Capsiate, a Nonpungent Capsaicin-Like Compound, Inhibits Angiogenesis and Vascular Permeability via a Direct Inhibition of Src Kinase Activity

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Abstract
Capsiate, a nonpungent capsaicin analogue, and its dihydroderivative dihydrocapsiate are the major capsaicinoids of the nonpungent red pepper cultivar CH-19 Sweet. In this study, we report the biological actions and underlying molecular mechanisms of capsiate on angiogenesis and vascular permeability. In vitro, capsiate and dihydrocapsiate inhibited vascular endothelial growth factor (VEGF)-induced proliferation, chemotactic motility, and capillary-like tube formation of primary cultured human endothelial cells. They also inhibited sprouting of endothelial cells in the rat aorta and formation of new blood vessels in the mouse Matrigel plug assay in response to VEGF. Moreover, both compounds blocked VEGF-induced endothelial permeability and loss of vascular endothelial (VE)-cadherin–facilitated endothelial cell-cell junctions. Importantly, capsiate suppressed VEGF-induced activation of Src kinase and phosphorylation of its downstream substrates, such as p125<sub>FAK</sub> and VE-cadherin, without affecting autophosphorylation of the VEGF receptor KDR/Flk-1. In vitro kinase assay and molecular modeling studies revealed that capsiate inhibits Src kinase activity via its preferential docking to the ATP-binding site of Src kinase. Taken together, these results suggest that capsiate could be useful for blocking pathologic angiogenesis and vascular permeability caused by VEGF. [Cancer Res 2008;68(1):227–35]

Introduction
Vascular endothelial growth factor (VEGF), expressed by most cancer cell types and certain tumor stromal cells, is a potent proangiogenic factor that functions in tumor vascular development (1, 2). Additionally, VEGF may play a role in several human diseases, such as rheumatoid arthritis, psoriasis, hyperthyroidism, atherosclerosis, and diabetic retinopathy (3). VEGF exerts its biological effects by binding to two receptor tyrosine kinases, KDR/Flk-1 and Flt-1, expressed on endothelial cells. The biologically relevant VEGF signaling events are mediated mainly via KDR.

Activation of KDR leads to the activation of various downstream signal transduction proteins, including extracellular signal-regulated kinases, protein kinase C, and phosphoinositide 3-kinase/Akt/ENOS pathway components (4). VEGF also increases Src kinase activity, leading to angiogenesis and breakdown of the endothelial barrier (5, 6). KDR-mediated Src activation induces phosphorylation of vascular endothelial (VE)-cadherin at Tyr<sup>665</sup>, resulting in the induction of angiogenesis and an increase in vascular permeability (7). Moreover, VEGF increases FAK phosphorylation at Tyr<sup>861</sup> via an Src-dependent pathway, and Src inhibition impairs VEGF-induced cell migration and survival (8).

A variety of phenolic substances, particularly those present in dietary and medicinal plants, are hypothesized to exhibit a preventive effect on a variety of diseases, including cancers (9, 10). Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a pungent, pungent compound present in some peppers of the Capsicum family. Because of its analgesic and antiinflammatory activities, topical capsaicin has been used in clinical practice for the treatment of a variety of neuropathic conditions (11). Capsaicin is recognized for its pharmacologic and toxicologic properties in the treatment of cancer and is known to inhibit the growth of or kill various types of human tumor cells (12). Epidemiologic studies have suggested that capsaicin consumption may reduce the risk of colon cancer (13). Recently, capsaicin was shown to inhibit tumor-induced or VEGF-induced new vessel formation in vivo (14). However, because of its highly pungent nature, it is rarely considered for treating angiogenesis-related diseases. Specific capsaicin molecular mechanisms and structural characteristics that are directly relevant to vascular functions are understudied.

In this study, we attempted to further characterize the biological actions of capsaicinoids (Supplementary Fig. S1) in the vascular- and their underlying molecular targets. We show that capsiate and dihydrocapsiate, the nonirritating capsaicinoids from the nonpungent red pepper cultivar CH-19 Sweet, potently inhibit VEGF-induced angiogenesis and act as antipermeability agents. Surprisingly, biochemical experiments revealed that capsiate inhibits the catalytic activity of Src kinase, which is a prominent target of the VEGF receptor KDR/Flk-1. Molecular modeling revealed that capsiate and structurally related capsaicinoids efficiently dock to the ATP-binding region of Src through hydrogen bonding and hydrophobic interactions. Our results indicate that capsiate has considerable potential as a novel pharmacologic model compound for the design and development of Src antagonists and angiogenesis inhibitors. Functional studies of capsiate will provide insights into the biological consequences of the dietary intake of capsaicin-related compounds.
Materials and Methods

