression of proangiogenic growth factor.
secretion and inactivation of Akt. We also found that PEITC is a potent inhibitor of ex vivo angiogenesis in chicken chorioallantoic membrane (CAM) assay. We propose that inhibition of angiogenesis may be an important mechanism in cancer chemoprevention by PEITC.

Materials and Methods

Reagents and antibodies. PEITC and D,L-sulforaphane (SFN) were purchased from LKT Laboratories (St. Paul, MN), whereas benzyl-ITC (BITC) was obtained from Aldrich (St. Louis, MO). Cell culture reagents and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY). An antibody specific for detection of phosphorylated Akt (Ser\(^{177}\)) was from Cell Signaling Technology (Beverly, MA), and the antibodies against total Akt and VEGF receptor 2 (VEGF-R2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Matrigel (growth factor reduced) was procured from BD PharMingen (Bedford, MA). The ELISA kits for measurements of VEGF, epidermal growth factor (EGF), granulocyte colony-stimulating factor (G-CSF), interleukin-17 (IL-17), matrix metalloproteinase (MMP)-2, and MMP-9 were from R&D Systems (Minneapolis, MN) or BioSource (Camarillo, CA). The kit for determination of Akt kinase activity and Akt-1/2 inhibitor [1.3-dihydro-1-[(4-(6-phenyl-1H-imidazol-4-yl)quinolin-2-yl)phenyl]methyl-4-piperidinyl]-2H-benzimidazol-2-one] was purchased from Calbiochem (La Jolla, CA).

Cell culture and cell survival assay. HUVECs were purchased from Clonetics (Walkersville, MD) and maintained in endothelial cell growth medium-2 (EGM2 MV SingleQuots, Clonetics) supplemented with 5% FBS. Monolayer cultures of PC-3 cells were maintained in F-12K Nutrient Mixture (Kaighn's modification) supplemented with 7% (v/v) non–heat-inactivated FBS and antibiotics. Cells were maintained in an atmosphere of 95% air and 5% CO\(_2\) at 37°C and incubated with 7% (v/v) non–heat-inactivated FBS and antibiotics. Cells were maintained in an atmosphere of 95% air and 5% CO\(_2\) at 37°C.

In vitro capillary-like tube structure formation assay. The effect of PEITC treatment on in vitro angiogenesis was determined by Matrigel capillary-like tube structure formation assay as described by us previously (25). The HUVECs were seeded on Matrigel differentiate and form capillary-like tube structures. Similar to neovascularization (angiogenesis), the tube formation on Matrigel requires cell-matrix interaction, cellular communication, and cellular motility. To examine the effect of PEITC on in vitro angiogenesis, HUVECs were seeded in 96-well culture plates precoated with Matrigel and exposed to different concentrations of PEITC (0.5, 1, and 2 \(\mu\)mol/L) for 24 h. The tube formation was visualized under an inverted microscope. Enclosed network of tube structures from three randomly chosen fields was scored using a Leica DC300F microscope (Leica, Cambridge, United Kingdom). In some tube formation experiments, the HUVECs were exposed to 0.5 \(\mu\)mol/L PEITC for 24 h in the presence or absence of 1 \(\mu\)mol/L of the Akt-1/2 inhibitor.

In vitro migration (invasion) assay. The effect of PEITC treatment on in vitro migration (invasion) by HUVECs or PC-3 cells was determined using Transwell Boyden chamber (Corning, Acton, MA) containing a polycarbonate filter with a pore size of 8 \(\mu\)m as described by us previously (25). Briefly, 0.2 mL HUVEC or PC-3 cell suspension (4 \(\times\) 10\(^5\) cells) in complete medium was mixed with desired concentrations of PEITC or DMSO (control), and the suspension was added to the upper compartment of the chamber. The lower compartment of the chamber contained 0.6 mL of complete medium containing the same concentrations of PEITC or DMSO. Following incubation at 37°C for 24 h, the nonmigrant cells from the upper face of the Transwell membrane were removed using a cotton swab. The membrane was washed with PBS. The migrated cells on the bottom face of the membrane were fixed with 90% ethanol and stained with eosin. Three randomly selected fields were scored for migrated cells. In some migration assays, HUVECs were treated with 0.5 \(\mu\)mol/L PEITC for 24 h in the presence or absence of 1 \(\mu\)mol/L Akt-1/2 inhibitor.

