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

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Abbreviations: VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells; Src, steroid receptor coactivator; FAK, focal adhesion kinase; NF-κB, nuclear factor-kappa B; ECM, endothelial cell culture medium; CAM, chick embryo chorioallantoic membrane; DMSO, dimethyl sulfoxide.
Abstract

Anacardic acid (6-pentadecylsalicylic acid), a natural inhibitor of histone acetyltransferase from *Amphipterygium adstringens*, has been implicated in anti-inflammatory, anticancer, antioxidative 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 its tumor inhibition. In this study, we found that vascular endothelial growth factor (VEGF)-induced cell proliferation, migration, 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 the vascular development in chick embryo chorioallantoic membrane *ex vivo* (*n*=10) and VEGF-triggered corneal neovascularization *in vivo* (*n*=10). 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. Notably, when subcutaneously administrated with anacardic acid (2 mg/kg/d) 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 imunohistochemistry 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 Src/FAK/Rho GTPases 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 the destabilization of integrated blood vessel, endothelial cell proliferation, migration and tubulogenesis. Nowadays, 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 anti-cancer therapy (Folkman, 2004). Among various pro-angiogenic growth factors and cytokines, vascular endothelial growth factor (VEGF) is the most well-characterized one 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. Src/FAK signaling pathway is the one of them.

Steroid receptor coactivator (Src) and focal adhesion kinase (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 regulating Ras/mitogen-activated protein kinases 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 guanosine triphosphatase (GTPases) and belong to the Rho-GTPases 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 α3β1-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 Src’s activation (Liu and Senger, 2004; Schenone et al., 2007). On the basis of these vital biological functions, small Src/FAK inhibitors, such as AZD0530, SKI-606 and Dasatinib have been developed and are undergoing clinical settings (Schenone et al., 2010). Therefore, novel and safe chemical agents targeting dual Src/FAK kinase show promising in cancer treatment.

Anacardic acid (6-pentadecylsalicylic acid) is the active and possible anti-inflammatory component in *Amphipterygium 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 of *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 antioxidant property (Trevisan et al., 2006) by serving as a mitochondrial uncoupler of oxidative phosphorylation (Toyomizu et al., 2000). Anacardic acid also exhibits antitumor activities (Itoh et al., 1987; Rea et al., 2003) by blockade of numerous enzymes and molecules, especially histone acetyltransferase (Balasubramanyam et al., 2003; Mai et al., 2006; Sun et al., 2006) and transcriptional factor nuclear factor-kappa B (NF-κB) (Sung et al., 2008). Recent reports further demonstrate that anacardic acid can sensitize tumor cells to ionizing radiation in cancer therapy (Sun et al., 2006) and inhibit protein SUMOylation by blocking formation of the E1-SUMO intermediate (Fukuda et al., 2009). However, the function and the mechanism of this compound in tumor angiogenesis is still an enigma. Thus we hypothesize that anacardic acid exerts antiangiogenic actions to contribute to its antitumor function.

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 Src/FAK/Rho GTPases signaling pathway, leading to the suppression of tumor angiogenesis and tumor growth in vivo.
Methods

Reagents. Anacardic acid (95% purity, Fig. 1A) was purchased from MERCK (Darmstadt, Germany). A 50-mmol/L stock solution was prepared in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO), stored at -20°C and then diluted as needed in cell culture medium. Bacteria-derived recombinant human VEGF (VEGF_{165}) was obtained from NIH Experimental Branch (NIH; Bethesda, MD). Growth factor-reduced Matrigel was purchased from BD Biosciences (San Jose, CA). Antibodies against CD31 (Cat.# AC-0083) and Ki-67 (Cat.# AC-0009) were from Epitomics (Burlingame, CA). Antibody against RhoA (Cat.# sc-418) was from Santa Cruz Biotechnology (Santa Cruz, CA); antibody against Rac1 (Cat.# 05-389) was bought from Millipore (Atlanta, GA); other antibodies [Cdc42 (Cat.#2462), pTyr^{1175}-VEGFR2 (Cat.#2948), VEGFR2 (Cat.# 2749), pTyr^{416}-Src (Cat.#6943), Src (Cat.#2109), pTyr^{397}-FAK (Cat.#3283) , FAK (Cat.#3285), pTyr^{118}-Paxillin (Cat.#2541)] 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 kindly gifted from Dr. Xinli Wang (Cardiothoracic Surgery Division of Michael E. DeBakey Department of Surgery at Baylor College of Medicine in Houston) in 2008 and 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 (ATCC; Manassas, VA) and cultured in RPMI 1640 or DMEM supplemented with 10% fetal bovine serum (FBS;
HyClone Laboratories, Logan, Utah). Western blotting using epithelial markers authenticated that they were of epithelial origin. All the cells were cultured at 37°C under a humidified 95%:5% (v/v) mixture of air and CO2.