Cell culture and reagents. Human umbilical vascular endothelial cell (HUVEC) were isolated from human umbilical cord veins by collagenase treatment, as described previously (15), and used in passages 2 to 7. The cells were grown in M199 medium (Invitrogen) supplemented with 20% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin, 3 ng/mL basic fibroblast growth factor (Upstate Biotechnology), and 5 units/mL heparin at 37°C under a humidified 95%:5% (v/v) mixture of air and CO2. Capsiate and dihydrocapsiate were dissolved in DMSO and diluted in cell culture medium. Recombinant human VEGF was purchased from Upstate Biotechnology. Matrigel, cell culture reagents, and most other biochemical reagents were purchased from Sigma-Aldrich, unless otherwise specified.

Endothelial cell proliferation assay. [3H]Thymidine incorporation assays were carried out as described previously (16). Briefly, HUVECs were seeded at a density of 2 × 10³ cells per well in gelatin-coated 24-well plates. After 24 h, they were washed twice with M199 and incubated for 6 h in M199 containing 1% FBS. The cells were preincubated for 30 min with various concentrations of capsiate or dihydrocapsiate and stimulated by incubating with 10 ng/mL VEGF for 30 h, followed by 0.5 µCi/mL of [3H]Thymidine (Amersham) for 6 h. High–molecular weight DNAs were precipitated with 10% trichloroacetic acid at 4°C for 30 min. After two washes with ice-cold H2O, labeled DNA was solubilized in 0.2 N NaOH/0.1% SDS and counted by liquid scintillation counter (Perkin-Elmer/Wallace).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was carried out as described previously (17). Briefly, HUVECs were seeded at a density of 2 × 10³ cells per well in gelatin-coated 24-well plates. After 24 h, cells were washed twice with M199 and incubated for 6 h in M199 containing 1% FBS. Cells were preincubated for 30 min with various concentrations of capsiate or dihydrocapsiate and stimulated by the addition of 10 ng/mL VEGF for 48 h, followed by a colorimetric assay based on the uptake of MTT by viable cells.

Endothelial cell migration assay. Chemotactic motility of HUVECs was assayed using Transwell (Corning Costar) with 6.5-mm diameter polycarbonate filters (8-mm pore size) as described previously (16). Briefly, the lower surface of the filter was coated with 10 µg of gelatin. Fresh M199 medium (1% FBS) containing VEGF was placed in the lower wells. The cells were trypsinized and suspended at a final concentration of 1 × 10⁵ cells/mL in M199 containing 1% FBS. Various concentrations of capsiate or dihydrocapsiate were given to the cells for 30 min at room temperature before seeding. One hundred microliters of the cell suspension were loaded into each of the upper wells, and the chamber was incubated at 37°C for 4 h. The cells were fixed and stained with H&E. Nonmigrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was quantified with an optical microscope (200×) by counting cells that had migrated to the lower side of the filter.

In vitro capsaicin-like tube formation assay. Tube formation was assessed as described previously (16). Briefly, 250 µL of growth factor–reduced Matrigel (10 mg protein/mL; Collaborative Biomedical Products) was pipetted into a 16-mm diameter tissue culture well and polymerized for 30 min at 37°C. HUVECs incubated in M199 containing 1% FBS for 6 h were harvested after trypsin treatment and resuspended in M199 containing 1% FBS. Various concentrations of capsiate or dihydrocapsiate were added to the cells for 30 min at room temperature before seeding. Cells were plated onto the layer of Matrigel at a density of 1.8 × 10³ cells per well, followed by the addition of 10 ng/mL VEGF. After 20 h, the cultures were photgraphed (200×). The area covered by the tube network was determined using an optical imaging technique, in which pictures of the tubes were scanned in Adobe Photoshop, and quantified using Image-Pro Plus (Media Cybernetics).

[14C]Sucrose permeability assay. HUVECs were plated onto a Transwell filter. After reaching confluence, HUVECs were incubated with M199 containing 1% FBS for 3 h, treated for 30 min with various concentrations of capsiate or dihydrocapsiate (5, 10, and 25 µmol/L), and stimulated by the addition of 50 ng/mL VEGF for 1 h. [14C]Sucrose (50 µL at 0.8 µCi/mL; Amersham Pharmacia) was added to the upper compartment. The amount of radioactivity that diffused into the lower compartment after 30 min was determined using a liquid scintillation counter.

