Secalonic Acid-D Represses HIF1α/VEGF-Mediated Angiogenesis by Regulating the Akt/mTOR/p70S6K Signaling Cascade

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Abstract

Tumor angiogenesis is a validated target for therapeutic intervention, but agents that are more disease selective are needed. Here, we report the isolation of secalonic acid-D (SAD), a mycotoxin from a novel source that exhibits potent antiangiogenic antitumor activity. SAD inhibited multiple HIF1α/VEGF-arbitrated angiogenesis dynamics as scored in human umbilical vascular endothelial cells and human MCF-7 breast tumor xenografts. Similarly, SAD suppressed VEGF-induced microvessel sprouting from rat aortic ring and blood vessel formation in the Matrigel plug assay in C57/BL6J mice. Under normoxic or hypoxic conditions, SAD inhibited cell survival through the Akt/mTOR/p70S6K pathway, with attendant effects on key proangiogenesis factors, including HIF1α, VEGFR, and MMP-2/MMP-9. These effects were reversed by cotreatment with the Akt inhibitors perifosine and GS69069 or by the addition of neutralizing VEGF antibodies. The apoptotic properties of SAD were determined to be both extrinsic and intrinsic in nature, whereas the cell-cycle inhibitory effects were mediated by altering the level of key G1-S transition-phase proteins. In experimental mouse models of breast cancer, SAD dosing produced no apparent toxicities (either orally or intraperitoneal) at levels that yielded antitumor effects. Taken together, our findings offered a preclinical validation and mechanistic definition of the antiangiogenic activity of a novel mycotoxin, with potential application as a cancer-selective therapeutic agent. Cancer Res; 75(14); 1–11. ©2015 AACR

Introduction

Cancer is a leading cause of death all over the world, and approximately 6 million new cases are reported every year (1). Angiogenesis is one of the common hallmark manifestations of all cancers and is an elementary event in the development of tumor growth and malignancy (2, 3). Currently, several antiangiogenesis drugs have been approved by the FDA that target VEGF, MMP, HIFα, etc. They may be useful as an appendage to chemotherapy, radiation, and surgical therapy and also useful for nonneoplastic diseases associated with angiogenesis. Therefore, angiogenesis inhibitors act as a dual-edge sword that target angiogenesis and potentiate the usefulness of chemotherapeutic drugs (3). Antiangiogenesis therapy is target-selective and relatively free from drug resistance and side effects, because it targets normal dividing endothelial cells, which are genetically stable with leaky, disorganized, and immature tumor vasculature (4). For these reasons, antiangiogenic drugs are highly selective toward tumor vascular lining and make it an attractive target for cancer therapy (5). Numerous growth factors promote tumor angiogenesis, among them is the VEGF family of proteins that plays a pivotal role in normal and pathologic angiogenesis. The activation of the Akt/mTOR/p70S6K mediated HIF1α/VEGF-receptor (VEGFR) alliance triggers endothelial and cancer cell functions toward protein synthesis, tumor genesis, angiogenesis, metastasis, migration, proliferation, and apoptosis (6, 7). Consequently, the discovery of novel HIF1α/VEGF and Akt/mTOR/p70S6K pathway inhibitors provides great magnetism in anticancer therapeutics.

Nature is an attractive source of novel therapeutic agents, and plants play an important role in the discovery and development of several clinically approved anticancer drugs. Taking into consideration the limitations coupled with the yield and susceptibility of plant-derived novel metabolites, microorganisms
provide a decisive, willingly renewable, and infinite source of novel therapeutic entities (8). The microorganisms, such as endophyte (fungi, yeast, bacteria), are relatively unexplored, interesting, and promising niches for the production of novel therapeutic agents. Among these, endophytic fungi are the concealed members of the microbial world and have received less attention due to their asymptomatic existence (9). In this perspective, we have for the first time isolated secalonic acid-D (SAD) from a novel source of endophytic fungus (Penicillium oxalicum) of Catharanthus roseus. SAD, a mycotoxin, was initially isolated from several foods intuitive fungi, but to date there is no report of its isolation from endophytic (P. oxalicum) of C. roseus (10). SAD has several biological activities, such as mycotoxin, protein kinase A and C inhibitor, cytotoxic, and K562 cell-cycle inhibitor (11). Conversely, there was no testimony on its antiangiogenesis potential and effect on the Akt/mTOR/ 

