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# Inhibition of angiogenesis by *S*-adenosylmethionine

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ABSTRACT

Metastasis is a leading cause of mortality and morbidity in cancer. One of the steps in metastasis process is the formation of new blood vessels. Aberrant DNA methylation patterns are common in cancer cells. In recent studies, *S*-adenosylmethionine (SAM), which is a DNA methylating agent, has been found to have inhibitory effects on some carcinoma cells *in vivo* and *in vitro*. In the present study, we have used SAM to investigate whether it is effective against angiogenesis *in vitro*. Our results have shown that SAM can reduce the formation and organization of capillary-like structures of endothelial cells in tumoral environment. Besides, we have found SAM can block endothelial cell proliferation and the migration of cells towards growth factors-rich media. In conclusion, our study suggests that SAM may be used against angiogenesis as a natural bio-product.



## 1. Introduction

Angiogenesis is a complex multi-step process involving extensive interplay between cells, soluble factors, and extracellular matrix components. There are four distinct and sequential steps in angiogenesis: Degradation of basement membrane by proteases; migration of endothelial cells (ECs) into the interstitial space and sprouting; ECs proliferation at the migrating tip; lumen formation, generation of new basement membrane with the recruitment of pericyte, formation of anastomoses and finally blood flow [1].

Angiogenesis is an essential stage in the growth and metastasis of solid tumors. Epigenetically altered genes should be involved in all these processes. Thus the epigenome has been gaining a great attention as a target for anticancer therapy. Reversibility of epigenetic modifications gives scientists an advantage to target the epigenome with drug [2,3].

DNA methyltransferases (DNMTs) catalyzes the transfer of a methyl group from *S*-adenosyl-methionine (SAM) to cytosine residues to form 5-methylcytosine which is found mostly at CpG sites in the genome. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes can block their expression. However, these silenced genes can be reactivated by DNA methylation inhibitors [4], thus terminal differentiation and senescence of neoplastic cells may be induced by demethylation [5].

Growing evidence now indicates that demethylation of some genes (tumor-promoting genes) can support the invasion of cancer cells. Some studies have reported that SAM protected cells from regional hypomethylation as a methyl donor and repressed metastasis-promoting genes [6].

In contrast to the increasing knowledge on epigenetic aberrations in tumor cells, there is almost nothing known about the role of DNA methylation in the regulation of gene expression in ECs. Recently, several studies have shown that DNMT and histone deacetylase (HDAC) inhibitors are potent angiostatic agents that inhibit growth of ECs *in vitro* and *in vivo* [7,8].

We had hypothesized that methylation inhibitors and methylating agents, e.g., SAM, may be used consecutively to ensure genome stability because not only methylation inhibitors but also SAM exhibits anti-proliferative and anti-metastatic characteristics in cancer cells [3]. We have not found any studies in the literature suggesting the effects of SAM on ECs and angiogenesis. Here, it is reported for the first time that SAM may block *in vitro* angiogenesis process in human ECs.

*Abbreviations:* DNMT, DNA methyltransferase; EBM-2, endothelial basal medium; ECs, endothelial cells; EGM-2, endothelial growth medium; FBS, fetal bovine serum; HDAC, histone deacetylase; HUVEC, human umbilical vein endothelial cell; MTT, (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide); SAM, *S*-adenosylmethionine; uPA, urokinase-type plasminogen activator.

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## 2. Materials and methods

### 2.1. Reagents

Human umbilical vein endothelial cells (HUVECs) and Matrigel™ were obtained from BD Biosciences (BD-354234, Two Oak Park, Bedford, MA). EGM-2® BulletKit® (CC-3162, EBM-2 + supplements) for HUVEC culture was purchased from Lonza (Walkersville, MD, USA). *S*-adenosylmethionine and MTT were purchased from Sigma–Aldrich (St. Louis, MO, USA) and AppliChem (AppliChem, Darmstadt, Germany), respectively.

### 2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2-complete medium consisting of EBM-2 basal medium and supplements (ascorbic acid, hydrocortisone, heparin, GA-1000 and 2% FBS, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, insulin-like growth factor). Cell culture flasks or well plates were coated with gelatin (Sigma–Aldrich, Irvine, Ayrshire, UK). Cells were grown in a humidified atmosphere (95% air, 5% $CO_2$ at 37 °C) and passaged every 4–6 days. Cells between the fourth and the sixth passage were used for experiments. SAM was dissolved in growth medium and applied to the cells at 0.5 and 1 mM concentrations. Before treated with SAM, cells in all groups had been serum-starved in EBM-2 medium containing 1% FBS for 24 h.