Immunofluorescence microscopy. HUVECs were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100/PBS, then incubated with blocking solution of PBS containing 5% normal goat serum and 0.05% Tween 20. Cells were labeled with anti–VE-cadherin antibody (Santa Cruz Biotechnology) for 2 h at room temperature, rinsed in PBS, and incubated with FITC-conjugated secondary antibody (Vector Lab) for 90 min at room temperature. Samples were examined with a fluorescence microscope (Zeiss).

Animal studies. Animals were maintained in a laminar airflow cabinet under specific pathogen-free conditions in a 12-h light-dark cycle. All animal experiments were carried out in accordance with University Institutional Animal Care and Use Committee guidelines.

Aortic ring assay. Aortas were harvested from 6-week-old male Sprague-Dawley rats as previously described (17). Plates (48-well) were coated with 120 µL of Matrigel, and after it had gelled, the aortic rings were placed in the wells and sealed in place with an overlay of 50 µL of Matrigel. VEGF, with or without capsiate or dihydrocapsiate, was added to the wells in a final volume of 200 µL of human endothelial serum-free medium (Invitrogen). As a control, medium alone was assayed. On day 6, cells were fixed and stained with Diff-Quick. The assay was scored from 0 (least positive) to 5 (most positive) in a double-blinded manner. Each data point was assayed in sextuplet.

In vivo Matrigel plug assay. Matrigel plug assay was performed as previously described (18). Briefly, 7-week-old C57/BL/6 mice (Orient Co.) were injected s.c. with 0.6 mL of Matrigel containing the indicated amount of capsiate or dihydrocapsiate, 100 ng VEGF, and 10 units heparin. The injected Matrigel rapidly formed a single, solid gel plug. After 6 days, the skin of the mouse was easily pulled back to expose the Matrigel plug, which remained intact. To identify infiltrating endothelial cells, immunohistochemistry was performed with anti–CD31 antibody. Seven mice were used for each group.

Immunoprecipitation. HUVECs were lysed in 1 mL of lysis buffer [20 mmol/L Tris/HCl (pH 8.0), 2 mmol/L EDTA, 137 mmol/L NaCl, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 10% glycerol, and 1% Triton X-100]. Lysates were chlorinated by centrifugation at 15,000 × g for 10 min, and the resulting supernatants were immunoprecipitated with either 1 µg/mL anti-KDR/FK-1 antibody, anti-pY253AK antibody, anti-VE-cadherin antibody (Santa Cruz Biotechnology), and antiphosphotyrosine (PY-20) antibody (BD Biosciences) at 4°C overnight, followed by the addition of protein A–agarose beads (Upstate Biotechnology) at 4°C for 1 h. Immunoprecipitates were washed thrice with lysis buffer, solubilized in SDS-PAGE sample buffer containing β-mercaptoethanol, and further analyzed by Western blotting.

In vitro Src kinase assay. The kinase activity of endogenous Src kinase was assayed as described according to the manufacturer's instructions (Upstate Biotechnology). In brief, supernatant containing 150 µg of protein per sample from endothelial cells stimulated with VEGF in the absence or presence of inhibitors was incubated with 1 µg of Src monoclonal antibody at 4°C overnight in a final volume of 500 µL. Immunoprecipitated Src was incubated under optimized buffer (25 mmol/L Tris-HCl buffer (pH 7.2), 10 mmol/L MgCl2, 150 µmol/L of substrate peptide (KVEKIGETGYVGYYK), 100 µmol/L ATP, 5 mmol/L glycero phosphate, 0.1 mmol/L sodium orthovanadate, 2 mmol/L DTT, and 0.2 µmol of [γ-32P] ATP) in a total volume of 30 µL for 10 min at 30°C with agitation. The reaction was stopped by adding 20 µL of 40% trichloroacetic acid, and a 25-µL aliquot was transferred onto the center of a numbered P81 paper square. The assay squares were washed thrice for 5 min each with 0.75% phosphoric acid and once with acetone. The assay squares were transferred to a scintillation vial, 5 mL of scintillation mixture were added, and the level of radioactivity was determined in a scintillation counter. The activity of the recombinant enzyme was determined by using 20 µg of purified active...
form of Src kinase (Upstate Biotechnology). The rest of the assay was conducted as described above.

**Molecular modeling.** The coordinates of human protooncogene tyrosine-protein kinase Src were obtained from the refined X-ray crystal structure of 2H8H.pdb (19), which is available from the Protein Data Bank. The protein structure was prepared using the Biopolymer Structure Preparation Tool in the SYBYL molecular modeling program (Tripos) and used for flexible docking studies with Surflex (20), implemented in SYBYL, after extraction of the crystal ligand. The crystal ligand was used to define the active site for Surflex (using ligand mode), which uses an idealized active site called a protomol, built from the hydrogen-containing protein mol2 file and based on protein residues that line the active site using standard variables. The three-dimensional structures of tested compounds were generated with Concord and energy minimized using the MMFF94s force field (method, Powell; termination gradient, 0.05 kcal/mol Å; max iterations, 1,000,000) in SYBYL. Surflex docking of ligand was run using default settings (except additional starting conformations per molecule of 5), and 30 poses for each ligand were analyzed. All computational studies were performed with the Tripos SYBYL molecular modeling program package, version 7.3.3, on a Linux (RHEL 4.0 Intel Xeon processor 5050) workstation.