ELISA assays for secretion of growth factors, IL-17, and MMPs. HUVECs or PC-3 cells (2 \(\times\) 10\(^5\)) were plated in 24-well plates and allowed to attach by overnight incubation at 37°C. Cells were treated with desired concentrations of PEITC or DMSO (control) for 24 h as described above. Subsequently, the culture medium was collected and used to determine secretion of VEGF, EGF, G-CSF, IL-17, MMP-2, and MMP-9 using commercially available kits according to the manufacturers' recommendations. Akt kinase assay. Akt kinase activity was determined using reagents supplied by the manufacturer. Briefly, 4 \(\times\) 10\(^6\) HUVECs cultured in the presence of 1% FBS were plated and allowed to attach by overnight incubation. The cells were then treated with 4 \(\mu\)mol/L of PEITC or DMSO (control) for 4 h and lysed with a solution containing 20 \(\mu\)mol/L Tris (pH 7.5), 150 \(\mu\)mol/L NaCl, 1 \(\mu\)mol/L EDTA, 1 \(\mu\)mol/L EGTA, 1% Triton X-100, 2.5 \(\mu\)mol/L sodium pyrophosphate, 1 \(\mu\)mol/L \(\beta\)-glycerophosphate, 1 \(\mu\)mol/L Na\(_2\)VO\(_4\), 1 \(\mu\)g/mL leupeptin, and 1 \(\mu\)mol/L phenylmethylsulfonyl fluoride. Equal amounts of lysate proteins (300 \(\mu\)g) from each treatment group were incubated overnight at 4°C with agaroase-conjugated anti-Akt antibody. Immunoprecipitates were washed four times with the lysis buffer and twice with kinase assay buffer [25 \(\mu\)mol/L Tris (pH 7.5), 5 \(\mu\)mol/L \(\beta\)-glycerophosphate, 2 \(\mu\)mol/L DTT, 0.1 \(\mu\)mol/L Na\(_2\)VO\(_4\), 10 \(\mu\)mol/L Mg\(_2\)Cl\(_2\)]. The immunoprecipitates were resuspended in 40 \(\mu\)L of kinase buffer supplemented with 200 \(\mu\)mol/L ATP and 1 \(\mu\)g of glycogen synthase kinase-3\(\alpha\)/\(\beta\) (GSK-3\(\alpha\)/\(\beta\)) fusion protein. After incubation at 30°C for 30 min, the kinase reaction was terminated with the addition of 40 \(\mu\)L of 2x SDS sample buffer. An aliquot of the immunoprecipitate (30 \(\mu\)L) was subjected to SDS-PAGE followed by immunoblotting using anti–phosphorylated GSK-3\(\alpha\)/\(\beta\) (Ser\(^{21}\)) antibody (25).

Ectopic expression of constitutively active Akt. PC-3 cells were transiently transfected with pcMV6 vector containing constitutively active Akt-1 (Myr-Akt1-HA; kindly provided by Dr. Daniel Altschuler, University of Pittsburgh, Pittsburgh, PA) or empty vector using Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s recommendations. Briefly, PC-3 cells were plated in six-well plates at a density of 2 \(\times\) 10\(^4\) cells/mL and allowed to attach by overnight incubation. Cells were transfected with the expression vector encoding constitutively active Akt (CA-Akt) or empty vector. Twenty-four hours after transfection, cells were treated with 2 or 4 \(\mu\)mol/L of PEITC or DMSO (control) for 24 h and processed for immunoblotting of total or phosphorylated Akt and migration assay.