### 2.3. Cell proliferation assay

The effects of SAM on cell viability were determined by MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) as described previously with some modifications [9]. MTT stock solution (5 mg/ml) was diluted by 10 times with RPMI-1640 without phenol red (Sigma–Aldrich, St. Louis, MO, USA) as working solution. The cells were plated at a density of $\sim 2.5 \times 10^4$ cells/well into 24-well plate. HUVECs were serum-starved for 24 h and then were treated with SAM for 24 h. Medium was exchanged with 500 μl MTT solution and cells were incubated at 37 °C for 3 h. At the end of the incubation period, the converted dye is solubilized with 500 μl acidic isopropanol (0.04 M HCl in absolute isopropanol). Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 650 nm by colorimetric plate reader (Thermo Labsystem, Model No: 1500, Finland). Experiments were repeated four times and the results were expressed as mean ± standard deviation (SD).

### 2.4. In vitro angiogenesis assay

*In vitro* angiogenesis was assayed by using Matrigel™ matrix (BD-354234, Two Oak Park, Bedford, MA). Matrigel™ Basement Membrane Matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. EHS sarcoma is a tumor rich in extracellular matrix proteins including various growth factors e.g., TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator and other growth factors which occur naturally in the EHS tumor.

After thawed at 4 °C, Matrigel™ matrix (250 μl) was quickly added to each well of a 24-well plate and allowed to solidify for 1 h at 37 °C. Serum-starved cells for 24 h were added to each well at a density of $\sim 3 \times 10^4$ cells/well in EGM-2-complete medium. After adhering to the Matrigel, cells were incubated with SAM containing medium at 37 °C for 24 h. Tubular structures of HUVECs were visualized and photographed using an Olympus Photomicroscope (Olympus IX81S1F-2, Japan) with ×4 objective. Tube lengths at three different areas were calculated as pixel values by means of UTHSCSA ImageTool Version 3.0 software program (The University of Texas Health Science Center, San Antonio, Texas). Experiments were repeated three times.

### 2.5. Cell migration assay

Endothelial cell migration was assayed by means of a modified Boyden chamber measuring endothelial cell movement towards chemoattractants (Millipore QCM™ (3 μm) Endothelial Migration Assay Kit, ECM201, Temecula, California). The bottom of the insert is coated with fibronectin, which provide an optimal condition for endothelial cell migration and adhesion. Experiments were carried out as manufacturer's kit assay procedures. Briefly, migrated cells at the bottom of the insert were dyed with CyQUANT® GR fluorescent dye (Millipore, Temecula, California). Green-fluorescent was assayed with a fluorescence plate reader using 480/520 nm filter set (Thermo Fluoroskan Ascent FL, Type: 374, Finland). Results were expressed as mean ± SD of three different experiments.

### 2.6. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Comparison of groups according to the parameters was performed using the student *t*-test in Prism Program of GraphPad Software (San Diego, CA). *p* values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. SAM suppresses endothelial cell proliferation

The percentage of the proliferating cells in groups treated with SAM at 0.5, 1 and 1.5 mM concentrations was found to significantly decrease (86.13 ± 4.67, 54.60 ± 5.57, 33.96 ± 4.49, respectively) (Fig. 1). Results were expressed as the percentage of control cells. Besides, SAM was seen to inhibit the proliferation of cells as a dose

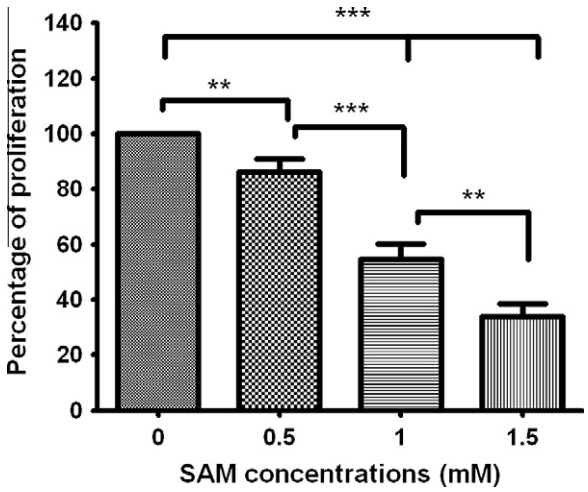


**Fig. 1.** Percentage of endothelial cells proliferation. The cells were plated at $\sim 2.5 \times 10^4$ cells per well. 70–80% confluent HUVECs in 24-well plate were serum-starved for 24 h. After this period, cells in growth medium were treated with SAM. After incubated at 37 °C for 24 h, cells were treated with MTT solution and the converted dye was measured at 570 nm. Values represent means ± SD of four different experiments. ***: $p < 0.001$, **: $p < 0.01$.