**Statistical analysis.** The data are presented as mean ± SE, and statistical comparisons between groups were performed using one-way ANOVA followed by Student’s *t* test.

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**Results**

Capsiate and dihydrocapsiate inhibit VEGF-induced proliferation of endothelial cells. To assess the antiangiogenic activity of capsiate and dihydrocapsiate *in vitro*, their inhibitory effects on VEGF-induced proliferation of endothelial cells were first evaluated by MTT assay. Both compounds inhibited VEGF-induced HUVEC proliferation in a dose-dependent manner, with half-maximal inhibition at ~10 μmol/L (Fig. 1A). Furthermore, both compounds significantly blocked VEGF-induced DNA synthesis in HUVECs (Fig. 1B). These inhibitory effects were not due to cytotoxicity because capsiate and dihydrocapsiate had no effect on the normal growth of HUVECs devoid of VEGF stimulation up to 50 μmol/L (data not shown). The activity of capsiate and dihydrocapsiate were comparable with those of capsaicin and dihydrocapsaicin (data not shown). We further examined the effects of capsiate and dihydrocapsiate on cell cycle progression by fluorescence-activated cell sorting. VEGF induced HUVEC S-phase entry, whereas addition of capsiate and dihydrocapsiate markedly reduced S-phase entry (Supplementary Fig. S2A). The transition of cells from G1-S is, in part, regulated by cyclin D1. Consistently, when HUVECs were treated with VEGF, the protein levels of cyclin D1 increased, and

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**Figure 1.** Capsiate and dihydrocapsiate inhibit VEGF-induced proliferation, migration, and tube formation of endothelial cells. A, HUVECs were pretreated for 30 min with various concentrations (1, 5, 10, or 25 μmol/L) of capsiate (Cap) or dihydrocapsiate (D-cap) before exposure to VEGF (10 ng/mL). After 48 h, proliferation was quantified by MTT assay. B, HUVECs were treated the same as in A, except for 36-h stimulation. [3H]Thymidine was present during the last 6 h of incubation. C, HUVECs were pretreated for 30 min with various concentrations (1, 5, 10, or 25 μmol/L) of capsiate or dihydrocapsiate before treatment with 10 ng/mL VEGF. After 4 h, chemotaxis was quantified by counting the cells that migrated to the lower side of the filter with optical microscopy at 200× magnification. D, HUVECs were pretreated for 30 min with various concentrations of capsiate or dihydrocapsiate (1, 5, 10, or 25 μmol/L). Cells were collected and replated on Matrigel-coated plates at a density of 1.8 × 10⁶ cells per well and then incubated in the absence (control) or presence of 10 ng/mL VEGF. Microphotographs were taken after 20 h (magnification, 200×). Columns, mean from three different experiments with duplicate; bars, SE (*, *P < 0.05; **, *P < 0.01 versus VEGF alone).
this effect was blocked by capsiate and dihydrocapsiate (Supplementary Fig. S2B). Taken together, these results suggest that capsiate and dihydrocapsiate block VEGF-induced cell cycle progression from G1-S via down-regulation of cyclin D1 expression.

Capsiate and dihydrocapsiate inhibit VEGF-induced endothelial cell migration and tube formation. The effects of capsiate and dihydrocapsiate on the chemotactic motility of HUVECs were measured by Transwell assay. Both capsiate and dihydrocapsiate inhibited VEGF-induced migration of HUVECs in a concentration-dependent manner, with half-maximal inhibition at ~10 μmol/L (Fig. 1C). Next, the effects of capsiate and dihydrocapsiate on endothelial cell differentiation were investigated using two-dimensional Matrigel. When HUVECs were placed on growth factor–reduced Matrigel in the presence of VEGF, elongated and robust tube-like structures were formed, and many more cells were observed compared with control. Capsiate reduced both the width and length of the endothelial tubes induced by VEGF (Fig. 1D and Supplementary Fig. S3). Half-maximal inhibition was achieved below 5 μmol/L capsiate. Dihydrocapsiate exhibited a similar effect (Fig. 1D and Supplementary Fig. S3). These results show that capsiate and dihydrocapsiate block VEGF-induced in vitro angiogenesis.

