Anacardic Acid (6-Pentadecylsalicylic Acid) Inhibits Tumor Angiogenesis by Targeting Src/FAK/Rho GTPases Signaling Pathway

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ABSTRACT

Anacardic acid (6-pentadecylsalicylic acid), a natural inhibitor of histone acetyltransferase from Anaphitrygium adstringens, has been shown to have anti-inflammatory, anticancer, antitoxicative, and antimicrobial functions. However, whether this salicylic acid could block angiogenesis has not been elucidated to date. Here, we postulate that anacardic acid affects multiple steps of tumor angiogenesis to contribute to tumor inhibition. In this study, we found that vascular endothelial growth factor (VEGF)-induced cell proliferation, migration, and adhesion and capillary-like structure formation of primary cultured human umbilical vascular endothelial cells (HUVECs) could all be significantly suppressed by anacardic acid in vitro, without detectable cellular toxicity. Furthermore, anacardic acid effectively inhibited vascular development in chick embryo chorioallantoic membrane ex vivo (n = 10) and VEGF-triggered corneal neovascularization in vivo (n = 10). A mechanistic study revealed that anacardic acid blocked activities of Src and FAK kinases in concentration- and time-dependent manners in HUVECs, resulting in activation of RhoA-GTPase and inactivation of Rac1- and Cdc42-GTPases. Of note, when anacardic acid (2 mg/kg per day) was subcutaneously administrated to mice bearing human prostate tumor xenografts (n = 6–7), the volume and weight of solid tumors were significantly retarded. Src, Ki-67, and CD31 immunohistochemical staining further revealed that Src protein expression, tumor cell proliferation, and microvesSEL density could be remarkably suppressed by anacardic acid. Taken together, our findings demonstrate for the first time that anacardic acid functions as a potent tumor angiogenesis inhibitor by targeting the Src/FAK/Rho GTPase signaling pathway, leading to significant suppression of prostate tumor growth.

Introduction

Angiogenesis, defined as the sprouting of new capillaries from preexisting vasculature, is a multistep process including destabilization of the integrated blood vessel, endothelial cell proliferation, migration, and tubulogenesis. At this time, tumor angiogenesis is considered as a pivotal process for tumor growth and invasiveness, as the tumor cells have an absolute requirement for new blood vessels to nourish their growth and to facilitate metastasis (Liotta et al., 1974). Angiogenesis-targeted therapies (biotherapy) has been called the fourth modality of anticancer therapy (Folkman, 2004). Among various proangiogenic growth factors and cytokines, vascular endothelial growth factor (VEGF) is the most well characterized and plays an essential role by specifically affecting the biological functions of vascular endothelial cells (Ferrara, 2002). Many receptors, kinases, and transcriptional factors have been involved in tumor angiogenesis, making complex networks. The ste-
roid receptor coactivator (Src)/focal adhesion kinase (FAK) signaling pathway is one of them.

Src and FAK are intracellular (nonreceptor) tyrosine kinases that physically and functionally interact (Bolos et al., 2010). Modulation of their activities alters cell responses that are often perturbed in cell proliferation and survival through regulation of the Ras/mitogen-activated protein kinase signaling pathway and signal transducer and activator of transcription 3 (Brunton and Frame, 2008). Src family kinase also affects cell adhesion and migration via interaction with GTPase-activating proteins and integrins (Boureux et al., 2007). FAK is overexpressed and highly activated in invasive tumor cells (Agochiya et al., 1999), and epidermis-specific knockout of FAK suppresses tumor formation and progression in vivo (McLean et al., 2004). Rhoa, Rac1, and Cdc42 are well known GTPases and belong to the Rho-GTPase family, which is a subfamily of the Ras superfamily (Boureux et al., 2007). Rho-GTPases have been implicated in many basic cellular processes that influence cell proliferation, motility, chemotaxis, and adhesion (Etienne-Manneville and Hall, 2002). The active Src/FAK complex stimulates Rac1 activity through the recruitment and phosphorylation of the scaffolding protein p130Cas (Chodniewicz and Klemke, 2004). Another study showed that cells treated with PP2 (Src kinase family inhibitor) showed a 5-fold reduction of active Rac1. Blockade of either Src kinase activity or FAK signaling interfered with α5β1-mediated Rac1 activation and polarized cell spreading (Choma et al., 2007). In addition, during the process of angiogenesis, the formation of capillary cords and vascular sprouting also require activation of Src (Liu and Senger, 2004; Schenone et al., 2007). On the basis of these vital biological functions, small Src/FAK inhibitors, such as N-(5-chloro-1,3-benzodioxol-4-yl)-7-[2-(4-methylpiperaizin-1-yl) ethoxy]-5-(tetrahydro-2H-pyran-4-yl)quinazolin-4-amine (AZD0530), 4-|2,4-dichloro-5-methoxyphenyl|aminoo]-6-methoxy-7-[3-(4-methyl-1-piperazinylo)propoxy]-3-quinolinecarbonitrile (SKI-606), and dasatinib have been developed and are undergoing testing in clinical settings (Schenone et al., 2010).

