Secalonic acid-D represses HIF-1α/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 anti-angiogenic antitumor activity. SAD inhibited multiple HIF-1α/VEGF-arbitrated angiogenesis dynamics as scored in human umbilical vascular endothelial cells (HUVEC) 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 pro-angiogenesis factors including HIF-1α, VEGFR and MMP-2/9. These effects were reversed by co-treatment with the Akt inhibitors perifosine and GSK69069 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 anti-angiogenic activity of a novel mycotoxin, with potential application as a cancer-selective therapeutic agent.

Keywords: Secalonic acid-D; endophyte; angiogenesis; Akt/mTOR/p70S6K; HIF-1α/VEGF

Abbreviations: SAD, Secalonic acid-D; HUVEC, Human umbilical vascular endothelial cells; VEGF, Vascular endothelial growth factor; HIF-1α, Hypoxia-inducible factor-1α; MMP, Matrix metalloproteinase; mTOR, Mammalian target of rapamycin
Introduction

Cancer is a foremost assassin disease all over the world and around six million new cases are reported every year (1). Angiogenesis is one of the common hallmark manifestations of all cancers and it is an elementary event in the development of tumor growth and malignancy (2,3). Currently, several anti-angiogenesis drugs have been approved by the FDA that target VEGF, MMP, HIF-α etc. They may be useful as an appendage to chemotherapy, radiation, and surgical therapy and also useful for non-neoplastic diseases associated with angiogenesis. Therefore, angiogenesis inhibitors act as a dual edge sword that target angiogenesis and potentiate the usefulness of chemotherapeutic drugs (3). Anti-angiogenesis 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, anti-angiogenic drugs are highly selective toward tumor vascular lining and make it an attractive target for cancer therapy (5). There are numerous growth factors that promote tumor angiogenesis, among them vascular endothelial growth factor (VEGF) family of proteins plays a pivotal role in normal and pathological angiogenesis. The activation of Akt/mTOR/p70S6K mediated HIF-1α/VEGF-receptor (VEGFR) alliance trigger endothelial and cancer cell functions toward protein synthesis, tumor genesis, angiogenesis, metastasis, migration, proliferation and apoptosis (6,7). Consequently, discovery of novel HIF-1α/VEGF and Akt/mTOR/p70S6k pathway inhibitors are great magnetism in anticancer therapeutics.

Nature is an attractive source of novel therapeutic agents and plants play an important role in discovery and development of several clinically approved anti-cancer 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 like 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. Secalonic acid D (SAD), a mycotoxin was initially isolated from several foods intuitive fungi but till date there was no report of its isolation from endophytic (Penicillium oxalicum) of Catharanthus roseus (10). SAD has several biological activities such as mycotoxin, protein kinase A,-C inhibitor, cytotoxic and K562 cell cycle inhibitor (11). Conversely, there was no testimony on its anti-angiogenesis potential and effect on Akt/ mTOR/p70S6K signalling cascade. Here, we
report first time the repression of VEGF induces in-vitro, ex-vivo and in-vivo angiogenesis and Akt/mTOR/ p70S6k signaling cascade by SAD under both normoxic and hypoxic condition in Human umbilical vascular endothelial cells (HUVEC) and human breast cancer MCF-7 cells.

Materials and Methods

Isolation of SAD

Secalonic acid-D was isolated from Penicillium oxalicum, an endophytic fungus of Catharanthus roseus. The pure endophytic fungal isolate (EF-VR2) was isolated from the roots of Catharanthus roseus (Family: Apocynaceae) (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 software MEGA5, subsequently analyzed for evolutionary distances by the neighbor joining method (14). The contiguous rDNA sequences of the representative isolate was submitted to 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 NCI protocol. The organic extract was then subjected to column chromatography to obtain pure secalonic acid-D (SAD). The compound was characterized by the various spectroscopic methods viz., 1H and 13C NMR, HRMS, optical rotation and IR and was found to be in accordance to the literature (16).