CAM assay in fertilized chicken eggs. The effect of PEITC on ex vivo angiogenesis was determined by CAM assay. Briefly, fertilized chicken eggs were incubated at 37.5°C in a humidified incubator with forced air circulation. On embryonic day 4, eggs were cracked open and embryos were carefully transferred into 100-mm Petri dishes to continue their development in an incubator at 37.5°C with 1.5% CO\(_2\). PEITC was mixed with 0.5% methylcellulose in water. Drops of this solution (10 \(\mu\)L per drop) were allowed to dry on a Teflon-coated surface forming methylcellulose discs of \(\sim 2\) mm in diameter. The methylcellulose discs containing different concentrations of PEITC (5 or 20 \(\mu\)mol/egg) were gently implanted on top of chicken CAM on embryonic day 6, and the embryos were incubated for 2 more days. The chicken CAMs were examined on embryonic day 8 and photographed under a stereomicroscope. Effect of PEITC on angiogenesis was quantitatively evaluated by scoring vascular density index (VDI) from control and treated groups. The VDI of each CAM represents the number of intersections made by the blood vessels with three equidistant concentric circles on the area covered by methylcellulose discs. The VDI was scored using Image-Pro Plus software.

Results

PEITC treatment decreases viability of HUVEC. To test the hypothesis that ITCs may inhibit angiogenesis, initially we determined the effects of three widely studied ITC compounds (PEITC, SFN, and BITC; compare Fig. L4 for structures) on survival of HUVEC by trypan blue dye exclusion assay, and the results are summarized in Fig. 1B and C. The viability of HUVEC was decreased significantly in the presence of PEITC in a concentration- and
time-dependent manner (Fig. 1B). The viability of HUVEC was also decreased significantly on treatment with SFN and BITC, but the IC$_{50}$ for these ITC analogues was between 3- and 10-fold higher compared with that for PEITC. These results suggested that even a subtle change in ITC structure (e.g., the alkyl chain length in PEITC versus BITC) could have a significant effect on its antiangiogenic activity. Because of higher potency of PEITC against HUVEC proliferation compared with BITC or SFN, we focused on this compound for further investigation to fully characterize its antiangiogenic response.

PEITC inhibits capillary-like tube structure network. Because PEITC treatment significantly decreased HUVEC viability, we proceeded to test whether PEITC inhibits in vitro angiogenesis. We explored this possibility by determining the effect of PEITC treatment on formation of capillary-like tube structures by HUVEC on growth factor–reduced Matrigel, which is a well-accepted technique to measure in vitro angiogenesis. Figure 2A depicts capillary-like tube structure formation by HUVEC following a 24-h treatment with DMSO (control) or different concentrations of PEITC. The capillary-like tube structures were clearly visible in DMSO-treated control HUVEC. On the other hand, PEITC treatment caused disruption of the capillary-like tube network in a concentration-dependent manner (Fig. 2A). Capillary-like tube structures were scored from control and PEITC-treated samples, and the results are summarized in Fig. 2B. The formation of capillary-like tube structures was inhibited by about 54% and 75% in the presence of 0.5 and 1 µmol/L of PEITC, respectively, compared with control (Fig. 2B). The capillary-like tube structure formation by HUVEC was completely abolished (100% inhibition) in the presence of

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**Figure 1.** A, chemical structures of the ITC analogues used in the present study. B, concentration- and time-dependent effect of PEITC on HUVEC viability as determined by trypan blue dye exclusion assay. C, trypan blue dye exclusion assay for effect of BITC and SFN on HUVEC viability following a 24-h drug exposure. Points, mean (n = 3); bars, SE. *P < 0.05, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test.