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 “Guidelines for the Care and Use of Experimental Animals”. All of the experimental protocols were approved by the Animal Investigation Committee of East China Normal University.

Endothelial cell wound-healing migration assay. HUVECs were allowed to grow to full confluence in 6-well plates precoated with 0.1% gelatin (Sigma, St. Louis, MO). 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, 50 μmol/L). Images were taken after 8~10 h of incubation (Olympus; magnification, 100×) and migrated cells in random eight fields were quantified by manual counting. Each concentration 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 (4×10⁴) suspended in 100 μL of ECM containing 0.5% FBS plus 0.1% DMSO and various concentrations of anacardic acid (5, 10, 20, 50 μmol/L) were seeded in the top chambers. Cells were allowed to migrate for 8~10 h. Non-migrated cells were removed with cotton swabs, and migrated cells were fixed with cold 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an inverted microscope (Olympus; magnification, 100×), 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 previously described (Pang et al., 2009b). Growth factor-reduced Matrigel was pipetted into prechilled 96-well plates (50 μL Matrigel/well) and polymerized for 30 min. HUVECs (2×10⁴/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, 50 μmol/L), followed by the addition of 20 ng/mL of VEGF. After 6~8 h of incubation, the endothelial cells were photographed using an inverted microscope (Original magnification, 100×; Olympus) and quantified by measuring the tube length of random eight fields. Three independent experiments were performed.

**Endothelial cell adhesion assay.** The *de novo* attachment of endothelial cells was accessed by ECM Cell Adhesion Array Kit (Cat.# ECM540; Millipore, Billerica, Massachusetts).
HUVECs suspension was prepared with 5 mmol/L EDTA/PBS buffer and pretreated with or without anacardic acid (5, 20 µmol/L) for 30 min in the assay buffer. Consecutively, experimental cells at a density of 2×10^5 cells/well were plated into ECM Array Plate and incubated with 20 ng/mL of VEGF for about 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 is determined using absorbance readings. The absorbance at 540~570 nm was recorded by microplate reader (Molecular Devices; Sunnyvale, CA) and the values of optical density (O.D) indicated relative adhesion cell number. Three independent experiments were performed.

**Immunofluorescent staining.** HUVECs were allowed to grow to confluence on glass coverslips. After being pretreated with anacardic acid (5, 10 µmol/L) for 30 min and stimulated by VEGF (50 ng/mL) for 10 min, cells were washed, fixed and permeabilized. These cells were then incubated with p-Paxillin (Tyr^{118}) antibody (Cell Signaling Technology, Danvers, MA) overnight at 4°C, followed by incubation with a secondary antibody conjugated to FITC for 1 h at room temperature. Filamentous actin was stained with FITC-phalloidin (Molecular Probes, Invitrogen) in PBS for 1 hour and nuclei were stained with DAPI for 5 min at room temperature. Coverslips were mounted onto microscope slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence was observed and photographed using a Laser Confocal Microscope (Leica TCS SP5; magnification, 600×). The densitometry of p-Paxillin is quantified by Image Pro Plus software. Three independent experiments were performed.
Chick embryo chorioallantoic membrane (CAM) assay. Fertilized chicken eggs were purchased from Poultry Breeding Co. Ltd (Shanghai, China). According to previous method (Mangieri et al., 2009), embryonic eggs were incubated in 38.5~39°C with the relative humidity at 65~70%. Five days later, a 1~2 cm² window was opened and the shell membrane was removed to expose the chorioallantoic membrane. As a carrier, a 6 mm-diameter Whatman filter disk pre-treated by 20 µg/disk hydrocortisone with or without anacardic acid was put onto the CAM. The window was sealed and eggs were incubated for another 5 d. At last, the CAM was observed under stereomicroscope and the neovascularization was quantified by Image Pro Plus software. Two independent experiments were performed (n=10 per group).