Therefore, novel and safe chemical agents targeting dual Src/FAK kinase show promise in cancer treatment.

Anacardic acid (6-pentadecylsalicylic acid) is the active and possible anti-inflammatory component in Anaphysetium adstringens, the bark of which is widely used in traditional medicine for treatment of gastric ulcers, gastritis, and stomach cancers (Acevedo et al., 2006). It also exists in the plants Ozoroa insignis (Rea et al., 2003), Anacardium occidentale (Kubo et al., 1994), and Ginkgo biloba (Itokawa et al., 1987).

Functional studies reveal that anacardic acid exerts antimicrobial (Muroi and Kubo, 1996; Kubo et al., 1999) and anti-inflammatory actions (Trevisan et al., 2006) by serving as a mitochondrial uncoupler of oxidative phosphorylation (Toyomizu et al., 2006). Anacardic acid also exhibits antitumor activities (Kubo et al., 1994), and epidermis-specific knockout of FAK suppresses tumor formation and progression in vivo (McLean et al., 2004). Rhoa, Rac1, and Cdc42 are well known GTPases and belong to the Rho-GTPase family, which is a subfamily of the Ras superfamily (Boureux et al., 2007). Rho-GTPases have been implicated in many basic cellular processes that influence cell proliferation, motility, chemotaxis, and adhesion (Etienne-Manneville and Hall, 2002). The active Src/FAK complex stimulates Rac1 activity through the recruitment and phosphorylation of the scaffolding protein p130Cas (Chodniewicz and Klemke, 2004). Another study showed that cells treated with PP2 (Src kinase family inhibitor) showed a 5-fold reduction of active Rac1. Blockade of either Src kinase activity or FAK signaling interfered with α5β1-mediated Rac1 activation and polarized cell spreading (Choma et al., 2007). In addition, during the process of angiogenesis, the formation of capillary cords and vascular sprouting also require activation of Src (Liu and Senger, 2004; Schenone et al., 2007). On the basis of these vital biological functions, small Src/FAK inhibitors, such as N-(5-chloro-1,3-benzodioxol-4-yl)-7-[2-(4-methylpiperaizin-1-yl) ethoxy]-5-(tetrahydro-2H-pyran-4-yl)quinazolin-4-amine (AZD0530), 4-|2,4-dichloro-5-methoxyphenyl|aminoo]-6-methoxy-7-[3-(4-methyl-1-piperazinylo)propoxy]-3-quinolinecarbonitrile (SKI-606), and dasatinib have been developed and are undergoing testing in clinical settings (Schenone et al., 2010). Therefore, novel and safe chemical agents targeting dual Src/FAK kinase show promise in cancer treatment.

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In this study, we showed for the first time that anacardic acid could significantly inhibit endothelial cell viability, migration, tube formation and adhesion via blocking of the Src/FAK/Rho GTPase signaling pathway, leading to the suppression of tumor angiogenesis and tumor growth in vivo.

**Materials and Methods**

**Reagents.** Anacardic acid (95% purity) (Fig. 1A) was purchased from Merck (Darmstadt, Germany). A 50 mM stock solution was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO), stored at −20°C, and then diluted as needed in cell culture medium. Bacteria-derived recombinant human VEGF (VEGF165) was obtained from the National Institutes of Health (NIH) Experimental Branch (Bethesda, MD). Growth factor-reduced Matrigel was purchased from BD Biosciences (San Jose, CA). Antibodies against CD31 and Ki-67 were from Epitomics (Burlingame, CA). Antibody against RhoA was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against Rac1 was from Millipore Corporation (Billerica, MA), and other antibodies (CD42, phospho-VEGFR2 (Tyr1175), VEGFR2, phospho-Src (Tyr416), Src, phospho-FAK (Tyr397), FAK, and phospho-paxillin (Tyr118)) used in the present study were purchased from Cell Signaling Technology (Danvers, MA).