Materials and Methods

Isolation of SAD

SAD was isolated from P. oxalicum, an endophytic fungus of C. roseus. The pure endophytic fungal isolate (EF-VR2) was isolated from the roots of C. roseus (family: Apocynaceae; ref. 12). The isolation of pure genomic DNA of fungal endophyte (EF-VR2) was done as described earlier with slight modifications (13). The PCR amplification of ITS1-5.8S rDNA-ITS2 regions was performed, and a phylogenetic tree was constructed by using MEGA5 software and was subsequently analyzed for evolutionary distances by the neighbor joining method (14). The contiguous rDNA sequences of the representative isolate were submitted to the GenBank database using SEQUIN program with accession no. KC189890. The single spore of the P. oxalicum was obtained, lyophilized, and maintained at 4°C for long-term preservation (15). The fermentation was performed, and broth was extracted according to the NCI protocol. The organic extract was then subjected to column chromatography to obtain pure SAD. The compound was characterized by various spectroscopic methods, viz., 1H and 13C NMR, high-resolution mass spectrometry, optical rotation, and IR, and was found to be in accordance to the literature (16).

Cell culture, animal, and reagents

HUVEC, breast cancer MCF-7 and MDA-MB-231, prostate cancer PC-3, pancreatic cancer LnCaP, and human breast epithelial F82 cells were obtained from Sigma-Aldrich India (ECACC). The HUVECs were grown in complete EndoGRO-LS media with VEGF (#SCME001; Millipore), and the remaining cells were grown in Minimal Essential Medium with 10% fetal bovine serum and 20 ng/mL VEGF supplement only for MCF-7 cells. All the cells were cultured at 37°C with 9% humidity and 5% CO2 gas environment. All animal experiments were approved by the animal ethics committee of the Indian Institute of Integrative Medicine, CSIR, Jammu, India (IEAC No. 34/8/14). Animal euthanasia was performed by carbon dioxide inhalation (10%-30% vol/min) in the Matrigel plug assay and cervical dislocation for the EAT tumor model and aortic ring assays. The primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Cell Signaling Technology, and BD Biosciences (Supplementary Table S1). The Matrigel and in vitro angiogenesis assay kits were procured from Millipore (India) Pvt. Ltd. All other biochemicals and reagents used in this study were AR grade and purchased from Sigma-Aldrich.

Cell-proliferation assay

The HUVEC, MCF-7, MDA-MB-231, PC-3, LnCaP, and F82 cells at around 70% confluence were treated with SAD at various concentrations from 1 to 100 μmol/L for 6, 12, 24, and 48 hours. MTT dye (2.5 mg/mL in PBS) was added 4 hours prior to the experiment's termination. MTT formazan crystals were dissolved in 150 μL of DMSO and absorbance was measured at 570 nm with a reference wavelength of 620 nm (17).

Cellular and nuclear morphology

The HUVEC and MCF-7 cells were treated with SAD at 10 to 50 μmol/L concentrations for 24 hours and were observed under a microscope for any morphologic changes that occurred during apoptosis. Simultaneously, the nuclear morphology was analyzed through Hoechst staining as described earlier (18).

Annexin V–FITC assay

HUVEC and MCF-7 cells were treated with SAD at 10 to 50 μmol/L concentrations for 24 hours. The cells were collected at 400 × g and stained with Annexin V–FITC antibody as per instructions provided with the Apoptosis Detection Kit (Santa Cruz Biotechnology; ref. 17).

Loss of mitochondrial membrane potential (Δψmt)

HUVEC and MCF-7 cells were exposed to 10, 30, and 50 μmol/L concentrations of SAD for 24 hours. Rhodamine-123 (200 nmol/L) was added 40 minutes before termination and washed with PBS and attain on flow cytometer (17).

Preparation of cell lysates and Western blot analysis

The HUVEC and MCF-7 cells were treated with SAD at 10, 30, and 50 μmol/L concentrations for 6 to 24 hours in the presence and absence of VEGF (20 ng/mL) and CoCl2 (100 μmol/L). The whole cells, cytosolic, and mitochondrial fractions were prepared as described earlier (17). An equal amount of protein (40–70 μg) was subjected to SDS-PAGE analysis and transferred to the polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat milk-TBS or 3% BSA-TBS (for phosphorylated antibodies) and probed with respective primary and secondary antibodies mentioned in Supplementary Table S1.