Mouse corneal micropocket assay. The mouse corneal assay was performed as previously described (Ziche and Morbidelli, 2009). Before surgery, 4-week-old mice were randomly divided into 3 groups: blank control, VEGF, or VEGF with anacardic acid. Micropellets were made of sucrose aluminum sulfate and Hydron pellets (polyhydroxyethyl-methacrylate) containing VEGF (100 ng) with or without anacardic acid (5 µg/pellet). An intrastromal micropocket (1×0.5 mm) was dissected toward the limbus with a modified needle. A single micropellet (~0.35×0.35 mm) was implanted into mouse corneal micropockets (n=10 each group). The eyes of mice were photographed with a digital camera at day 6 after pellet implantation. Maximal vessel length was recorded by Image Pro Plus software. Clock hours of circumferential neovascularization were measured and one clock hour equals 30 degrees of
arc. The area of neovascularization was calculated according the formula $0.2 \times 3.14 \times VL (\text{mm}) \times CN (\text{mm})$, 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, western blotting assay was performed. HUVECs were first starved in serum-free ECM for 4~6 h and then pretreated with or without indicated concentrations of anacardic acid (1, 5, 10, 20 µmol/L) for 30 min, followed by the stimulation with 50 ng/mL of VEGF for 2~10 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 previously described (Pang et al., 2009b). About 40~50 microgram of cellular protein from each sample was resolved by SDS-PAGE 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 qualified by Image J software (NIH; Bethesda, MD).

**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 Rho family of small GTPases. After that, cells were lysed with lysis buffer (20 mmol/L Tris-HCL, 2.5 mmol/L EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mmol/L NaF, 10 mmol/L Na$_3$P$_2$O$_7$, and 1 mmol/L PMSF) supplemented with proteinase inhibitor cocktail (Calbiochem, San
Diego, CA). For GTPases activation experiment, GST-PBD (Pak Rac/Cdc42-binding domain) or GST-RBD (Rho-binding domain) attached to beads (Santa Cruz Biotechnology) were added to the cell lysates. After incubation at 4°C for 2 h, the beads were washed 5 times with lysis buffer and boiled at 100°C for 10 min with 2×SDS sample buffer. Immunoblot for Rac1, Cdc42 and RhoA was done with specific antibodies. Three independent experiments were performed. Relative optical density of blotting bands was qualified by Image J software.

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

**Cell viability analysis.** HUVECs, PC-3, DU 145 and LNCap cells were plated in 96-well plates (5×10^3 per well) and treated with different concentrations of ancardic acid (1, 10, 20, 50, 100 μmol/L) for 48 h. To determine cell viability, we used the kit of CellTiter96 AQueous One Solution Cell Proliferation (MTS; Promega, Madison, WI), and the optical density was measured with VERSAmax microplate reader (Molecular Devices; Sunnyvale, CA). Three independent experiments with six duplicates were performed.

**Xenograft human prostate tumor mouse model.** Xenograft mouse model assay was
performed as previously described (Yi et al., 2008). Five-week-old male BALB/cA nude mice (~25 g) were randomly divided into 2 groups (n=6~7 each group). PC-3 cells were subcutaneously injected into the left flank of mice (3×10⁶ per mouse). After tumors grew to ~70 mm³, tumor-bearing mice were treated with or without anacardic acid (2 mg/kg/d) via subcutaneous injection for consecutive 30 d. 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 d. 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. The anti-CD31, Src, 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 immunohistochemistry. Images were taken using a Leica DM 4000B photomicroscope (magnification, 400x). Immunoreactive spots from random five fields of each tumor section were further selected for analysis and quantified by the Image-Pro plus 6.0 software package (Media Cybernetics Inc., Bethesda, MD).