**Cell Lines and Cell Culture.** Primary human umbilical vascular endothelial cells (HUVECs) were a kind gift from Dr. Xindi Wang (Cardiothoracic Surgery Division of Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, TX) in 2008 and were cultured in endothelial cell culture medium (ECM) as described previously (Pang et al., 2009a). HUVECs were confirmed by their typical microscopic morphology: homogeneous, large, polygonal, and cobblestone-like. The human prostate cancer cell lines PC-3, DU145, and LNCap were purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, Utah). Western blotting using epithelial markers authenticated that these were of epithelial origin. All the cells were cultured at 37°C under a humidified mixture of air and CO2 (95%/5%; v/v).

**Animal Studies.** C57BL/6 mice and nude mice were purchased from National Rodent Laboratory Animal Resources (Shanghai, China) and maintained according to the NIH standards established in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). All of the experimental protocols were approved by the Animal Investigation Committee of East China Normal University (Shanghai, China).

**Endothelial Cell Wound-Healing Migration Assay.** HUVECs were allowed to grow to full confluence in six-well plates precoated with 0.1% gelatin (Sigma-Aldrich). HUVECs were first starved in low-serum medium and then wounded with pipette tips. ECM containing 0.5% FBS was added into the wells with 0.1% DMSO, 20 ng/ml VEGF, and various concentrations of anacardic acid (5, 10, 20, and 50 μM). Images were taken after 8 to 10 h of incubation (magnification, 100×; Olympus, Tokyo, Japan) and migrated cells in random eight fields were quantified by manual counting. Each con-
centration of anacardic acid had three repeats. Three independent experiments were performed.

**Endothelial Cell Transwell Migration Assay.** The chemotactic motility of HUVECs was determined using a Transwell migration assay (BD Biosciences) with 6.5-mm-diameter polycarbonate filters (8-μm pore size) as described previously (Pang et al., 2009a). In brief, the filter of the Transwell plate was coated with 0.1% gelatin. The bottom chambers were filled with 500 μl of ECM containing 0.5% FBS supplemented with 20 ng/ml VEGF. Inactivated HUVECs (4 × 10^5 cells/well) suspended in 100 μl of ECM containing 0.5% FBS plus 0.1% DMSO and various concentrations of anacardic acid (5, 10, 20, and 50 μM) were seeded in the top chambers. Cells were allowed to migrate for 8 to 10 h. Nonmigrated cells were removed with cotton swabs, and migrated cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an inverted microscope (magnification, 100×; Olympus), and migrated cells in random eight fields were quantified by manual counting. Three independent experiments were performed.

**Endothelial Cell Capillary-Like Structure Formation Assay.** Tube formation was assessed as described previously (Pang et al., 2009b). Growth factor-reduced Matrigel was pipetted into prechilled 96-well plates (50 μl of Matrigel/well) and polymerized for 30 min. HUVECs (2 × 10^5/well) were collected and placed onto the layer of Matrigel in 200 μl of ECM containing 0.5% FBS plus 0.1% DMSO and various concentrations of anacardic acid (5, 10, 20, and 50 μM), followed by the addition of 20 ng/ml VEGF. After 6 to 8 h of incubation, the endothelial cells were photographed using an inverted microscope (magnification, 100×; Olympus) and quantified by measuring the tube length of eight random fields. Three independent experiments were performed.

**Endothelial Cell Adhesion Assay.** The de novo attachment of endothelial cells was accessed with an ECM Cell Adhesion Array Kit (Millipore Corporation). A HUVEC suspension was prepared with 5 mM EDTA-phosphate-buffered saline buffer and pretreated with or without anacardic acid (5 and 20 μM) for 30 min in the assay buffer. Experimental cells at a density of 2 × 10^5 cells/well were consecutively plated into an ECM array plate and incubated with 20 ng/ml VEGF for approximately 2 h at 37°C in CO2 incubator. After being gently washed 3 times, those cells were stained with staining buffer. After stain extraction, the relative cell attachment was determined using absorbance readings. The absorbance at 540 to 570 nm was measured; 1 clock hour equals 30° of arc. The area of neo-vascularization was calculated according the formula 0.2 × 3.14 × VL (millimeters) × CN (millimeters), where VL is the maximal vessel length extending from the limbal vasculature toward the pellet, and CN is the clock hour as described previously (Rogers et al., 2007). Two independent experiments were performed.