**Figure 2.** A, representative images depicting formation of capillary-like tube structures by HUVEC following a 24-h treatment with DMSO (Control) or the indicated concentrations of PEITC. B, percentage of capillary-like tube structure formation following a 24-h exposure of HUVEC to the indicated concentrations of PEITC relative to DMSO-treated control. Columns, mean (n = 3); bars, SE. *P < 0.05, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test. Similar results were observed in two independent experiments carried out in triplicate.
2 μmol/L PEITC (Fig. 2A). These results indicated that PEITC treatment inhibited formation of capillary-like tube structures by HUVEC.

**PEITC inhibits HUVEC migration.** Next, we determined the effect of PEITC treatment on invasion potential (migration) of HUVEC using a modified Boyden Chamber assay. Figure 3A depicts eosin-stained images of migrated HUVEC. In DMSO-treated controls, a large fraction of HUVEC migrated to the bottom face of the membrane, which was decreased markedly in the presence of PEITC. For instance, compared with DMSO-treated control, the migration potential of HUVEC was inhibited by about 58%, 72%, 81%, and 99% in the presence of 0.5, 1, 2, and 4 μmol/L of PEITC, respectively (Fig. 3B). These results indicated that the migration by HUVEC was inhibited significantly in the presence of PEITC.

**PEITC treatment suppresses VEGF secretion and VEGF-R2 protein level in HUVEC.** VEGF plays an important role in angiogenesis by promoting endothelial cell proliferation, migration, and differentiation (24). To gain insights into the mechanism by which PEITC inhibits in vitro angiogenesis, we determined its effect on VEGF secretion and the results are shown in Fig. 4A. The PEITC treatment caused a dose-dependent and statistically significant decrease in VEGF secretion into the medium. For instance, relative to DMSO-treated control, the secretion of VEGF into the medium was inhibited by about 45%, 62%, and 65% on a 24-h treatment with

![Figure 3](image-url)

**Figure 3.** A, representative images depicting effect of PEITC treatment (24 h) on HUVEC migration. B, percentage of HUVEC migration following a 24-h exposure to the indicated concentrations of PEITC relative to DMSO-treated control. Columns, mean (n = 3); bars, SE. *, P < 0.05, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett’s test.

![Figure 4](image-url)

**Figure 4.** A, VEGF secretion into the medium following a 24-h treatment of HUVEC with DMSO (control) or the indicated concentrations of PEITC. Experiment was repeated twice in duplicate with similar results; error bars are shown to indicate data scatter. *, P < 0.05, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett’s test. B, immunoblotting for VEGF-R2 (top) using lysates from HUVEC treated with 4 μmol/L PEITC for the indicated times. The blot was stripped and reprobed with anti-actin antibody to ensure equal protein loading. Immunoblotting was done twice using independently prepared lysates, and the results were comparable. Akt kinase activity (bottom) using lysates from HUVEC treated with 0.01 % DMSO (control) or 4 μmol/L PEITC for 4 h. C, percentage of capillary-like tube structure formation in the presence of PEITC and/or Akt-1/2 inhibitor. Similar results were obtained in two independent experiments. D, percentage of HUVEC migration in the presence of PEITC and/or Akt-1/2 inhibitor. C and D, columns, mean (n = 3); bars, SE. a, P < 0.05, significantly different compared with DMSO-treated control; b, P < 0.05, significantly different compared with PEITC treatment alone by one-way ANOVA followed by Bonferroni’s multiple comparison test.
1, 2, and 4 μmol/L of PEITC, respectively (Fig. 4A). The VEGF-mediated prosurvival signal transduction to the nucleus is caused by its interaction with receptors, including VEGF-R1, VEGF-R2, and VEGF-R3. Of the three receptors, VEGF-R2 is believed to play an important role in angiogenesis. Next, we tested the possibility whether PEITC-mediated inhibition of HUVEC migration and tube formation was caused by a decrease in the protein level of VEGF-R2. As can be seen in Fig. 4B, PEITC treatment caused a time-dependent decrease in the levels of VEGF-R2 protein. The PEITC-mediated down-regulation of VEGF-R2 protein was evident as early as 2 h after treatment and increased progressively with increasing drug exposure time (Fig. 4B, top).