Cell culture, animal, and reagents

Human umbilical vein endothelial cells (HUVEC), breast cancer MCF-7 and MDA-MB-231, prostate cancer PC-3, pancreatic cancer LNCaP and human breast epithelial fR2 cells were obtained from Sigma Aldrich India (ECACC). The HUVEC were grown in complete EndoGRO-LS media with VEGF (#SCME001, Millipore) and other remaining cells were grown in minimal essential medium (MEM) with 10% fetal bovine serum and 20ng/ml VEGF supplement only for MCF-7 cells. All the cells were cultured at 37°C with 95% humidity and 5% CO2 gas environment. All animal experiments were approved by the animal ethics committee of Indian Institute of Integrative Medicine, CSIR, Jammu, India (IEAC NO. 34/8/14). Animals euthanasia were performed by carbon dioxide inhalation (10-30% volume/min) in matrigel plug assay and cervical dislocation for EAT tumor model and aortic ring assays. The primary and secondary antibodies were purchased from Santa Cruz biotechnology, cell signalling technology and BD biosciences, USA (Table S1). The matrigel and in vitro angiogenesis assay kits were
procured from Millipore (India) Pvt. Ltd. All other bio-chemicals and reagents used in this study were AR grade and purchased from Sigma Aldrich, India.

Cell proliferation assay
The HUVEC, MCF-7, MDA-MB-231, PC-3, LNCaP and fR2 cells at around 70% confluence were treated with SAD at various concentrations from 1-100 µM for 6, 12, 24 and 48 h. MTT dye (2.5 mg/ml in PBS) was added 4 hours prior to the experiment termination. MTT formazan crystals were dissolved in 150 µL of DMSO and absorbance was measured at 570 nm with reference wavelength 620 nm (17).

Cellular and nuclear morphology
The HUVEC and MCF-7 cells were treated with SAD at 10-50 µM concentrations for 24h and observed under microscope for any morphological changes that occur during apoptosis. Simultaneously, the nuclear morphology was analysed through Hoechst staining as described earlier (18).

AnnexinV-FITC assay
HUVEC and MCF-7 cells were treated with SAD at 10-50 µM concentrations for 24h. The cells were collected at 400×g, and stained with annexinV-FITC antibody as per instructions provided with the apoptosis detection kit (Santa Cruz Biotechnology) (17).

Loss of mitochondrial membrane potential (Ψmt)
HUVEC and MCF-7 cells were exposed to 10, 30 and 50 µM concentrations of SAD for 24 h. Rhodamine-123 (200 nM) was added 40min 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 µM concentrations for 6-24 h in the presence and absence of VEGF (20ng/ml) and Cocl2 (100µM). The whole cells, cytosolic and mitochondrial fractions were prepared as describe earlier (17). An equal amount of protein (40-70 µg) was subjected to SDS-PAGE analysis and transferred to PVDF membrane. The membrane was blocked with 5% non-fat milk-TBS or 3% BSA-TBS (for phospholyrated antibodies) and probed with respective primary and secondary antibodies mentioned in Table S1.

Cell cycle analysis
The breast cancer MCF-7 cells and HUVEC were treated with SAD at 10, 30, and 50 µM concentrations for 24 h in the presence of VEGF (20ng/ml). The cell cycle was analysed on flow cytometer as described earlier (19).
Colony formation assay

The MCF-7 cells were treated with different concentration of SAD (10, 30 and 50µM) for 24 h in the presence of VEGF (20ng/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/ well. Cells were treated with different concentration of SAD (10-50µM) for 24h, 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 µM) for 24h. 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 RevertAid 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 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 h and the culture medium was electrophoresed (80V for 120 min) on 8% SDS-PAGE gel containing 0.1% gelatin. The gel were assess 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 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 provide by in vitro angiogenesis assay kit (Millipore, Cat No-ECM 625). Briefly, HUVECs were seeded on the surface of the polymerized EC Matrix and incubate at 37°C for 12h. The cells were treated with different concentration of SAD for 24h and capillary-like tube sprouting was observed under an inverted microscope at 10Xmagnification.
**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 concentration of SAD for 24h. The cell migration and invasion percentage was calculated as per the protocol of the respective kits (Millipore, USA; Cat No-ECM509 and ECM554).