**Western Blotting Analysis.** To test the anacardic acid-mediated cell signaling pathway, a Western blotting assay was performed. HUVECs were first starved in serum-free ECM for 4 to 6 h and then pretreated with or without the indicated concentrations of anacardic acid (1, 5, 10, and 20 μM) for 30 min, followed by the stimulation with 50 ng/ml VEGF for 2 to 10 min (2 min for VEGFR2 activation, 5 min for FAK activation, and 10 min for Src kinase activation). The whole-cell extracts were prepared as described previously (Pang et al., 2009b). Approximately 40 to 50 μg of cellular protein from each sample was resolved by SDS-polyacrylamide gel electrophoresis and probed with specific antibodies, followed by exposure to a horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody. Three independent experiments were performed. Relative optical density of blotting bands was quantified by ImageJ software (NIH).

**GTPase Activation Assay.** HUVECs were starved, pretreated with anacardic acid for 30 min, and stimulated with VEGF (100 ng/ml) for another 30 min to activate the Rho family of small GTPases. After that, cells were lysed with lysis buffer (20 mM Tris-HCl, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 10 mM Na3P2O7, and 1 mM phenylmethylsulfonyl fluoride) supplemented with proteinase inhibitor cocktail (Calbiochem, San Diego, CA). For the GTPase activation experiment, GST-PBD (Pak Rac/Cdc42-binding domain) or GST-RBD (Rho-binding domain) attached to beads (Santa Cruz Biotechnology, Inc.) were added to the cell lysates. After incubation at 4°C for 2 h, the beads were washed five times with lysis buffer and boiled at 100°C for 10 min with 2× SDS sample buffer. Immunoblotting for Rac1, Cdc42, and RhoA was done with specific antibodies. Three independent experiments were performed. Relative optical density of the blotting bands was qualified by ImageJ software.

**Molecular Modeling.** The coordinates of human protooncogene Src and FAK kinases were obtained from the refined X-ray crystal structures of 2H8H (Hennequin et al., 2006) and 2IJM, which are available from the Protein Data Bank. The anacardic acid was then docked to the human Src and FAK by Glide software (version 5.5, 2009; Schrödinger, LLC, New York, NY). The protein structures were prepared in Protein Preparation Wizard, and anacardic acid was prepared in LigPrep.

**Cell Viability Analysis.** HUVECs, PC-3, DU145, and LNCap cells were plated in 96-well plates (5 × 10^3/well) and treated with different concentrations of anacardic acid (1, 10, 20, 50, and 100 μM) for 48 h. To determine cell viability, we used the CellTiter 96 AQue-
Scous One Solution Cell Proliferation (MTS; Promega, Madison, WI), and the optical density was measured with a VersaMax microplate reader (Molecular Devices). Three independent experiments with six duplicates were performed.

**Xenograft Human Prostate Tumor Mouse Model.** The xenograft mouse model assay was performed as described previously (Yi et al., 2008). Five-week-old male BALB/cA nude mice (~25 g) were randomly divided into two groups (n = 6–7 each group). PC-3 cells were subcutaneously injected into the left flank of the mice (3 × 10^6/mouse). After tumors grew to ~70 mm³, tumor-bearing mice were treated with or without anacardic acid (2 mg/kg per day) via subcutaneous injection for a consecutive 30 days. Anacardic acid (dissolved in DMSO) was delivered through one or two injection sites around the tumors, depending on tumor size at the time of injection. The body weight of mice and tumor size were recorded every 5 days. The tumor volume was determined by Vernier caliper measurements and calculated as length × width² × 0.52.

**Histology and Immunohistochemistry.** Solid tumors were removed, fixed with 10% formaldehyde, and embedded in paraffin. Anti-CyD31, Src, and Ki-67 antibodies were used to stain blood vessels, molecular target, and tumor cell proliferation on 5-µm tumor sections, respectively. In our present study, all the solid tumors (n = 6 each group) were sectioned, and we randomly choose three sections of each tumor to perform immunohistochemical analysis. Images were taken using a Leica DM 4000B photomicroscope (magnification, 400×). Immunoreactive spots from a random five fields of each tumor section were further selected for analysis and quantified by Image-Pro Plus 6.0 software.

**Statistical Analysis.** Statistical comparisons between groups were performed using the standard Student’s t test. Data are presented as means ± S.D. P < 0.05 is considered statistically significant.