**PeITC treatment causes inactivation of Akt in HUVEC.** Akt (also known as protein kinase B) is implicated in promotion of endothelial cell survival and VEGF-stimulated endothelial cell migration and differentiation (26–29). Activation of Akt is mediated by receptor tyrosine kinase–mediated phosphorylation (30). The Ser\(^{473}\) phosphorylation of Akt occurs in response to growth factor stimulus and is necessary for its activation (reviewed in ref. 30). As can be seen in Fig. 4B (bottom), a 4-h treatment of HUVEC with 4 μmol/L PEITC caused a marked decrease in kinase activity of Akt as determined by immunoprecipitation-kinase assay using GSK-3α/β as a substrate. To further examine the relationship between PEITC-mediated suppression of angiogenesis and Akt inactivation, we did tube formation assay in HUVEC exposed to 0.5 μmol/L PEITC in the presence or absence of a selective inhibitor of Akt-1/2 (31, 32). The Akt-1/2 inhibitor used in the present study is cell active and blocks phosphorylation of Akt at Thr\(^{308}\) and Ser\(^{473}\) and reduces the level of active Akt in human prostate cancer LNCaP cells (31, 32), human cervical carcinoma C33A cells (31), human breast carcinoma MCF-7 and MDA-MB-468 cells (32), and human colon cancer HT29 cells (32). As can be seen in Fig. 4C, the capillary-like tube structure formation by HUVEC was inhibited by about 33% and 54% on a 24-h treatment with 0.5 μmol/L PEITC and 1 μmol/L of Akt-1/2 inhibitor alone, respectively. On the other hand, combined treatment of HUVEC with PEITC and Akt-1/2 inhibitor resulted in near complete loss of capillary-like tube network (Fig. 4C). We determined the effects of PEITC and/or Akt-1/2 inhibitor on HUVEC migration (invasion potential), and the results are shown in Fig. 4D. Similar to tube formation assay, combined treatment of HUVEC with PEITC (0.5 μmol/L) and Akt-1/2 inhibitor (1 μmol/L) resulted in a much greater inhibition of migration compared with single agent alone. Together, these results indicated that the PEITC-mediated inhibition of HUVEC capillary-like tube formation and migration was mediated by suppression of VEGF secretion and VEGF-R2 protein level and inactivation of Akt.

**PeITC treatment inhibits PC-3 cell migration.** Cell migration and invasion are fundamental to tumor metastasis (33). Next, we raised the question of whether PEITC-mediated suppression of migration (Fig. 3) was an effect restricted to endothelial cells

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**Figure 5.** A, percentage of PC-3 migration following a 24-h exposure to the indicated concentrations of PEITC relative to DMSO-treated control. Columns, mean (n = 3); bars, SE. *, P < 0.05, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett’s test. Similar results were observed in two independent experiments carried out in triplicate. B, immunoblotting for phosphorylated Akt (Ser\(^{473}\); P-Akt) using lysates from PC-3 treated with 4 μmol/L PEITC for the indicated times. The blot was stripped and reprobed with anti-actin antibody to ensure equal protein loading. C, immunoblotting for phosphorylated Akt (Ser\(^{473}\)) and total Akt using lysates from PC-3 cells transiently transfected with empty vector or expression vector carrying CA-Akt. The blot was stripped and reprobed with anti-actin antibody to ensure equal protein loading. D, percentage migration in PC-3 cells transiently transfected with empty vector or vector containing CA-Akt following a 24-h exposure to the indicated concentrations of PEITC relative to DMSO-treated control. Columns, mean (n = 3); bars, SE. *, P < 0.05, significantly different compared with empty vector–transfected control by paired t test. Similar results were observed in two independent experiments.
(HUVEC). We addressed this question by determining the effect of PEITC treatment on migration potential of PC-3 human prostate cancer cells, and the results are shown in Fig. 5A. Similar to HUVEC, migration by PC-3 cells was inhibited significantly in the presence of PEITC in a concentration-dependent manner (Fig. 5A). The PEITC-mediated suppression of PC-3 cell migration correlated with a decrease in phosphorylated Akt (active Akt) level, which was clearly evident at 16 and 24 h after treatment (Fig. 5B). Inhibition of Ser\(^{273}\) phosphorylation of Akt by PEITC treatment was dose dependent (results not shown). These results suggested that PEITC-mediated inhibition of PC-3 cell migration might be caused by inactivation of Akt.