**Aortic ring assay**

The aortas isolated from Sprague Dawley rats were cleaned and cut into rings of 1 to 1.5mm circumference. The aortic rings were randomized into matrigel coated wells and sealed with a 100μl overlay of Matrigel (23). The aortic rings were cultured in EndoGRO LS Media with and without 20ng/ml of VEGF for 4 days. On day 5th, various concentration of SAD (10-30μM), were added to the wells and further incubated for 96h. On day 9th, the micro vessel sprouting was fixed and photographed. The aortic ring tissue was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 5μm and stained with H&E (haematoxylin and eosin).

**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 (0.5x10⁶), was injected sub-cutaneously into the ventral area of 4-6 week old C57/BL6J mice (IIIM, central animal house). Animals were dosed daily with SAD (10 & 20 mg/Kg b.w; oral/i.p) from second to sixth day. On the day seventh, animals were sacrificed to remove intact matrigel plugs with different treatments and photographed that show the extent of vascularization. The neovascularisation of matrigel plugs was quantified by measuring their size and Hb content through Drabkin’s reagent.

**Ehrlich Tumor (ET) Solid**

Ehrlich Ascites Carcinoma (EAC) cells (1x10⁷) were collected from 8-10 day old Swiss albino mice and were injected (i.m.) in right thigh on day 0. On day 1, the animals were randomized and divided into different groups. SAD was given intraperitoneally at 10 mpk and 20 mpk of body weight from day 1 to day 9. Similarly, Control group received vehicle, whilst 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 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 intra group using one way and two ways ANOVA with post Bonferroni test through GraphPad Prism 5.00.288 statistical analysis software.

**Result**

**Isolation, characterisation and cytotoxic profile of SAD**

Scalonic acid-D (SAD) was isolated from the pure endophytic fungal isolate (EF-VR2) of *Penicillium oxalicum* from *Catharanthus roseus*. The morphology and sequencing of *Penicillium oxalicum*, endophyte fungi isolate (EF-VR2) was characterized with a neighbor-joining rooted tree (Fig. 1S A-E). SAD inhibits cell proliferation of HUVEC and different cancer cells in a dose and time dependent manner (Fig.1S F). SAD was found to be non-toxic at the experimental concentration in normal human breast epithelial FR2 cells, where the IC50 value was to 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 dependant manner and decrease wound closure percentage from 100% to 10% at 50 µM concentrations (Fig. 1A and 1B). To further assess the effect of SAD on angiogenesis, we examined how SAD regulates capillary tube formation of endothelial cells. HUVEC showed robust tubular like structures when seeded on two-dimensional 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 1D).

*SAD inhibits chemotaxis cell migration and invasion*

Boyden Chamber assays enable to simulate 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 dependant manner. The migration inhibition percentage was reduce from 100% to 10% at 50 µM concentration of SAD, whereas the invasion inhibition percentage was reduce from 100% to 30% in both experimental cell lines (Fig. 1E and 1F).
**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 to traditional cell-based assays. VEGF (20 ng/mL) significantly stimulated microvessel sprouting, and this effect was completely inhibits by SAD in a dose-dependent manner (Fig. 1G). The histological analysis of rat aortic ring further reveals that SAD inhibits the micro-vessel sprouting in 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 intraperitoneally with SAD (10-20mg/kg/b.w.) 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/ b.w) show a complete reduction in the degree of vascularization and size and appear as the white color appearance and negligible Hb content (Fig. 2B) indicating that SAD significantly inhibited tumor angiogenesis. We also got the same results when animals were treated with SAD at 10-20mg/kg/b.w intraperitoneally in the presence and absence of human breast cancer MCF-7 cells (Fig. 2C, 2D, 2E).