**Results**

**Anacardic Acid Inhibits VEGF-Induced Chemotactic Motility and Capillary-Like Structure Formation of Endothelial Cells.** To assess the antiangiogenic activity of anacardic acid in vitro, its inhibitory effect on endothelial cell migration and differentiation was first evaluated. To identify the effects of anacardic acid on cell migration, we used two types of migration models: a wound-healing migration assay and Transwell migration assay. As shown in Fig. 2, anacardic acid inhibits HUVEC migration in a concentration-dependent manner, with significant effectiveness at 5 µM in the wound-healing assay (Fig. 2A) and Transwell assay (Fig. 2B). The highest concentration of anacardic acid (50 µM) completely blocked endothelial cell motility. However, with the same experimental conditions, anacardic acid did not affect cell viability of VEGF-activated HUVECs (Supplemental Fig. 1).

Although angiogenesis is a very complex process, tube formation of endothelial cells is substantially required (Patan, 2004). To examine the potential effects of anacardic acid on tubular structure formation of endothelial cells, we used a two-dimensional Matrigel assay. As shown in Fig. 2C, anacardic acid potently inhibited tube formation in HUVECs, with a functional concentration at 5 µM. Of interest, anacardic acid had little effect on cell viability of HUVECs at this process (Supplemental Fig. 1). All of these results revealed that anacardic acid could block angiogenesis in vitro by inhibiting multiple biological processes in endothelial cells.

**Anacardic Acid Abolishes Cell Adhesion and Paxillin Phosphorylation in HUVECs.** Adhesion of cells to the extracellular matrix is crucial for development and tissue homeostasis (Huveneers and Danen, 2009). Integrins are α/β heterodimeric cell surface receptors that play a pivotal role in cell adhesion and migration, as well as in growth and survival (Liu et al., 2000; Hood and Cheresh, 2002). We next examined the adhesion ability of HUVECs mediated by integrins after treatment of anacardic acid. As shown in Fig. 3A, cell adhesion activity was dramatically reduced by anacardic acid in a dose-dependent manner. The integrin family is composed of 18 α subunits and 8 β subunits that can assemble into 24 different heterodimers. Our results showed that the effect of anacardic acid on integrin ligands is not specific.

Paxillin is a multidomain protein that localizes primarily to focal adhesion sites to the extracellular matrix, and tyrosine phosphorylation of paxillin is required for integrin-

![Fig. 2](https://example.com/f2.png) Anacardic acid (AA) inhibits VEGF-induced cell migration and differentiation of endothelial cells. HUVECs were treated with vehicle (0.1% DMSO), VEGF (20 ng/ml), and indicated concentrations of anacardic acid (5, 10, 20, and 50 µM). Representative images and the quantitative result are shown. A, anacardic acid inhibited HUVEC migration. HUVECs were grown into full confluence in six-well plates and treated with anacardic acid. The migrated cells were quantified by manual counting. B, anacardic acid inhibited HUVEC Transwell migration. HUVECs were seeded in the upper chamber of a Transwell and treated with anacardic acid. After 8 to 10 h, the number of HUVECs that migrated through the membrane was quantified by manual counting. C, anacardic acid inhibited the tube formation of HUVECs. HUVECs (2 × 10⁵/well) were placed in 96-well plates coated with Matrigel. Tubular structures were photographed. Columns represent the mean from three independent experiments with triplicates; bars represent S.D. **P < 0.01 versus VEGF alone.**
mediated cytoskeletal reorganization (Burridge et al., 1992). Paxillin plays a central role in coordinating the spatial and temporal action of the Rho family of small GTPases (Deakin and Turner, 2008). Therefore, we examined the VEGF-triggered phosphorylation of paxillin in endothelial cells. HUVECs were pretreated with anacardic acid, followed by stimulation with VEGF for 10 min. After that, cells were fixed, permeabilized, and incubated with p-paxillin (Tyr118) antibody. Immunofluorescence was recorded with a confocal microscope (original magnification, 600×). The densitometry of p-paxillin was quantified by Image-Pro Plus 6.0 software. Columns represent the mean from three independent experiments with triplicate; bars represent S.D. ***, P < 0.001 versus control. B, anacardic acid inhibited phosphorylation of paxillin in endothelial cells. HUVECs were pretreated with anacardic acid, followed by stimulation with VEGF for 10 min. After that, cells were fixed, permeabilized, and incubated with p-paxillin (Tyr118) antibody. Immunofluorescence was recorded with a confocal microscope (original magnification, 600×). The densitometry of p-paxillin was quantified by Image-Pro Plus 6.0 software. Columns represent the mean from three independent experiments with triplicate; bars represent S.D. ***, P < 0.001 versus VEGF alone.