**Statistical analysis.** Statistical comparisons between groups were performed using the standard Student's t-test. Data were presented as means ± standard deviation. P values ≤ 0.05
were 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 were first evaluated. To identify the effects of anacardic acid on cell migration, we used two types of migration models, 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 μmol/L in wound-healing assay (Fig. 2A) and Transwell assay (Fig. 2B). The highest concentration of anacardic acid (50 μmol/L) completely blocked endothelial cell motility. However at the same experimental condition, anacardic acid didn’t affect cell viability of VEGF-activated HUVEC (Supplemental Figure 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 two-dimensioned Matrigel assay. As shown in Fig. 2C, anacardic acid potently inhibited the tube formation in HUVECs, with functional concentration at 5 μmol/L. Interestingly, anacardic acid had little effect on cell viability of HUVEC at this process (Supplemental Figure 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 $\alpha/\beta$ 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 $\alpha$ subunits and 8 $\beta$ 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 multi-domain protein that localizes primarily to focal adhesion sites to the extracellular matrix, and tyrosine phosphorylation of paxillin is required for integrin-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 by immunofluorescent analysis in HUVECs. Our results showed that VEGF could significantly induce the activation of paxillin; however, anacardic acid abolished this process to a great extent (Fig. 3B), which indicated that anacardic acid had ability to destroy endothelial cell focal adhesion to extracellular matrix. All of these results suggested that anacardic acid was functional in multiple steps of angiogenesis in vitro.

**Anacardic acid inhibits vascular development ex vivo and corneal neovascularization in**
The chick chorioallantoic membrane was used to examine the inhibitory activity of anacardic acid on vascular development. As shown in Fig. 4A, the vascular generation in the treated group was significantly suppressed compared with that in the control group, suggesting anacardic acid had ability to inhibit CAM angiogenesis.

To further confirm whether anacardic acid could affect VEGF-induced angiogenesis in vivo, the corneal angiogenesis model was performed. As shown in Fig. 4B, addition to VEGF in the pellet significantly triggered neovascularization in the mouse corneal via angiogenesis, whereas supplement with anacardic acid could potently block this process. Statistical analysis showed that the vessel length and clock number were diminished after anacardic acid’s treatment (Fig. 4C, left and middle). Accordingly, the areas of cornea angiogenesis could also be rectified (Fig. 4C, right). These results indicated that anacardic acid could block angiogenesis ex vivo and in vivo.

**Anacardic acid blocks dual Src/FAK kinase in HUVECs without affecting VEGFR2’s activity.** The Src/FAK pathway is well-known to be involved in cell proliferation, migration and adhesion. Using Western blotting assay, we examined the activities of Src and FAK kinases in treated HUVECs. As shown in Fig. 5B, anacardic acid could significantly inhibit the phosphorylation of Src/FAK complex induced by VEGF in a concentration-dependent manner, effective at the concentration of 5 μmol/L (Fig. 5B1). Similar results were also observed that anacardic acid time-dependently suppressed the constitutive activities of Src/FAK kinases (Fig. 5B2). However, VEGFR2’s activation appeared to be not affected by
the treatment (Fig.5A). Relative optical density of blotting bands was qualified by Image J software and the results were shown in Supplemental Figure 2A and Supplemental Figure 2B.