Capsiate and dihydrocapsiate inhibit VEGF-induced endothelial permeability and VE-cadherin phosphorylation. We next examined the effect of capsiate and dihydrocapsiate on VEGF-induced endothelial permeability. VEGF increased [14C]sucrose diffusion through the pores of Transwell membranes in HUVEC monolayer culture, and this effect was blocked by capsiate and dihydrocapsiate in a dose-dependent manner (Fig. 2A). The half-maximal inhibition was achieved below 10 μmol/L for both compounds. Vascular endothelial permeability is maintained by the endothelial junction proteins VE-cadherin and occludins (21). In confluent endothelial cells, VE-cadherin is located at cell-cell contacts. When HUVECs were treated with 50 ng/mL VEGF, the level of VE-cadherin at cell-cell junctions was markedly decreased, and pretreatment with capsiate or dihydrocapsiate blocked this effect (Fig. 2B). VEGF induces VE-cadherin tyrosine phosphorylation in endothelial cells, and this event may be involved in the loosening of cell-cell contacts in established vessels to modulate transendothelial permeability (22). Treatment of confluent HUVECs with VEGF significantly induced VE-cadherin phosphorylation (Fig. 2C). Both capsiate and dihydrocapsiate inhibited this VEGF-induced effect (Fig. 2C). Together, these results suggest that capsiate and dihydrocapsiate may inhibit VEGF-induced vascular hyper-permeability by blocking tyrosine phosphorylation of VE-cadherin.

Capsiate and dihydrocapsiate inhibit VEGF-induced vessel sprouting ex vivo and angiogenesis in vivo. We further explored the antiangiogenic activity of capsiate and dihydrocapsiate in ex vivo and in vivo angiogenesis models. First, the sprouting of vessels from aortic rings was investigated to determine whether capsiate and dihydrocapsiate inhibit VEGF-induced angiogenesis ex vivo. VEGF (20 ng/mL) significantly stimulated vessel sprouting, and this effect was antagonized by capsiate and dihydrocapsiate in a dose-dependent manner (Fig. 3A). To determine the effect of capsiate and dihydrocapsiate on VEGF-induced angiogenesis in vivo, an established in vivo angiogenesis model, the mouse Matrigel plug assay, was performed. Matrigel containing VEGF (100 ng/mL), with or without 60 μg/mL capsiate or dihydrocapsiate, was s.c. injected into C57/BL/6 mice. Six days later, plugs containing VEGF alone appeared dark red (Fig. 3B). The vessels were abundantly filled with intact RBCs, indicating the formation of a functional vasculature inside the Matrigel via angiogenesis induced by VEGF. In contrast, plugs containing VEGF and capsiate or VEGF and dihydrocapsiate were pale in color, similar to Matrigel alone, indicating no or less blood vessel formation (Fig. 3B). The endothelial cell content inside the Matrigel plugs was quantified by

![Figure 2](image-url)

**Figure 2.** Capsiate and dihydrocapsiate inhibit VEGF-induced endothelial permeability and VE-cadherin phosphorylation. A, HUVECs were preincubated for 30 min with various concentrations (5, 10, or 25 μmol/L) of capsiate or dihydrocapsiate before treatment with 50 ng/mL VEGF. [14C]Sucrose permeability assay was then performed as described in Materials and Methods. Three independent experiments were performed in duplicate. Columns, means; bars, SE (*, P < 0.05; **, P < 0.01 versus VEGF alone). B, effects of capsiate or dihydrocapsiate on VEGF-induced alteration in location of VE-cadherin at cell-cell contacts. Confluent HUVECs were starved for 6 h and treated with vehicle control, 50 ng/mL VEGF alone, VEGF plus capsiate, and VEGF plus dihydrocapsiate for 6 h. Cells were fixed, permeabilized, and then stained sequentially with anti–VE-cadherin antibody and FITC-conjugated secondary antibody. C, HUVECs were preincubated for 30 min with various concentrations (5, 10, or 25 μmol/L) of capsiate or dihydrocapsiate and treated with 50 ng/mL VEGF for further 30 min. VE-cadherin protein was immunoprecipitated as described under Materials and Methods. Precipitated proteins were analyzed by SDS-PAGE followed by immunoblotting with antibody to VE-cadherin. The levels of VE-cadherin in whole-cell lysates were used as an internal control.

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CD-31 staining (Fig. 3C). These results indicate that capsaicin and dihydrocapsiate are capable of inhibiting VEGF-induced neovessel formation in vivo.