**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 mouse (Fig.4F). SAD when administered intraperitoneally at doses of 10 and 20 mpk body weight daily for nine days produced a significant tumor growth inhibition of 44% in ET (solid) mouse tumor model (Fig.2F). SAD inhibits the tumor growth in a dose dependant manner. Interestingly, treatment of SAD at all experimental doses was non toxic 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 signalling cascade**

Akt/mTOR serves as a central regulator of endothelial and cancer cell metabolism. SAD inhibits Akt/mTOR signalling cascade in both HUVEC and MCF-7 cells, which was evident through both immunoblot and immunofluorescence (Fig. 3A and 3B). SAD drastically inhibits the expression of ubiquitation factor Skp-1, Skp-2 and UBC-3B, however the inhibition was found to be at translational level (Fig. 3D). SAD significantly inhibits mTOR activated downstream targets such as p70S6K, 4EBP1 and HIF-1α (Fig. 3A), which are crucial to the
regulation of protein synthesis and angiogenesis (26). Hypoxia-inducible factor (HIF-1α) induced VEGF expression, which promotes endothelial nitric oxide synthase (eNOS) expression that plays a critical role in VEGF-induced angiogenesis and vascular hyper permeability (27). SAD inhibits VEGF-1,-2 and eNOS expression in both cell lines that results in repressing VEGF-induced angiogenesis and vascular permeability (Fig. 3A). We further assess the time dependent inhibition of Akt/ VEGF signaling cascade, which confirm that SAD first target the VEGFR signaling (Fig. 3C). SAD significantly inhibits the HIF-1α and VEGF expression at both transcriptional and translational level in MCF-7 cells (Fig. 3A and 3D). We further validate our hypothesis by means of protective effect of different Akt inhibitors, perifosine, GSK69069 and neutralizing VEGF antibodies on the SAD induces HUVECs and MCF-7 cell death (Fig. 3E, 3F). SAD at 50 µM concentration induces 59% and 72% cells death, which was significantly revert back by pretreatment of Akt inhibitor, GSK69069 (500nM), up to 27% and 18%, in MCF-7 and HUVEC, respectively (Fig. 3F). We were got the same result with other inhibitors like rapamycin (mTOR) and sunitinib (VEGF) (data not shown). We also got the same protective effect of the key targeted proteins (VEGFR2, Akt, mTOR,p70S6K) via VEGF neutralizing antibody (Fig. 3E). All these experiments prove that SAD induced VEGF arbitrated angiogenesis via Akt/mTOR signaling cascade. Interestingly, SAD also inhibits the expression of ERK, IGFR and EGFR (Fig. 3A), that collectively linked with tumor angiogenesis and Akt/mTOR pathway (24).

**SAD inhibits CoCl₂ induced hypoxia mediated angiogenic factors**

We further investigate the effect of SAD on the outcome of pro-angiogenic environment such as hypoxia on 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 were significantly enhanced in CoCl₂ pretreated samples as compared to untreated cells (Fig. 4A). The HIFα and p-VEGFR2 expression, a critical target of anti angiogenic drugs, was completely diminished by SAD after 24 h treatment in MCF-7 cells (Fig. 4A). Interestingly, SAD also inhibits the CoCl₂ induced elevated level of Akt/mTOR/p70S6K signaling cascade as well as levels of MMP-9 and MMP-2 (Fig. 4A and 4B). Therefore SAD inhibit 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**

Akt/mTOR pathway regulates cell cycle and cell proliferation through CDK inhibitors p21/p27, cyclin D1 and p53 levels (30). Both the cell lines when exposed to SAD showed chalk and cheese like retort in cell cycle distribution.
SAD significantly induces concentration independent G1 arrests in MCF-7 cells (Fig. 5A). The story in HUVEC was different, here SAD significantly increase the subG0 DNA fraction (<2n DNA, apoptotic population) (Fig. 5A). SAD alters the expression of cell cycle regulated key proteins like 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 inhibits the colony formation of MCF-7 cells (Fig. 5C).

*SAD induced Akt/mTOR mediated morphological amendment, mitochondrial dysfunction with concomitant apoptotic signaling cascade*