Cell-cycle analysis

The breast cancer MCF-7 cells and HUVECs were treated with SAD at 10, 30, and 50 μmol/L concentrations for 24 hours in the presence of VEGF (20 ng/mL). The cell cycle was analyzed on flow cytometer as described earlier (19).

Colonies-formation assay

The MCF-7 cells were treated with different concentrations of SAD (10, 30, and 50 μmol/L) for 24 hours in the presence of VEGF (20 ng/mL). Colony-formation assay was carried out as described earlier (20). Colonies of 50 or more cells were counted from three independent experiments.
Immunofluorescence
MCF-7 cells supplemented with fresh medium with or without VEGF (20 ng/mL) were cultured on the sterile cover slips placed on the surface of 6-well plates at a density of 10,000 cells per well. Cells were treated with different concentrations of SAD (10–50 μmol/L) for 24 hours, fixed with 4% paraformaldehyde, and incubated with mTOR primary antibody.

RT-PCR analysis
Human breast cancer MCF-7 cells were treated with SAD (10–50 μmol/L) for 24 hours. Total RNA was extracted by using Tri-reagent (Sigma) and incubated with RNase free DNase. cDNAs were synthesized from equal amount of RNA (3 μg) using Revert Aid cDNA Synthesis Kit according to manufacturer’s instruction. SYBR Green PCR amplification was performed using the StepOne Real-time PCR System. Two set of primers as listed in Supplementary Table S2 were used to amplify housekeeping genes GAPDH and β-actin.

Gelatin zymography
The MCF-7 cells were treated with indicated concentrations of SAD for 24 hours, and the culture medium was electrophoresed (80 V for 120 minutes) on 8% SDS-PAGE gel containing 0.1% gelatin. The gel was assessed for two gelatin-degrading matrix metalloproteinase, MMP-2 and MMP-9, as described earlier (21).

Wound-healing migration assay
The wound-healing migration assay was performed as described previously (22). Briefly, HUVECs were treated with mitomycin C to inactivate cell proliferation, wounded by a micro-tip, washed with PBS, supplemented with fresh medium with or without VEGF (20 ng/mL) and treated with SAD.

Capillary-like tube formation assay
The tube formation assay was performed according to the instructions provided by the in vitro Angiogenesis Assay Kit (Millipore, Cat. No. ECM 625). Briefly, HUVECs were seeded on the surface of the polymerized EC Matrix and incubated at 37°C for 12 hours. The cells were treated with different concentrations of SAD for 24 hours, and capillary-like tube sprouting was observed under an inverted microscope at ×10 magnification.

Chemotaxis cell migration and invasion assays
Microporous membrane inserts are widely used for cell migration and invasion assays. MCF-7 and HUVECs were incubated on the surface of the insert and treated with different concentrations of SAD for 24 hours. The cell migration and invasion percentage was calculated as per the protocol of the respective kits (Millipore; cat. nos. ECM509 and ECM554).

Aortic ring assay
The aortas isolated from Sprague–Dawley rats were cleaned and cut into rings of 1- to 1.5-mm circumference. The aortic rings were randomized into Matrigel-coated wells and sealed with a 100 μL overlay of Matrigel (23). The aortic rings were cultured in Endo-GRO LS Media with and without 20 ng/mL of VEGF for 4 days. On day 5, various concentrations of SAD (10–30 μmol/L) were added to the wells and further incubated for 96 hours. On day 9, the microvessel sprouting was fixed and photographed. The aortic ring tissue was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin (H&E).

Tumor angiogenesis Matrigel plug assay
The Matrigel plug assay was performed as described previously (23). Briefly, 0.5 mL of Matrigel with VEGF (100 ng), in the presence and absence of human breast cancer MCF-7 cells (0.5 × 10^6), was injected subcutaneously into the ventral area of 4- to 6-week-old C57/BL6j mice (IIIM, central animal house). Animals were dosed daily with SAD (10 and 20 mg/kg body weight; oral/i.p.) from day 2 to day 6. On day 7, animals were sacrificed to remove intact Matrigel plugs with different treatments and photographed that show the extent of vascularization. Neovascularization of Matrigel plugs was quantified by measuring their size and hemoglobin (Hb) content through the Drabkin reagent.