**Ectopic expression of CA-Akt attenuates PEITC-mediated suppression of PC-3 cell migration.** To firmly establish the role of Akt in our model, we determined the effect of overexpression of CA-Akt on PEITC-mediated inhibition of PC-3 cell migration. Cells transfected with the empty vector were used as control. Overexpression of CA-Akt in transfected PC-3 cells was confirmed by immunoblotting using antibodies against total Akt and phosphorylated Akt (Ser\(^{273}\)). As can be seen in Fig. 5C, the levels of total Akt and phosphorylated Akt (Ser\(^{273}\)) were markedly higher in PC-3 cells transfected with CA-Akt compared with empty vector–transfected control cells. As expected, ectopic expression of CA-Akt potentiated PC-3 cell migration when compared with empty vector–transfected control cells (Fig. 5D). Similar to untransfected PC-3 cells (Fig. 5A), PEITC treatment inhibited migration in vector-transfected control PC-3 cells in a concentration-dependent manner (Fig. 5D). The PEITC-mediated inhibition of PC-3 cell migration was statistically significantly attenuated by ectopic expression of CA-Akt compared with empty vector–transfected control cells (Fig. 5D). These results confirmed that Akt was a critical determinant of PEITC-mediated inhibition of PC-3 cell migration.

**PEITC treatment suppresses VEGF, EGF, and G-CSF secretion by PC-3 cells.** Angiogenic potential of cancer cells is regulated by multiple growth factors, cytokines, and MMPs (24, 34–37). We therefore determined the levels of VEGF, EGF, G-CSF, IL-17, MMP-2, and MMP-9 in medium from PC-3 cells cultured for 24 h in the presence of 0.01% DMSO (control) or 2, 4, and 8 μmol/L of PEITC. Compared with DMSO-treated control, secretion of VEGF was decreased by about 32% and 59% in the presence of 4 and 8 μmol/L of PEITC, respectively. Similarly, secretion of EGF and G-CSF was reduced by 27% and 30% in the presence of 4 μmol/L PEITC and by 32% and 50% in the presence of 8 μmol/L PEITC, respectively, compared with DMSO-treated control (P < 0.05 between DMSO control and PEITC group by one-way ANOVA followed by Dunnett’s test). On the other hand, PEITC treatment did not have any appreciable effect on secretion of IL-17, MMP-2, or MMP-9 (results not shown). The results indicated that PEITC treatment decreased levels of VEGF, EGF, and G-CSF in PC-3 cells.

**PEITC treatment inhibits ex vivo angiogenesis in CAM assay.** We used an ex vivo CAM assay to further examine antiangiogenic effect of PEITC. The concentrations of the PEITC for CAM assay were selected from preliminary dose-finding studies and were well within the PEITC concentrations effective against PC-3 xenograft growth in nude mice in vivo (21). We have shown previously that PC-3 xenograft growth in nude mice is significantly inhibited by p.o. administration (five times weekly) of 12 μmol PEITC (21). Microscopic examination of the CAM revealed highly vascularized structure among trabeculae of the control group (Fig. 6A). Exposure to PEITC (20 nmol/egg) drastically reduced the

![Figure 6. A. representative images depicting antiangiogenic response to PEITC in CAM assay. Numerous allantoic vessels converged radially in the control group, whereas fewer vessels were seen in eggs exposed to 20 nmol PEITC/egg. B, VDI in eggs exposed to the indicated concentrations of PEITC or DMSO (Control). Columns, mean (n = 4–7); bars, SE. *, P < 0.05, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett’s test. Similar results were observed in three independent experiments.](https://cancerres.aacrjournals.org/article-fig9)
PEITC treated versus control). However, 5 nmol PEITC/egg did not have any appreciable effect on VDI. These results confirmed anti-angiogenic potential of PEITC through an \textit{ex vivo} assay.