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

**Discussion**

It is estimated that more than 90% of cancer deaths that occur are due to angiogenesis, invasion, and metastasis of cancer to vital organs. Angiogenesis is one of the key processes that mediate metastasis via HIF-1α/VEGF signals and Akt/mTOR signaling cascade (33). There are several angiogenesis inhibitors that are used clinically however novel ones still need to be explored from renewable and infinite sources, like endophyte. In that concern, we have for the first time isolated secalonic acid-D (SAD) from a novel source of endophytic fungus (*Penicillium oxalicum*) of *Catharanthus roseus* and explored its anti-cancer and anti-angiogenesis potential. The previous studies have shown the cytotoxic potential of SAD in human leukaemia cells (11). But its anti-angiogenesis potential and effect on Akt/mTOR/p70S6K signalling cascade remained to be unexplored. The 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 HIF-1α/VEGF mediated *in-vitro/ex-vivo/in-vivo* angiogenesis, suggesting that SAD exaggerated angiogenesis by targeting manifold aspects of endothelial cells. The 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 intraperitoneal route 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, 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 Akt/mTOR/p70S6K pathway. We found that SAD consistently condenses signaling from Akt and mTOR; that stands in a central position on the crossroad of various cell signal pathways (Ras, PI3-K/Akt, VEGF, HIF) (26). Over activation of mTOR downstream p70S6K and 4E-BP1 is frequently associated with activation of hypoxia inducing factor (HIF), which regulates tumor genesis, angiogenesis and tumor growth through VEGF (27,35). The vascular endothelial growth factor (VEGF), which is a ligand for VEGFR1 and R2 is the most potent angiogenic factor till date and plays a major role in tumour and hypoxia induced angiogenesis. Commencement of the VEGF/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) initiate 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. There are various anti-VEGF/VEGFR therapies that potently inhibit angiogenesis and tumor growth in preclinical models. Therefore, VEGF/VEGFR pathway has been a major focus of basic research and drug development in the field of oncology. Consecutively, SAD also inhibits ERK phosphorylation, eNOS inhibition and MMP degradation which are in addition a fundamental aspect for regulating the angiogenesis (24). Further, we also tried to explore the implication of the CoCl2 induces angiogenic microenvironment on Akt/mTOR and VEGF signaling cascade. HIF-1α is a key regulatory protein in hypoxic response, which is downstream of mTOR signaling and 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 above hypothesis by means of protective effect of different Akt inhibitors, perifosine, GSK69069 and neutralizing VEGF antibodies on the SAD induces HUVECs and MCF-7 cell death. All these experiments prove that SAD induced VEGF mediated angiogenesis by regulating the Akt/mTOR signaling cascade.
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). By extension of this fact, 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 cells death and SAD induces apoptosis, which is a hallmark of cancer (2). The rate and extent of apoptosis was much higher in HUVEC in comparison to MCF-7 cell. Apoptosis can be triggered by various stimuli by extrinsic or intrinsic pathways. Extrinsic pathway involved the signal transduction from death receptors and caspase-8 while the intrinsic apoptotic pathway involves mitochondrial apoptotic proteins (Bcl-2, Cyt c, Bax), which are activated downstream of mitochondrial pro-apoptotic events (17). The early event which was responsible for SAD induces apoptosis, found to be loss of mitochondrial potential that might be linked with the drastic reduction in Bcl-2/Bax ratio. Subsequently, SAD treatments also induce caspase-8, which was part of the extrinsic apoptosis pathway. So 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 pathways induce by SAD was intrinsic or mitochondrial dependant. In conclusion our results are first to show that SAD inhibits HIF-1α/VEGF mediated angiogenesis by regulating the Akt/mTOR/p70S6K signaling pathway in HUVEC and MCF-7 cells (Fig. S2). Hence, our discovery of this novel mechanism of SAD not only gives further insights into the anti-angiogenesis and anti-cancer potential of SAD but also contributes the role of endophyte in novel drug discovery and developments against diseases associated with angiogenesis.