Capsiate and dihydrocapsiate inhibit VEGF-induced Src kinase and p125FAK tyrosine phosphorylation without affecting KDR/Flk-1 autophosphorylation. VEGF induces proliferation, migration, tube formation, and permeability mainly through activation of its cell surface receptor KDR/Flk-1 (23). To understand the molecular mechanism by which capsaicin inhibits VEGF-induced angiogenesis, we investigated the effect of capsiate and dihydrocapsiate on VEGF-induced KDR/Flk-1 autophosphorylation. However, neither compound had an effect on VEGF-induced KDR/Flk-1 autophosphorylation up to 25 μmol/L (Fig. 4A), suggesting that capsiate may inhibit angiogenesis by curbing downstream signaling molecules of KDR/Flk-1. Previous reports have suggested that VEGF-induced angiogenesis and endothelial permeability involve activation of Src kinase, downstream of KDR/Flk-1 (24, 25). Because capsiate inhibits VEGF-induced angiogenesis and tyrosine phosphorylation of VE-cadherin, we speculated that capsiate could exert antiangiogenic and antipermeability actions through inhibition of Src kinase activity. Therefore, the effect of capsiate on VEGF-induced Src kinase activity in HUVECs was determined by measuring phosphorylation of Src at Tyr416, which leads to autoactivation. VEGF significantly increased Tyr416 phosphorylation, and this was blocked by capsiate or dihydrocapsiate in a dose-dependent manner (Fig. 4B). Half-maximal effects were obtained at a concentration of ~10 μmol/L. Consistently, p125FAK, which is a known substrate of Src kinase, was tyrosine phosphorylated by VEGF, and this effect was suppressed by capsiate or dihydrocapsiate (Fig. 4C). Similar to capsiate, capsaicin also inhibited VEGF-induced Tyr416 phosphorylation and Src kinase activity (Fig. 4D). These data suggest that capsiate and dihydrocapsiate are able to block the VEGF-induced Src/p125FAK signaling pathway in endothelial cells.

Capsiate inhibits Src kinase activity. HUVECs were pretreated with or without various concentrations of capsiate for 1 h before VEGF stimulation. Then, Src kinase from cell lysates was immunoprecipitated with anti-Src antibody and subjected to an in vitro kinase assay using the KVEKIGEYGTGVVK peptide substrate. VEGF significantly increased endogenous Src kinase activity, and this was inhibited by pretreatment of capsiate in a dose-dependent manner (Fig. 5A). Moreover, capsiate inhibited the
catalytic activity of purified Src kinase with an IC₅₀ of ~ 10 μmol/L (Fig. 5B), and capsaicin showed a similar effect (Fig. 5B). These data indicate that Src kinase may be a direct target for capsiate and capsaicin.

Docking studies. To examine the direct predicted interactions of our tested compounds with the target protein Src tyrosine kinase, docking studies were performed. The X-ray crystal structure of human protooncogene Src tyrosine kinase, 2H8H.pdb (19), was used for flexible docking studies, and Surflex (20) docking very nicely reproduced the binding mode of the crystal ligand with RMSD of ca. 0.85 Å. Figure 6 shows the active site of Src tyrosine kinase (with MOLCAD surface-colored by cavity depth) and representative binding modes of the other tested compounds. Docking studies of ATP showed that it binds to the ATP-binding site, with its adenine moiety making hydrogen bonds with Glu339 and Met341 (Fig. 6B, ii), as the previous crystal structure with an ATP analogue showed (26). Capsiate also successfully docked into the ATP-binding site, but interestingly, it was able to occupy the hydrophobic pocket (near to the purine binding site) and maintain a hydrogen bond with Met341, which seems to be a rational binding mode (Fig. 6B, ii). In the case of capsaicin, the major binding mode seemed to be very similar to that of capsiate (Fig. 6C, i); the minor binding mode seemed to be possible via binding to the purine binding site, like ATP (Fig. 6C, ii), maintaining hydrogen bonds with Glu339 and Met341. Moreover, other capsiate-related compounds, such as dihydrocapsiate, dihydrocapsaicin, and [6]-gingerol, were docked and showed similar binding modes to capsiate, occupying the hydrophobic pocket at the ATP-binding site (data not shown). This docking study confirmed that the tested compounds could bind to the ATP-binding site of Src tyrosine kinase. Capsiate and its analogues were able to occupy the hydrophobic pocket, which could be exploited to obtain selectivity among kinases. This information provides insight into future kinase inhibitor design for drug discovery.