**Discussion**

The present study reveals that PEITC inhibits angiogenic features of HUVEC \textit{in vitro} as revealed by capillary-like tube formation and migration (invasion) assays. In addition, PEITC treatment significantly inhibits neovascularization \textit{ex vivo} in CAM assay. To the best of our knowledge, the present study is the first published report to document inhibition of angiogenesis \textit{in vitro} and \textit{ex vivo} by PEITC. Statistically significant inhibition of HUVEC capillary-like tube structure formation (Fig. 2) and migration (Fig. 3) by PEITC is evident at concentrations (<1 μmol/L) well within the pharmacologically achievable range. For example, the maximal plasma concentration of PEITC (C\textsubscript{max}) following ingestion of 100 g watercress was shown to vary between 673 and 1,155 nmol/L (mean, 928 ± 250 nmol/L; ref. 38). A mean C\textsubscript{max} of 1.04 ± 0.22 μmol/L of total ITC in three subjects taking a single dose of PEITC (40 mg) has been noted in another study (39). We propose that inhibition of angiogenesis may be an important mechanism in cancer chemoprevention by PEITC.

VEGF is a proangiogenic growth factor most closely associated with aggressive human cancer cells (40). VEGF provides prosurvival signals to normal and tumor-derived endothelial cells (24), and this signal transduction to the nucleus is mediated by VEGF receptors, including VEGF-R1, VEGF-R2, and VEGF-R3. Whereas VEGF-R2 is believed to play an important role in angiogenesis, VEGF-R3 is responsible for lymphangiogenesis. VEGF-R2 is a receptor tyrosine kinase, which, on activation by ligand binding, phosphorylates and activates secondary messengers, including Akt, to regulate endothelial cell proliferation and migration (41). The present study reveals that exposure of HUVEC to PEITC results in suppression of VEGF secretion to the medium (Fig. 4A) as well as down-regulation of VEGF-R2 protein level (Fig. 4B). The PEITC-mediated suppression of VEGF secretion is not restricted to HUVEC because similar effects are evident in PC-3 cells. Together, these results suggest that the PEITC-mediated inhibition of \textit{in vitro} angiogenic features of HUVEC is most likely caused by suppression of VEGF and VEGF-R2.

Although the mechanism by which PEITC causes suppression of VEGF and VEGF-R2 expression in HUVECs or PC-3 cells remains to be elucidated, several possibilities exist to explain these effects. For instance, several transcription factors, including hypoxia-inducible factor (HIF)-1α, NF-κB, activator protein-1, and Sp-1, bind to the VEGF promoter to initiate and activate its transcription (reviewed in ref. 42). Likewise, HIF-2α is highly expressed by vascular endothelial cells and activates the transcription of endothelial cell–specific receptor tyrosine kinases and VEGF-R2 (42). It is possible that PEITC inhibits transcriptional activity of some of these transcription factors to suppress VEGF and VEGF-R2 expression. Suppression of NF-κB and NF-κB–regulated gene expression by PEITC treatment has been documented in human prostate cancer cells (23). We have also shown previously that BITC, a close structural analogue of PEITC, inhibits activity of NF-κB in human pancreatic cancer cells (43). Recent studies have also implicated reactive oxygen species in EGF-mediated regulation of VEGF and HIF-1α expression (44). Because PEITC is an antioxidant (9), the possibility that suppression of VEGF expression by this agent is linked to its antioxidative effect cannot be ignored and warrants further investigation.