**Fig. 3.** Anacardic acid (AA) abolishes cell adhesion and paxillin activation in HUVECs. A, anacardic acid remarkably inhibited HUVEC adhesion to the extracellular matrix. HUVECs pretreated with or without anacardic acid were added to an ECM cell adhesion array plate and incubated with 20 ng/ml VEGF for 2 h. Those cells were then washed and stained. After stain extraction, the absorbance at 540 to 570 nm was determined with a microplate reader. Columns represent the mean from three independent experiments with triplicate; bars represent S.D. *, P < 0.01; **, P < 0.001 versus control. B, anacardic acid inhibited phosphorylation of paxillin in endothelial cells. HUVECs were pretreated with anacardic acid, followed by stimulation with VEGF for 10 min. After that, cells were fixed, permeabilized, and incubated with p-paxillin (Tyr118) antibody. Immunofluorescence was recorded with a confocal microscope (original magnification, 600×). The densitometry of p-paxillin was quantified by Image-Pro Plus 6.0 software. Columns represent the mean from three independent experiments with triplicate; bars represent S.D. ***, P < 0.001 versus VEGF alone.
Fig. 5. Molecular basis of anacardic acid (AA) in angiogenesis. HUVECs were starved, pretreated with anacardic acid, and stimulated by 50 ng/ml VEGF for 2 to 10 min (2 min for VEGFR2 activation, 5 min for FAK activation, and 10 min for Src activation). In the GTPase pull-down assay, starved HUVECs were incubated with VEGF for 30 min to activate intracellular small Rho GTPases. Proteins from different treatments were analyzed by Western blotting. A, anacardic acid had little effect on the phosphorylation of VEGFR2. B, anacardic acid significantly inhibited inducible and constitutive activities of Src and FAK kinases in a concentration-dependent (B1) and a time-dependent (B2) manner. C, predicted binding model of compound anacardic acid to human Src (left) and FAK (right). Protein surfaces, oxygen atoms, and carbon atoms of anacardic acid are drawn in gray. Side chains of crucial residues in the binding site are labeled and shown as sticks. Hydrogen bonds between anacardic acid and proteins are depicted as a dotted line. Figures were generated by PyMOL. D, anacardic acid had suppressive effects on Rac1 and Cdc42-GTPases activities but increased the activation of RhoA-GTPase in HUVECs.

Fig. 4. Anacardic acid (AA) inhibits CAM angiogenesis ex vivo and VEGF-induced corneal neovascularization in vivo. A, angiogenic response on CAM occurred after the carrier was implanted with vehicle or anacardic acid (5 μg/disk). The statistical result is shown, and inhibition is expressed, using untreated wells as 100% (n = 10). Columns represent the mean from two independent experiments; bars represent S.D. **, P < 0.01 versus control. B, anacardic acid inhibited VEGF-induced corneal neovascularization. The micropellets containing 100 ng of VEGF with or without anacardic acid (5 μg) were seeded into the mouse corneal pocket. The photographs of mouse cornea were taken with a digital camera at day 6 after pellet implantation. C, maximal vessel length and clock hour of circumferential neovascularization were recorded, and the blood vessel area was calculated by the formula 0.2 × 3.14 × VL (millimeters) × CN (millimeters), where VL is the maximal vessel length extending from the limbal vasculature toward the pellet, and CN is the clock hour (1 clock hour equals 30° of arc). Columns represent the mean from two independent experiments; bars represent S.D. **, P < 0.01 versus VEGF alone.
showed that anacardic acid at a concentration of 5 µM significantly suppressed the activities of Rac1 and Cdc42 and increased the active form of RhoA. Relative optical density of blotting bands was qualified by ImageJ software, and the result is shown in Supplemental Fig. 2C.

**Endothelial Cells Are More Sensitive to Treatment with Anacardic Acid.** The viability of endothelial cells and prostate cancer cells was further evaluated by the MTS assay. As shown in Fig. 6, anacardic acid had the potential to block cell proliferation in different prostate cancer cells, including DU145, LNCap, and PC-3 (Fig. 6A). However, the half-inhibitory concentration (IC50) of anacardic acid in PC-3 was approximately 72 µM, which was relatively higher than that in other cancer cells. Next, we compared the sensitivity of HUVECs and PC-3 to the treatment, and the results showed that IC50 of anacardic acid in endothelial cells was much lower, approximately 50 µM in normal culture condition and 20 µM in VEGF-activated condition (Fig. 6B). These results indicated that anacardic acid was more effective in inhibiting viability of angiogenic endothelial cells. We thus postulated that activated endothelial cells might serve as the primary target of anacardic acid in cancer treatment.