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**Figure 4.** Capsiate and dihydrocapsiate inhibit VEGF-induced Src kinase and p125FAK tyrosine phosphorylation without affecting KDR/Fk-1 autophosphorylation. HUVECs were pretreated for 30 min with various concentrations (5, 10, or 25 μmol/L) of capsiate or dihydrocapsiate and stimulated with 10 ng/mL VEGF (or 20 ng/mL VEGF) for 10 min. A, B, and C, anti-KDR/Fk-1, anti-Src, and anti-p125FAK immunoprecipitates (IP) were analyzed by SDS-PAGE. Immunoblot analysis was performed with antiphosphotyrosine antibody (anti-PY20). B, blots were also probed with antibody specific to phosphorylated Src at Tyr416 (Src-pY416). D, the effect of capsaicin on phosphorylation of Src at Tyr416 (Src-pY416) was compared with that of capsiate. Blots are representative of three independent experiments. Densitometric analyses are presented as the relative ratio of p-KDR:KDR, p-Src:Src, and p-p125FAK:p125FAK (bottom). The relative ratio in untreated control is arbitrarily presented as 100.
Discussion

Phytochemicals, nonnutritive components in the plant-based diet, have been recognized as medicinally important natural products that possess medicinal properties to treat a variety of diseases (27). In particular, chemoprevention by consumption of plant-derived foods that contain edible phenolic compounds, such as resveratrol, curcumin, gingerol, epigallocatechin gallate, and capsaicin, is now widely considered to be an inexpensive, readily applicable, and accessible approach to cancer control and management (9, 28). Numerous previous studies have shown that these compounds could block the biochemical events that are associated with the multistage process of carcinogenesis (27, 28). However, little is known about the precise mechanism of action of most chemopreventive agents. Their biological effects are thought to be exerted by the sum of several distinct mechanisms.

Recently, capsaicin was shown to be a potent angioinhibitory compound in vitro and in vivo (14). Capsaicin possesses three distinct structural moieties: methoxyphenol, amide, and vanillyl groups. With the exception of the replacement of one nitrogen atom and one hydrogen atom in the capsaicin molecule with one oxygen atom, the capsaicin structure is identical to capsiate, a major capsaiacinoid of the sweet pepper (29). This minor variation reflects a critical difference in pungency, and capsiate becomes nonirritating. We showed that capsiate retains antiangiogenic activity that is comparable with capsaicin. Moreover, dihydrocapsaicin and dihydrocapsiate, which have hydrogenated carbons at the vanillyl group, also exhibit similar efficacy in inhibiting VEGF-induced angiogenesis. It is worth noting that [6]-gingerol, which is structurally similar to capsiate with respect to the methoxyphenol moiety and the hydrocarbon backbone structure, inhibits angiogenesis in a way that is similar to that of capsiate (16). These results suggest that this group of phenolic compounds may share a common target that is used for antiangiogenic activity.

This study shows, for the first time, that capsiate and capsaicin directly target Src kinase in endothelial cells. VEGF and VEGF receptors are essential not only for the development of the vasculature in mouse embryos (30, 31), but also for physiologic and pathologic angiogenesis in the adult. A large number of previous studies have shown that Src kinase is strongly involved in VEGF-induced angiogenesis (6, 32). Several angiogenic signaling molecules, including p125Fak (33) and eNOS (34), are predicted to lie downstream of Src, which is activated upon the interaction of VEGF with its receptor KDR/Flik-1. Blockage of Src kinase by either pharmacologic inhibitors (35) or a dominant-interfering mutant of Src (6) diminished VEGF-induced angiogenesis in vitro and in vivo (36). Importantly, we found that capsiate and capsaicin inhibit VEGF-induced Src kinase activity in endothelial cells. In agreement, these capsaiacinoids strongly inhibited VEGF-induced proliferation, migration, and tube formation of cultured endothelial cells (Fig. 1), which require Src activity. Src kinase also functions as a crucial component of the VEGF receptor signaling pathway engaged in endothelial permeability, which is an initial step for allowing vessel sprouting and the formation of capillary networks during angiogenesis and is important for VEGF-dependent vascular leakage under pathologic conditions (37). Genetic studies showed that VEGF-induced vascular permeability is impaired in Src−/− mice (6) and the absence of Src was associated with a reduction in VEGF-mediated vascular permeability (38). It is known that Src kinase–dependent tyrosine phosphorylation of VE-cadherin is involved in the loosening of cell-cell contacts in established vessels with VEGF stimulation (22, 36). KDR-mediated Src activation is also shown to regulate the availability of VE-cadherin at the cell surface by promoting its endocytosis through a Vav2/Rac/PAK signaling axis (5). In agreement with this view, our results show that capsiate inhibits tyrosine phosphorylation of VE-cadherin, the loss of VE-cadherin–facilitated endothelial cell-cell junctions, and the increased endothelial permeability triggered by VEGF. Therefore, these findings suggest that Src kinase is a rational molecular target of capsaiacinoids for modulating angiogenesis and permeability in endothelial cells.