Serine-threonine kinase Akt, a homologue of a retroviral oncoprotein v-Akt, is recruited during signal transduction from growth factor receptors and intracellular pathways (28). Vascular homeostasis and angiogenesis by different stimuli, including VEGF and angiopoietin-1, is regulated by the activation of Akt (28). Overexpression of CA-Akt and its upstream kinase phosphatidylinositol 3-kinase (PI3K) has been shown to induce angiogenesis in chicken embryo (29). The expression of VEGF in endothelial cells is also regulated by the PI3K-Akt signaling axis (29). Akt activation not only promotes endothelial cell survival but also regulates vasomotor responses via phosphorylation of endothelial nitric oxide synthase and VEGF-stimulated endothelial cell migration and differentiation (27–30, 45). The present study reveals that the antiangiogenic effect of PEITC is closely associated with inhibition of Akt kinase. This conclusion is based on the following observations: (a) PEITC treatment causes inhibition of Akt kinase in HUVEC as revealed by immunoprecipitation–kinase assay using GSK-3α/β as a substrate (Fig. 4B) and in PC-3 cells as revealed by immunoblotting for Ser\textsuperscript{473} phosphorylated (activated) Akt (Fig. 5B), (b) PEITC-mediated inhibition of HUVEC capillary-like tube structure formation and migration is potentiated in the presence of cell-permeable and selective inhibitor of Akt-1/2 (Fig. 4C and D), and (c) PEITC-mediated suppression of PC-3 cell migration is statistically significantly attenuated by ectopic expression of CA-Akt (Fig. 5D). Activation of Akt is mediated by receptor tyrosine kinases that, on ligand binding, are autophosphorylated and cause activation of PI3K (30). Activated PI3K generates lipid second messengers, which facilitate recruitment of Akt to the plasma membrane for its activation (30). It is possible that PEITC treatment causes inhibition of PI3K to inhibit Ser\textsuperscript{473} phosphorylation (activation) of Akt. It was shown recently that cellular responses to PEITC in Caco-2 human colon adenocarcinoma cell line are attenuated in the presence of PI3K inhibitor LY294002 (46).

Recent studies have implicated multiple growth factors and cytokines, including VEGF, EGF, G-CSF, and IL-17, in tumor angiogenesis and growth (24, 35–37, 47). For instance, IL-17 increases angiogenic activity and \textit{in vivo} growth of human non–small lung cancer cells in severe combined immunodeficient mice (37). Similarly, G-CSF promotes tumor angiogenesis by increasing circulating endothelial progenitor cells (47). Degradation of extracellular matrix is a critical component of tumor metastasis (48, 49). Degradation of extracellular matrix is orchestrated by MMPs, which are zinc-dependent proteolytic enzymes (48, 49). Type IV collagen, a basic component of extracellular matrix, is degraded by MMP-2 (gelatinase A) and MMP-9 (gelatinase B; ref. 48). MMP-2 in particular is believed to have an important role in the initial steps of tumor invasion (49). Expression of MMPs is associated with grade and stage of solid tumors (50). The present study reveals that PEITC treatment significantly inhibits secretion of VEGF, EGF, and G-CSF, but not IL-17, by PC-3 cells. Thus, it is reasonable to conclude that suppression of growth factor secretion may exacerbate antiangiogenic effect of PEITC. On the other hand, PEITC treatment does not affect secretion of either MMP-2 or MMP-9.

In conclusion, the present study shows that PEITC is a potent inhibitor of angiogenesis \textit{in vitro} and \textit{ex vivo}. It is important to note that the PEITC-mediated inhibition of angiogenesis occurs at concentrations achievable by dietary intervention or pharmacologic...
administration. The PEITC-mediated inhibition of angiogenesis and migration is associated with inactivation of Akt, suppression of proangiogenic growth factors, and down-regulation of VEGF-R2 protein expression. We are tempted to speculate that inhibition of angiogenesis may be an important mechanism in overall scheme of PEITC-mediated cancer prevention.

References

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