To provide insights into how anacardic acid affected the activity of both Src and FAK, we carried out docking simulation for binding modes of anacardic acid to human Src and FAK proteins. Src model was shown in Fig. 5C (Left), in which the salicylic acid moiety of anacardic acid formed two hydrogen bonds with Arg388, and the pentadecane of anacardic acid was buried in the hydrophobic pocket composed of Leu273, Val281, Ile336, Leu393 and Leu407. Similarly, FAK bound to salicylic acid moiety of anacardic acid by three hydrogen bonds with Glu430, Gln432, and Lys454 as well as 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 Src’s exact peripheral localization (Timpson et al., 2001). Our results in Fig. 5D showed that anacardic acid at concentration of 5 μmol/L significantly suppressed the activities of Rac1 and Cdc42 and increased the active form of RhoA. Relative optical density of blotting bands was qualified by Image J software and the result was shown in Supplemental Figure 2C.

Endothelial cells are more sensitive to anacardic acid’s treatment. The viability of
endothelial cells and prostate cancer cells were further evaluated by MTS assay. As shown in
Fig. 6, anacardic acid had potential to block cell proliferation in different prostate cancer cells,
including DU145, LNCap and PC-3 (Fig. 6A). However, the half inhibitory concentration
(IC_{50}) of anacardic acid in PC-3 was about 72 µmol/L, 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 IC_{50} of anacardic acid in endothelial cells was much
lower, about 50 µmol/L in normal culture condition and 20 µmol/L in VEGF-activated
condition (Fig. 6B). These results indicated that anacardic acid was more effective to inhibit
viability of angiogenic endothelial cells. We thus postulated that activated endothelial cells
might served as the primary target of anacardic acid in cancer treatment.

**Anacardic acid inhibits tumor growth and tumor angiogenesis.** Tumor angiogenesis
provides oxygen, nutrients, and main routes for tumor growth, invasiveness and metastasis,
which acts as a rate-limiting step in tumor progression (Tozer et al., 2005). Upwards of 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
performed a xenograft mouse prostate tumor model with PC-3 cancer cells, since relative
higher concentration of anacardic acid was required for PC-3 cells to reach IC_{50} value (Fig.
6A). As shown in Fig. 7A, tumor volume in the control group increased from 70.84±20.56
mm³ 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 taken out
(Fig. 7B), and the average weight of tumors of the control mice was 0.17±0.03 g, whereas that
of anacardic acid-treated mice was only 0.042±0.040 g (Fig. 7B), suggesting that anacardic acid strongly inhibited prostate tumor growth in mice. Notably, anacardic acid had little toxicity at 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 immunohistochemistry 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 additionally performed immunohistochemistry with Src antibody. Our results further revealed that anacardic acid had 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 examine whether anacardic acid inhibits tumor angiogenesis and how it works. We find 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, where the volume and weight of solid tumors are significantly inhibited (Fig. 7A and 7B).

Unlike those anticancer agents that have side effects or serve cytotoxicity in chemotherapy, traditional medicine is generally considered safe (Sung et al., 2008). In *in vitro* examination, we found that anacardic acid could suppress cell viability of several prostate cancer cells, such as PC-3, DU145 and LNCap. Relative higher concentration of anacardic acid was required for PC-3 cells to reach IC$_{50}$ value (Fig. 6A). To reveal the antiangiogenic action of anacardic acid *in vivo*, we therefore selected PC-3 cell for tumor xenograft experiments. Interestingly, in comparison with those control mice, anacardic acid at dosage of 2 mg/kg/d did not affect the body weight of the mice but showed significant inhibitory function on tumor growth and angiogenesis. When compared the antiproliferative effect of this salicylic acid on HUVECs and PC-3, we found that HUVECs, especially activated HUVECs (HUVECS+VEGF), are more sensitive to the treatment (Fig. 6). This convinces us that salicylic acid’s antiangiogenic activity to retard tumor growth *in vivo* is 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 about 5 μmol/L (Fig. 2), while that in blockade of endothelial cell viability was about 20 μmol/L (Fig. 6), indicating that anacardic acid was substantially more effective to regulate 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 deemed that the inhibitory action of anacardic acid might be not universal to tyrosine kinases on the cell membrane. Probably, anacardic acid might enter into the cell as a small molecular to dock to other non-receptor tyrosine kinases. Therefore, we pursued to study the mechanism of anacardic acid in regulation of cell motility and cytoskeleton. In recent years, a serious interest in Src/FAK kinases as therapeutic targets in cancer treatment has been evolved (Liu and Senger, 2004; Brunton and Frame, 2008; Kim et al., 2009). Src and FAK kinases physically and functionally interact together to regulate multiple cellular functions, such as cell-matrix and cell-cell adhesion, migration and invasion (Brunton and Frame, 2008) (Brown et al., 2005). 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. Consistently, Src protein expression in tumors was also inhibited by anacardic acid. Mechanistically, this salicylic acid could dock into the hydrophobic pocket of Src and FAK protein (Fig. 5C), and thus 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). Due to 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 (Fig. 2 and 3).