Ehrlich tumor solid
Ehrlich ascites carcinoma (EAC) cells (1 × 10^7) were collected from 8- to 10-day-old Swiss albino mice and were injected (intramuscularly) in the right thigh on day 0. On day 1, the animals were randomized and divided into different groups. SAD was given i.p. at 10 and 20 mpk of body weight from day 1 to 9. Similarly, the control group received vehicle, whereas treatment with 5-fluorouracil (5-FU), 22 mpk i.p., was given to a group of mice serving as positive control. On day 13, animals were sacrificed, and average tumor weight was calculated.

Statistical analysis
Data are expressed as mean ± SD or representative of one of three similar experiments unless otherwise indicated. Comparisons were made between control and treated groups or the entire intragroup using one-way and two-way ANOVA with the post-Bonferroni test through GraphPad Prism 5.00.288 statistical analysis software.

Results
Isolation, characterization, and cytotoxic profile of SAD
SAD was isolated from the pure endophytic fungal isolate (EF-VR2) of P. oxalicum from C. roseus. The morphology and sequencing of P. oxalicum, endophyte fungi isolate (EF-VR2), was characterized with a neighbor-joining rooted tree (Supplementary Fig. S1A–S1E). SAD inhibits cell proliferation of HUVEC and different cancer cells in a dose- and time-dependent manner (Supplementary Fig. S1F). SAD was found to be nontoxic at the experimental concentration in normal human breast epithelial rR2 cells, where the IC_{50} value was 13 times higher than in MCF-7 cells. The cytotoxic effect of SAD was more pronounced in HUVEC and MCF-7 cells; therefore, we have explored both these cells for further studies.

SAD inhibits VEGF-induced chemotactic wound-healing migration and microvessel sprouting
Chemotactic motility and microvessel sprouting are necessary for tumor growth and metastasis (24). SAD significantly inhibited VEGF-induced HUVEC migration in a dose-dependent manner and decreased the wound-closure percentage from 100% to 10% at 50 μmol/L concentrations (Fig. 1A and B). To further assess the effect of SAD on angiogenesis, we examined how SAD regulates capillary tube formation of endothelial cells. HUVECs showed robust tubular-like structures when seeded on two-dimensional...
Figure 1.
Effect of SAD on VEGF-mediated angiogenesis. A and B, SAD inhibited HUVEC migration in the wound-healing assay. C and D, SAD inhibited tube sprouting of HUVEC cells in the tube formation assay. The tube formation was observed as described in Materials and Methods. E and F, SAD inhibited chemotaxis cell migration and invasion. MCF-7 and HUVECs were incubated in the invasion and migration chamber, based on the Boyden chamber principle. Data, mean ± SD of three independent experiments; *, P < 0.001, compared with untreated control considered statistically significant. G, effect of SAD on VEGF-arbitrated ex vivo angiogenesis. Aortic segments were harvested from Sprague-Dawley rats, as described in Materials and Methods. H, histologic analysis of the rat aortic ring was performed as described in Materials and Methods. Data are representative of one of three similar experiments.
Matrigel containing VEGF, which was significantly abolished by SAD in a dose-dependent manner, suggesting its potential inhibitory efficacy of VEGF-induced angiogenesis (Fig. 1C and D).

**SAD inhibits chemotaxis cell migration and invasion**

Boyden chamber assays enable simulation of the barriers invaded by and conditions encountered by metastatic tumor cells in vivo. SAD significantly inhibits the invasion and migration of both the MCF-7 and HUVECs in a dose-dependent manner. The migration inhibition percentage was reduced from 100% to 10% at 50 μmol/L concentration of SAD, whereas the invasion inhibition percentage was reduced from 100% to 30% in both experimental cell lines (Fig. 1E and F).

**SAD inhibits VEGF-induced microvessel sprouting ex vivo angiogenesis**

The aortic ring assay allows analysis of cellular proliferation, migration, tube formation, microvessel branching, perivascular recruitment, which provide a more complete picture of angiogenic processes compared with traditional cell-based assays. VEGF (20 ng/mL) significantly stimulated microvessel sprouting, and this effect was completely inhibited by SAD in a dose-dependent manner (Fig. 1G). The histologic analysis of rat aortic ring further revealed that SAD inhibits the microvessel sprouting in the same manner (Fig. 1H).