**Anacardic Acid Inhibits Tumor Growth and Tumor Angiogenesis.** Tumor angiogenesis provides oxygen, nutrients, and the main routes for tumor growth, invasiveness, and metastasis and acts as a rate-limiting step in tumor progression (Tozer et al., 2005). Results showed that anacardic acid could inhibit angiogenesis in vitro and in vivo, but whether it could affect tumor angiogenesis and tumor growth is unknown. To determine this effect, we used a xenograft mouse prostate tumor model with PC-3 cancer cells, because a relatively higher concentration of anacardic acid was required for PC-3 cells to reach IC50 value (Fig. 6A). As shown in Fig. 7A, tumor volume in the control group increased from 70.84 ± 20.56 to 364.71 ± 71.13 mm³, whereas that in anacardic acid-treated group increased only from 83.55 ± 19.91 to 91.62 ± 41.01 mm³. At the end of experiment, the solid tumors were removed (Fig. 7B), and the average weight of tumors of the control mice was 0.17 ± 0.03 g, whereas that of which the salicylic acid moiety of anacardic acid formed two hydrogen bonds with Arg388 and the pentadecane moiety of anacardic acid was buried in the hydrophobic pocket composed of Leu273, Val281, Ile336, Leu393, and Leu407. Likewise, FAK bound to the salicylic acid moiety of anacardic acid by three hydrogen bonds with Arg388 and the pentadecane of anacardic acid. However, the hydrophobic interaction between the pentadecane moiety of anacardic acid and the hydrophobic patch including Ile428, Val436, Leu501, and Leu553 (Fig. 5C, right).

**Anacardic Acid Affects Rho Family of GTPases.** The ability of a cell to polarize and move is governed by remodeling of the cellular adhesion/cytoskeletal network. In turn, the cellular cytoskeletal is controlled by the Rho family of small GTPases, which specify the exact peripheral localization of Src (Timpson et al., 2001). Our results in Fig. 5D showed that anacardic acid at a concentration of 5 µM significantly suppressed the activities of Rac1 and Cdc42 and
anacardic acid-treated mice was only 0.042 ± 0.040 g (Fig. 7B), suggesting that anacardic acid strongly inhibited prostate tumor growth in mice. Of note, anacardic acid had little toxicity at the tested dosage (Fig. 7C).

To further investigate whether anacardic acid inhibited angiogenesis and tumor cell proliferation in solid tumors, we stained the tumor sections with specific anti-CD31 and anti-Ki-67 antibodies. As shown in Fig. 7D, the blood vessel area as indicated by CD31 immunohistochemical staining in anacardic acid-treated group was just 17% of that in the control group, indicating that anacardic acid significantly inhibited angiogenesis in solid tumors. Furthermore, we found that the expression of Ki-67 was obviously suppressed, which suggested that tumor cell proliferation could be slowed down by anacardic acid. To validate the molecular target of anacardic acid in vivo, we also performed immunohistochemical staining with Src antibody. Our results further revealed that anacardic acid had a notable ability to suppress the expression of Src protein in solid tumors, which was quite consistent with its in vitro mechanism.

Discussion

In this study, we examined whether anacardic acid inhibits tumor angiogenesis and how it works. We found that anacardic acid is a novel and potent angiogenesis inhibitor and inhibits multiple steps of angiogenesis, including endothelial cell viability, migration, adhesion, and differentiation (Fig. 2). As evidenced by our xenograft human prostate tumor mouse model, the angiogenic effect of anacardic acid contributes to its antitumor action to a great extent in vivo, with significant inhibition of the volume and weight of solid tumors (Fig. 7, A and B).

Unlike anticancer agents that have side effects or cause cytotoxicity, traditional medicine is generally considered safe (Sung et al., 2008). In an in vitro examination, we found that anacardic acid could suppress cell viability of several prostate cancer cell lines, such as PC-3, DU145, and LNCaP. A relatively higher concentration of anacardic acid was required for PC-3 cells to reach the IC_{50} value (Fig. 6A). To reveal the antiangiogenic action of anacardic acid in vivo, we therefore selected PC-3 cells for tumor xenograft experiments. Of interest, in comparison with control mice, anacardic acid at a dose of 2 mg/kg per day did not affect the body weight of the mice but showed significant inhibitory function on tumor growth and angiogenesis. When we compared the antiproliferative effect of this salicylic acid on HUVECs and PC-3 cells, we found that HUVECs, especially activated HUVECs (HUVECs + VEGF), are more sensitive to the treatment (Fig. 6). This convinced us that the antiangiogenic activity of salicylic acid to retard tumor growth in vivo occurs much earlier than its typical cytotoxic effects on tumor cells. Our finding suggested that anacardic acid might be a safe anticancer agent with low toxicity and high selectivity.