RhoA, Rac1, and Cdc42 are guanosine triphosphatase and have been implicated in many
basic cellular processes that influence cell motility, chemotaxis and adhesion (Etienne-Manneville and Hall, 2002). Interestingly, Src kinase is crucial mediator of 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 RhoA's active form (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 unclear 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 lower dosage (2 mg/kg/d), we concluded that it might have other signal pathway combined such as NF-κB. The resistance of tumor cells to anoikis (loss of matrix adhesion induced apoptosis) is important for their ability to survive, growth and 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 relative higher dosage
might have potential effects on tumor metastasis. However, this assumption needs further evaluation.

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

Participated in research design: Pang, Liu, Yi, and Wu

Conducted experiments: Wu, He, L. Zhang, Chen, J. Zhang, Pang, and Yi

Performed data analysis: Wu, J. Zhang, Pang, Yi, and Liu

Wrote or contributed to the writing of the manuscript: Pang, Liu, Wu, J. Zhang, and He
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Legends for Figures

Figure 1. Chemical structure of anacardic acid.

Figure 2. Anacardic acid 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, 50 μmol/L). Representative images (Left) and quantitative result (Right) were shown. A, anacardic acid inhibited HUVEC migration. HUVECs were grown into full confluence in 6-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 Transwell and treated with anacardic acid. After 8~10 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 (2×10^4 per well) were placed in 96-well plates coated with Matrigel. Tubular structures were photographed. Columns, mean from three independent experiments with triplicates; bars, standard deviation; *, P < 0.01 vs. VEGF alone.

Figure 3. Anacardic acid abolishes cell adhesion and paxillin activation in HUVECs.

A, anacardic acid remarkably inhibited HUVEC adhesion to extracellular matrix. HUVECs pretreated with or without anacardic acid were added to ECM cell adhesion array plate and incubate with 20 ng/mL of VEGF for 2 h. Those cells were then washed and stained. After stain extraction, the absorbance at 540~570 nm was determined by microplate reader. B,
anacardic acid inhibited phosphorylation of paxillin in endothelial cells. HUVECs were pretreated with anacardic acid, followed by stimulation of VEGF for 10 min. After that, cells were fixed, permeabilized and incubated with p-Paxillin (Tyr118) antibody. Immunofluorescence was recorded by confocal microscope (magnification, 600×). The densitometry of p-Paxillin is quantified by the Image-Pro plus 6.0 software. Columns, mean from three independent experiments with triplicate; bars, standard deviation; *, $P < 0.01$ vs. VEGF alone.

Figure 4. Anacardic acid inhibits CAM angiogenesis ex vivo and VEGF-induced corneal neovascularization in vivo. A, angiogenic response on CAM occurred after implanting the carrier with vehicle or anacardic acid (5 μg/disk). The statistical result was shown and inhibition was expressed using untreated wells as 100% ($n=10$). Columns, mean from two independent experiments; bars, standard deviation. **, $P < 0.01$ vs. control. B, anacardic acid inhibited VEGF-induced corneal neovascularization. The micropellets containing 100 ng 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 $0.2 \times 3.14 \times VL \times CN$, where VL is the maximal vessel length extending from the limbal vasculature toward the pellet, and CN is the clock hour (One clock hour equals 30 degrees of arc). Columns, mean from two independent experiments; bars, standard deviation. **, $P < 0.01$ vs. VEGF alone.
Figure 5. Molecular basis of anacardic acid in angiogenesis. HUVECs were starved, pretreated with anacardic acid and stimulated by 50 ng/mL of VEGF for 2~10 min (2 min for VEGFR2 activation, 5 min for FAK activation and 10 min for Src activation). In 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- (Fig.5B1) and a time- (Fig.5B2) dependent manner. C, predicted binding model of compound anacardic acid to the human Src (Left) and FAK (Right). Protein surface were drawn in gray. Oxygen atoms were shown in red and nitrogen atoms in blue. Carbon atoms of anacardic acid and proteins were shown in pink and gray, respectively. Side chains of crucial residues in the binding site were shown as stick and labeled. Hydrogen bonds between anacardic acid and proteins were depicted in dotted line in yellow. 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.