**SAD inhibits VEGF-induced tumor angiogenesis in the Matrigel plug assay**

Angiogenesis is the key stride in tumor growth and metastasis (25). To determine the effects of SAD on VEGF-induced angiogenesis in vivo, mice were dosed orally or i.p. with SAD (10–20 mg/kg/body weight) after Matrigel implantation in the presence and absence of MCF-7 cells. Matrigel plug of untreated animals revealed the marked increase in vascularization and size, seen as a deep red appearance and high Hb content (Fig. 2A). The Matrigel plugs of animals dosed with SAD (10 and 20 mg/kg/body weight) show a complete reduction in the degree of vascularization and size and appear as white and negligible Hb content (Fig. 2B), indicating that SAD significantly inhibited tumor angiogenesis. We also obtained the same results when animals were treated with SAD at 10 to 20 mg/kg/body weight i.p. in the presence and absence of human breast cancer MCF-7 cells (Fig. 2C–E).

**SAD induced inhibition of tumor growth in experimental mice**

SAD was evaluated for its efficacy in the inhibition of tumor growth in solid tumor models of mice (Fig. 4F). When
administered i.p. at doses of 10 and 20 mpk body weight daily for 90 days, SAD produced a significant tumor growth inhibition of 44% in an Ehrlich tumor (solid) mouse tumor model (Fig. 2F). Interestingly, treatment of SAD at all experimental doses was nontoxic in solid tumor model animals as no mortality (0/7) occurred during the course of the experiment.

SAD inhibits Akt/mTOR/p70S6K and VEGF-regulated angiogenesis signaling cascade

Akt/mTOR serves as a central regulator of endothelial and cancer cell metabolism. SAD inhibited the Akt/mTOR signaling cascade in both HUVEC and MCF-7 cells, which was evident through both immunoblot and immunofluorescence (Fig. 3A and B). SAD drastically inhibited the expression of ubiquitination factors Skp-1, Skp-2, and UBC-3B; however, the inhibition was found to be at translational level (Fig. 3D). SAD significantly inhibited mTOR-activated downstream targets such as p70S6K, 4EBP1, and HIF1α (Fig. 3A), which are crucial to the regulation of protein synthesis and angiogenesis (26). HIF1α induced VEGF expression, which promotes endothelial nitric oxide synthase (eNOS) expression, which plays a critical role in VEGF-induced angiogenesis and vascular hyperpermeability (27). SAD inhibited VEGF-1, -2, and eNOS expression in both cell lines that resulted in repression of VEGF-induced angiogenesis and vascular permeability (Fig. 3A). We further assessed the time-dependent inhibition of the Akt/VEGF signaling cascade, which confirmed that SAD first targets the VEGF signaling (Fig. 3C). SAD significantly inhibited the HIF1α and VEGF expression at both transcriptional and translational levels in MCF-7 cells (Fig. 3A and D). The protective effect of different Akt inhibitors (perifosine and GSK69069) and the induction of HUVECs and MCF-7 cell death (Fig. 3E and F) by neutralizing VEGF antibodies on the SAD further validated our hypothesis. SAD at 50 μmol/L induced 59%
and 72% of cell death, which was significantly reverted by the pretreatment of the Akt inhibitor GSK69069 (500 nmol/L), up to 27% and 18%, in MCF-7 and HUVEC, respectively (Fig. 3F). We obtained the same result with other inhibitors such as rapamycin (mTOR) and sunitinib (VEGF; data not shown). We also obtained the same protective effect for the key targeted proteins (VEGFR2, Akt, mTOR, and p70S6K) via VEGF-neutralizing antibody (Fig. 3A), which is collectively linked with tumor angiogenesis and the Akt/mTOR pathway (24). SAD inhibits CoCl₂-induced hypoxia-mediated angiogenic factors

We further investigated the effect of SAD on the outcome of proangiogenic environment, such as hypoxia on the Akt/mTOR/p70S6K and VEGF signaling cascade. We pretreated the cells with CoCl₂, which was widely used for the induction of hypoxia-induced angiogenesis (28, 29). The expression level of angiogenic factors such as HIFα, HIFβ, VEGFR2, p-VEGFR2, and all the key proteins of Akt/mTOR signaling cascade was significantly enhanced in CoCl₂-pretreated samples as compared with untreated cells (Fig. 4A). The HIFα and p-VEGFR2 expression, a critical target of antiangiogenic drugs, was completely diminished by SAD after 24-hour treatment in MCF-7 cells (Fig. 4A). Interestingly, SAD also inhibited the CoCl₂-induced elevated level of the Akt/mTOR/p70S6K signaling cascade as well as levels of MMP-9 and MMP-2 (Fig. 4A and B). Therefore, SAD inhibits the VEGF-linked Akt/mTOR/p70S6K signaling cascade in both normoxic and hypoxic conditions.