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References


**Legend for Figures**

**Figure 1:** Effect of SAD on VEGF mediated angiogenesis. (A-B) SAD inhibits HUVEC migration in the wound healing assay. (C-D) SAD inhibits tube sprouting of HUVEC cells in the tube formation assay. The tube formation was observed as described in Materials and Methods. (E-F) SAD inhibits chemotaxis cell migration and invasion. MCF-7 and HUVECs were incubated in the invasion and migration chamber, based on the Boyden chamber principle. Data were mean ± S.D. of three independent experiments and p-value: *<0.001 compared to untreated control considered significant. (G) Effect of SAD on VEGF arbitrated *ex vivo* angiogenesis. Aortic segments were harvested from Sprague-Dawley rat as described in Materials and Methods. (H) Histological analysis of rat aortic ring was performed as described in Materials & Methods. Data are representative of one of three similar experiments.
**Figure 2:** Effect of SAD on VEGF arbitrated *in vivo* angiogenesis. (A-D) Matrigel plug assay was performed as described in Materials & Methods. SAD inhibits VEGF-induced *in vivo* angiogenesis in C57/BL6J mice. (E) MCF-7 cells (0.5x10^6) were injected into C57/BL6J mice along with the matrigel (0.5 ml), other condition were remained the same as above. The data are presented as mean ± S.D. of three independent experiments and p-value: *<0.001 (Hb) or @<0.001 (size) compared to VEGF treated control considered significant. (F) *In-vivo* antitumor activity of SAD in ehrlich tumor (ET) solid mouse models. The antitumor potential of SAD was evaluated as described in Material & Methods. Data are mean ± SE (n = 7) and p-values *<0.001 were considered significant.

**Figure 3:** Effect of SAD on the PI-3K/Akt/mTOR pathway and angiogenesis. (A) MCF-7 and HUVEC cell lysates were prepared after 24h treatment of SAD at indicated concentrations and specific antibodies were used for detection of indicated proteins. (B) Immunofluorescence staining of MCF-7 cells for detection of mTOR localization. (C) Time dependant inhibition ofAkt/VEGFR expression by SAD. (D) RTPCR analysis of SAD targeted proteins. All samples were analysed in triplicate and data were analyse through StepOne software v 2.0. p-value: *<0.001 compared to untreated control. (E) Blocking of circulating VEGF reduces the expression of VEGFR2 and Akt. The MCF-7 cell were treated along with anti-p-VEGFR2 antibody (2 ng/ml) in the presence and absence of VEGF (50 ng/ml) and SAD (30µM) for 24h. The anti-p-VEGFR2 antibody was added 2h before of SAD treatment. (F) Effect of Akt inhibitors on the cell viability of SAD treated MCF-7 cells. All inhibitors were added 1h before of SAD treatment. The cell viability was determined by MTT assay as described in Materials and Methods. Data are Mean ± SD (n= 8 wells) of three similar experiments. p-value: *<0.001 were consider significant.

**Figure 4:** Effect of SAD on the hypoxia linked key angiogenesis protein and enzyme. (A) Hypoxia was induces with cobalt chloride (100µm) and treated with SAD for 24h. Cells were lysed as described in Materials and Methods and immunobloted with indicated antibodies. (B) Effects of SAD on MMP-2 and MMP-9 activities. The conditioned media from above experiment were collected, and MMP activities were determined by gelatin zymography as described in Materials and Methods. Data are Mean ± S.D. of relative density from three similar experiments. p-value: *<0.001(MMP-2) or @<0.001(MMP-9) compared to untreated control.

**Figure 5:** Effect of SAD on cell cycle and colony forming efficiency. (A) MCF-7 and HUVEC cells were stained with propidium iodide and acquire for cell cycle analyses on flow cytometer as described in Materials & Methods. Data are representative of one of three similar experiments. (B) Influence of SAD on the expression of key cell cycle
proteins of MCF-7 cells. (C) SAD inhibits the colony formation of MCF-7 cells. Data are Mean ± S.D. of calculated colonies percentages from three similar experiments. P-value: *<0.001 compared to untreated control.

**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 the apoptotic bodies are indicated by white arrows. (C) Flow cytometric analysis of apoptosis and necrosis by Annexin-V assay. (D) Influence of SAD on mitochondrial membrane potential (Ψmt) and apoptotic proteins. SAD induced loss of mitochondrial membrane potential (Ψmt) 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 are Mean ± S.D. from three similar experiments. p-value: *<0.001 compared to untreated control.
**Fig. 2**

(A) Control vs. VEGF Control

(B) Hb O.D. or matrigel size

(C) Control vs. VEGF Control

(D) Hb O.D. or matrigel size

(E) SAD, mg/kg x 10^6

(F) Tumor growth inhibition %

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Fig. 3
Fig. 4
Fig. 6
Secalonic acid-D represses HIF-1α/VEGF mediated angiogenesis by regulating the Akt/mTOR/p70S6K signaling cascade

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