Figure 6. Endothelial cells are more sensitive to anacardic acid’s treatment. The viability of endothelial cells and prostate cancer cells were further evaluated by MTS assay. A, anacardic acid inhibited viability of human prostate cancer cells. DU145, LNCap and PC-3 cells were incubated with indicated concentrations of anacardic acid (1, 10, 20, 50, 100 μmol/L) for 48 h. B, activated HUVECs were much more sensitive to the treatment. PC-3 and
HUVECs at normal or VEGF-inducible culture conditions were treated with various concentration of anacardic acid for 48 h. *Columns*, mean from three independent experiments with six duplicates; *bars*, standard deviation.

**Figure 7. Anacardic acid inhibits tumor growth and tumor angiogenesis in the xenograft mice.** PC-3 cells were first subcutaneously injected into the left flank of mice (3×10⁶ per mouse). After tumor volume reached about 70 mm³, mice were treated with or without anacardic acid (2 mg/kg/d; n=6-7 each group). *A*, anacardic acid inhibited solid tumor volume. *B*, tumor weight was significantly inhibited by anacardic acid. The picture of those xenografts (Left) and statistic results of tumor weight (Right) were presented. *C*, anacardic acid had little effect on the average body weight of mice. *D*, anacardic acid suppressed tumor angiogenesis and tumor growth. The 5-µm sections of the solid tumor were stained with CD31, Src and Ki-67 antibodies. The blood vessel density and positive staining area were calculated by using the Image-Pro plus 6.0 software. *Columns*, mean from random five fields of each tumor section; *bars*, standard deviation. **, P < 0.01 vs. control.
Anacardic acid (AA)

\[ C_{22}H_{36}O_3 \quad \text{FW. 348.5} \]
Fig. 2

A

Control | VEGF | VEGF+AA 50 μmol/L

Migrated cell number (% of control)

AA (μmol/L) | 0 | 0 | 5 | 10 | 20 | 50

VEGF | - | + | + | + | + | +

B

Control | VEGF | VEGF+AA 50 μmol/L

Migrated cell number (% of control)

AA (μmol/L) | 0 | 0 | 5 | 10 | 20 | 50

VEGF | - | + | + | + | + | +

C

Control | VEGF | VEGF+AA 50 μmol/L

Tube formation (% of control)

AA (μmol/L) | 0 | 0 | 5 | 10 | 20 | 50

VEGF | - | + | + | + | + | +
Fig. 3

A

Relative cell adhesion (OD.)

Control  AA 5 μmol/L  AA 20 μmol/L

Collagen I  Collagen II  Collagen IV  Fibronectin  Laminin  Tenasin  Vitronectin  Blank

*p  **

B

p-Paxillin (Tyr18)

Control  VEGF  VEGF+AA 5μmol/L  VEGF+AA 10μmol/L

Densitometry of p-Paxillin (% of control)

AA (μmol/L)  0  0  5  10

VEGF  -  +  +  +
Fig. 4

A

Control | AA 5 μg

Vessel area (% of control)

AA (μg) 0 1 5

B

Control | VEGF | VEGF+AA

C

Vessel area (mm²)

VEGF | VEGF+AA

Vessel length (mm)

VEGF | VEGF+AA

Clock number

VEGF | VEGF+AA

**