SAD induced G₁ arrest and distorted key cell-cycle–regulated proteins

The Akt/mTOR pathway regulates the cell cycle and cell proliferation through CDK inhibitors p21/p27, cyclin D1, and p53 levels (30). When exposed to SAD, both the cell lines showed a chalk and cheese-like retort in cell-cycle distribution. SAD significantly induced concentration-independent G₁ arrests in MCF-7 cells (Fig. 5A). The case in HUVEC was different; here SAD significantly increased the sub-G₁ DNA fraction (≤2n DNA, apoptotic population; Fig. 5A). SAD altered the expression of cell-cycle–regulated key proteins such as cyclin D1, E, A, c-Myc, p-Rb, p53, p21, and p27 (Fig. 5B). These proteins regulate the G₁/S phase transition, which represses the transcription of genes that govern cell-cycle progression (31, 32). Simultaneously, SAD also inhibited the colony formation of MCF-7 cells (Fig. 5C).

SAD induced Akt/mTOR-mediated morphologic amendment, mitochondrial dysfunction with concomitant apoptotic signaling cascade

The Akt/mTOR pathway has a significant role in induction of apoptosis (30). SAD significantly induced the apoptosis in both HUVEC and MCF-7 cells (Fig. 6A and B). The sensitivity, rate, and the extent of apoptosis were 33% higher in HUVEC than in MCF-7 cells (Fig. 6C). SAD robustly induced loss of mitochondrial membrane potential in both cells, and the lethality of SAD on mito-dysfunction was more pronounced in HUVEC than in MCF-7 cells (Fig. 6D). SAD significantly inhibited the Bcl-2/Bax ratio, cytochrome c, c-IAP-1, survivin, pro–caspase-9, -3, -8, and PARP cleavage (Fig. 6E and F). These data suggest that SAD induced apoptosis by both extrinsic and intrinsic pathways as evidenced by a decrease in pro–caspase-8 and -9.

Discussion

It is estimated that more than 90% of cancer deaths occur due to angiogenesis, invasion, and metastasis of cancer to vital organs. Angiogenesis is one of the key processes that mediates metastasis via HIF1α/VEGF signals and the Akt/mTOR signaling cascade (33). Several angiogenesis inhibitors are used clinically; however,
novel inhibitors still need to be explored from renewable and infinite sources, such as endophyte. In that regard, we have, for the first time, isolated SAD from a novel source of endophytic fungus (*P. oxalicum*) of *C. roseus* and explored its anticancer and anti-angiogenesis potential. Previous studies have shown the cytotoxic potential of SAD in human leukemia cells (11). However, its antiangiogenesis potential and effect on the Akt/mTOR/p70S6K signaling cascade remained to be unexplored. Angiogenesis and apoptosis are two universal hallmarks of cancer and play a very critical role in breast cancer treatment and management; therefore, we have explored the effect of SAD on these two events. The isolated SAD simultaneously targets multiple cancer and angiogenesis dynamics, such as proliferation, migration, growth factors signaling cascade, cell cycle, and apoptosis. SAD significantly hampered HIF1α/VEGF-mediated *in vitro/*ex vivo/*in vivo* angiogenesis, suggesting that SAD exaggerated angiogenesis by targeting manifold aspects of endothelial cells. Endothelial cell migration and tubular formation are necessary for invasion and metastasis (34). Moreover, SAD was found to be pharmacologically active through both oral and i.p. routes of drug administration, as evident in the Matrigel plug assay. Interestingly, SAD-treated C57/BL6J mice did not show any toxic effects such as loss of body weight and mortality (data not shown) at experimental doses. Furthermore, the SAD IC50 value in normal cell was 13 times...
higher than in breast cancer MCF-7 cells. These results suggested that SAD inhibited VEGF-mediated angiogenesis, with no toxic effects.

Importantly, our study is the first to explore the effect of SAD on the Akt/mTOR/p70S6K pathway. We found that SAD consistently condenses signaling from Akt and mTOR and stands in a central position on the crossroad of various cell signal pathways (Ras, PI3-K/Akt, VEGF, HIF; ref. 26). Overactivation of mTOR downstream p70S6K and 4E-BP1 is frequently associated with activation of HIF, which regulates tumor genesis, angiogenesis, and tumor growth through VEGF (27, 35). VEGF, which is a ligand for VEGFR1 and R2, is the most potent angiogenic factor to date and plays a major role in tumor and hypoxia-induced angiogenesis.