The present study further shows how capsiate inhibits Src kinase. The catalytic activity of Src kinase is regulated by phosphorylation of its tyrosine residues (39). It has two important phosphorylation sites: autophosphorylation of Tyr 416 at the activation loop is stimulatory and phosphorylation of Tyr527 at the COOH terminus is inhibitory (40). Our data show that capsiate inhibits tyrosine phosphorylation of VE-cadherin, the loss of VE-cadherin–facilitated endothelial cell-cell junctions, and the increased endothelial permeability triggered by VEGF. Therefore, these findings suggest that Src kinase is a rational molecular target of capsaiacinoids for modulating angiogenesis and permeability in endothelial cells.

Figure 5. Capsiate inhibits Src kinase activity. A, HUVECs were preincubated for 30 min with various concentrations (5, 10, or 25 μmol/L) of capsiate and stimulated with 20 ng/ml VEGF for 10 min. Src protein was immunoprecipitated and then subjected to in vitro kinase assay. Three independent experiments were performed. Columns, mean; bars, SE (**, P < 0.01 versus VEGF alone). B, effects of capsiate and capsaicin on the catalytic activity of purified Src kinase. In vitro Src kinase assay was performed as described under Materials and Methods. Three independent experiments were performed in duplicate.
pocket within the catalytic cleft of Src kinase, which is not directly involved in ATP binding. It is known that resveratrol, a phenolic compound, inhibits VEGF-induced angiogenesis primarily through blocking Src-dependent tyrosine phosphorylation of VE-cadherin (24). Unlike capsaicinoids, Src kinase was not a direct target for resveratrol (24). Interestingly, curcumin, which is structurally related to capsiate by possessing a methoxyphenol moiety, can directly inhibit Src kinase activity (42). However, the IC$_{50}$ value of curcumin for the Src kinase activity (42) was much higher (50 μmol/L) than that observed in capsiate (IC$_{50}$, 10 μmol/L). These results suggest that capsaicinoids, among the dietary antiangiogenic phytochemicals, are most likely to bind to the active site of Src kinase. Additionally, considering that capsiate was unable to block KDR autophosphorylation (Fig. 4), its inhibitory action may not be universal to tyrosine kinases. The binding mode of capsiate, which uses the hydrophobic pocket at the catalytic site of Src kinase, may deliver some degree of specificity toward this enzyme. Because of this specificity, we propose that capsiate and its analogues may be novel pharmacologic agents for directly targeting the catalytic site of Src kinase.

Many previous studies have shown that capsaicin induces decreased cell viability and apoptosis in various tumor cells, including HeLa, ovarian carcinoma, mammary adenocarcinoma, HepG2 human hepatoma, C6 glioma, and promyelocytic leukemia (HL-60) cells (43, 44). In addition, capsaicin inhibited tumor growth in a mouse xenograft model (45). In most cases, capsaicin effectively induced growth inhibition or apoptosis of cultured tumor cells only at concentrations over 50 μmol/L (44). In contrast, both capsiate and capsaicin sufficiently inhibited VEGF-induced angiogenic responses and permeability at 10 μmol/L in in vitro and ex vivo angiogenesis assays. Such concentrations did not affect endothelial cell viability. Therefore, capsiate and capsaicin could inhibit specific endothelial cell response to VEGF at much lower concentrations than required for the inhibition of normal cell growth. Because VEGF is a major inducer of the formation of tumor vasculature (1, 2), capsiate and capsaicin could inhibit tumor growth in vivo via their antiangiogenic activity, before their cytotoxic effects on tumor cells. It is noteworthy that Src kinase is involved in the expression of proangiogenic factors, such as VEGF and interleukin-8 in various human tumor cells (46–48). Therefore, in addition to disrupting Src kinase–mediated functions in host endothelial cells, capsiate may suppress tumor angiogenesis by directly acting on tumor cells to decrease production of proangiogenic molecules.

In conclusion, we showed that capsiate exhibited potent activity against VEGF-induced angiogenesis and vascular permeability. Through biochemical and structural analyses, capsiate and capsaicin were identified as specific inhibitors of Src kinase, a crucial intracellular mediator of KDR/Flik-1 in the vasculature. The nonpungent nature of capsiate, unlike capsaicin, could make this compound more attractive for treating angiogenesis-dependent and vascular leakage–dependent human diseases caused by unwanted generation of VEGF. We present important biological attributes of capsiate, a compound that can be enriched by dietary modification and daily consumption, that may prove to be essential to the prevention of cancer.
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References


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