Our in vitro results furthermore showed that the effective concentration of anacardic acid in inhibiting endothelial cell migration was approximately 5 μM (Fig. 2), whereas that in blockade of endothelial cell viability was approximately 20 μM (Fig. 6), indicating that anacardic acid was substantially more effective for regulating cell motility than cell survival. Considering that anacardic acid was unable to inhibit VEGF receptor 2 autophosphorylation (Fig. 5A) but efficiently blocked Src and FAK kinases (Fig. 5B), we determined that the inhibitory action of anacardic acid might be not universal to tyrosine kinases on the cell membrane. It is probable that anacardic acid might enter into the cell as a small molecule to dock to other nonreceptor tyrosine kinases. Therefore, we studied the mechanism of anacardic acid in regulation of cell motility and the cytoskeleton. In recent years, a serious interest in Src/FAK kinases as therapeutic targets in cancer treatment has evolved (Liu and Senger, 2004; Brunton and Frame, 2008; Kim et al., 2009). Src and FAK kinases physically and functionally interact to regulate multiple cellular functions, such as cell-matrix and cell-cell adhesion, migration, and invasion (Brown et al., 2005; Brunton and Frame, 2008). In the present study, we found for the first time that anacardic acid, as a histone acetyltransferase inhibitor, could suppress Src and FAK kinases in either a concentration- or a time-dependent manner. Src protein expression in tumors was also consistently inhibited by anacardic acid. Mechanistically, this salicylic acid could dock into the hydrophobic pocket of Src and FAK protein (Fig. 5C), and this might explain why it affected the phosphorylation levels of the kinases. It has been confirmed that Src is required downstream of VEGF signaling in endothelial cells and regulates both angiogenesis and vascular permeability (Eliceiri et al., 1999). Because of the potent inhibition of anacardic acid on Src/FAK signaling, VEGF-stimulated migration, capillary structure formation, attachment, and paxillin activation could all be inhibited by anacardic acid in endothelial cells (Figs. 2 and 3).

RhoA, Rac1, and Cdc42 are GTPases and have been implicated in many basic cellular processes that influence cell motility, chemotaxis, and adhesion (Etienne-Manneville and Hall, 2002). Of interest, Src kinase is a crucial mediator of the integrin signaling pathway to activate Rho-GTPases, and, sequentially, RhoA and Rac1 affect one another. During early stages of cell spreading, the FAK/Src complex activates several pathways that lead to protrusive activity via Rac and Cdc42 GTPases at sites of integrin ligation. At the same time, this complex, together with syndecans, mediates suppression of actomyosin contractility by keeping the activity of RhoA low (Huveneers and Danen, 2009). In this study, we found that anacardic acid significantly suppressed the activities of Rac1 and Cdc42 and increased the active form of RhoA (Fig. 5D). Thus, endothelial cell adhesion and spreading were substantially inhibited by anacardic acid (Fig. 3).

Evidence showed that Src family kinase members presented a common response to histone deacetylase inhibitors (Hirsch et al., 2006). However, it is still not clear whether histone acetyltransferase inhibitors could affect Src signaling. According to our molecular analysis and predictive modeling (Fig. 5), we speculated that the antiangiogenesis actions of anacardic acid might be independent of its suppression on post-translational modification as a histone acetyltransferase inhibitor. Considering that anacardic acid could suppress angiogenesis and tumor growth at such a lower dosage (2 mg/kg per day), we conclude that it might have other signal pathways combined such as nuclear factor-κB. The resistance of tumor cells to anoikis (loss of matrix adhesion-induced apoptosis) is important for their ability to survive, for growth, and for metastasis. During the process, the activation of FAK is known to act as a survival signal (Frisch et al., 1996). Given the potent inhibition on FAK
kinase, anacardic acid at a relatively higher dosage might potentially have effects on tumor metastasis. However, this assumption needs further evaluation.

In conclusion, we systemically demonstrated for the first time that anacardic acid is a novel angiogenesis inhibitor and we highlighted the roles of anacardic acid in the inhibition of tumor growth through suppressing angiogenesis. Our novel findings for anacardic acid not only confirm its ethnopharmacological value with multiple targets but also contribute to drug developments.

Authorship Contributions


References


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Anacardic Acid Inhibits Tumor Angiogenesis

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