Commencement of the VEGF/VEGF-receptor (VEGFR) axis triggers multiple signaling networks that result in endothelial cell survival, mitogenesis, migration, differentiation, vascular permeabilization, and mobilization. Binding to VEGF receptor-2 (VEGFR-2) initiates a tyrosine kinase signaling cascade that stimulates the production of factors that variously stimulate vessel permeability (eNOS), proliferation/survival (bFGF), migration (MMPs), and finally differentiation into mature blood vessels. Various anti-VEGF/VEGFR receptor therapies potently inhibit angiogenesis and tumor growth in preclinical models. Therefore, the VEGF/VEGFR pathway has been a major focus of basic research and drug development in the field of oncology. Consequently, SAD also inhibits ERK phosphorylation, eNOS inhibition,

Figure 6.
Apoptotic potential of SAD in HUVEC and MCF-7 cells. A and B, effect of SAD on cellular and nuclear morphology. Condensed nuclei and apoptotic bodies are indicated by white arrows. C, flow cytometric analysis of apoptosis and necrosis by the Annexin V assay. D, influence of SAD on the mitochondrial membrane potential (Δmit) and apoptotic proteins. SAD induced loss of mitochondrial membrane potential (Δmt), determined by Rhodamine-123 staining. Data are representative of one of three similar experiments. E, effect of SAD on the expression of mitochondrial-dependent apoptotic proteins. F, effect of SAD on the Bcl-2/Bax ratio. Data, mean ± SD from three similar experiments. *, P < 0.001, compared with untreated control.
and MMP degradation, which are in addition a fundamental aspect for regulating angiogenesis (24). Further, we also tried to explore the implication of the CoCl₂-induced angiogenic microenvironment on the Akt/mTOR and VEGF signaling cascade. HIF1α is a key regulatory protein in hypoxic response, which is downstream of mTOR signaling and is an important mediator of VEGF (27). SAD inhibits the elevated level of angiogenic and Akt/mTOR/p70S6K signaling cascade under both normoxic and hypoxic conditions. We have validated the above hypothesis by means of the protective effect of different Akt inhibitors, such as perifosine and GSK69069, and the induction of HUVECs and MCF-7 cell death by neutralizing VEGF antibodies on the SAD. All these experiments prove that SAD induced VEGF-mediated angiogenesis by regulating the Akt/mTOR signaling cascade.

The Akt/mTOR pathway can induce cell-cycle progression by modulating the protein stability of cyclin D, A, E, CDK-2/4, p21, and p27 (31). SAD significantly inhibits these cyclins as well as Skp2, an oncogene, which targets cell-cycle control elements, such as p21 and p27 (36). In addition, Skp2 inactivation profoundly restricts cancer development by triggering a massive cellular senescence, and/or apoptosis response that is surprisingly observed only in oncogenic conditions (36, 37). We further explored the final mode of cell death and SAD-induced apoptosis, which is a hallmark of cancer (2). The rate and extent of apoptosis was much higher in HUVEC than in MCF-7 cells. Apoptosis can be triggered by various stimuli by extrinsic or intrinsic pathways. Extrinsic pathway involved the signal transduction from death receptors and caspase-8, whereas the intrinsic apoptotic pathway involved mitochondrial apoptotic proteins (Bcl-2, Cyt c, and Bax), which are activated downstream of mitochondrial proapoptotic events (17). The early event that was responsible for SAD-induced apoptosis was found to be the loss of mitochondrial potential that might be linked to the drastic reduction in the Bcl-2/Bax ratio. Subsequently, SAD treatment also induced caspase-8, which was part of the extrinsic apoptosis pathway. Therefore, these findings suggest that SAD caused induction of apoptosis through both intrinsic and extrinsic apoptotic pathways in MCF-7 and HUVECs.

However, the most predominant apoptotic pathway induced by SAD was intrinsic or mitochondrial dependent. In conclusion, our results are first to show that SAD inhibits HIF1α/VEGF-mediated angiogenesis by regulating the Akt/mTOR/p70S6K signaling pathway in HUVEC and MCF-7 cells (Supplementary Fig. S2). Hence, our discovery of this novel mechanism of SAD not only gives further insights into the antiangiogenesis and anticancer potential of SAD but also contributes to the role of endophyte in novel drug discovery and developments against diseases associated with angiogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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