dependent manner. The difference between doses was found to be statistically significant (Fig. 1) ($p < 0.01$ and $p < 0.001$).

### 3.2. SAM inhibits the capillary-like tube formation

We found that SAM (0.5 and 1 mM) have significantly suppressed the endothelial tube formations (9177 ± 2120, 6142 ± 588, respectively) on Matrigel matrix as compared with control (15816 ± 1097) (Figs. 2 and 3) ($p < 0.01$). SAM inhibited not only tube lengths but also the number of branch points as a dose dependent manner ($p < 0.05$).

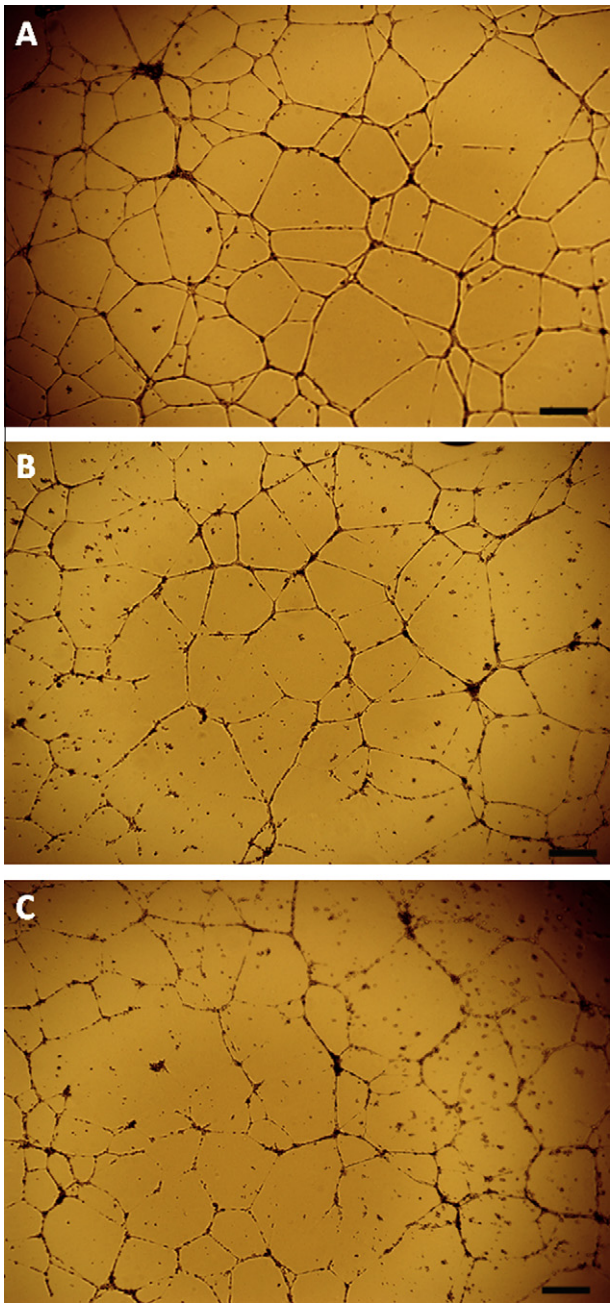


**Fig. 2.** SAM represses tube formation produced by human umbilical vein endothelial cells. Representative microscopy photographs of HUVECs cultured for 24 h on Matrigel in the presence of the indicated compound are illustrated. Serum-starved cells were seeded to each well of 24-well plate as $\sim 2.5 \times 10^4$ cells in EGM-2-complete medium. Pictures were taken at ×4 objective. HUVEC cells were incubated with (A); control (no treated any reagent), (B); SAM (0.5 mM), (C): SAM (1 mM). Scale bar = 250 μm.

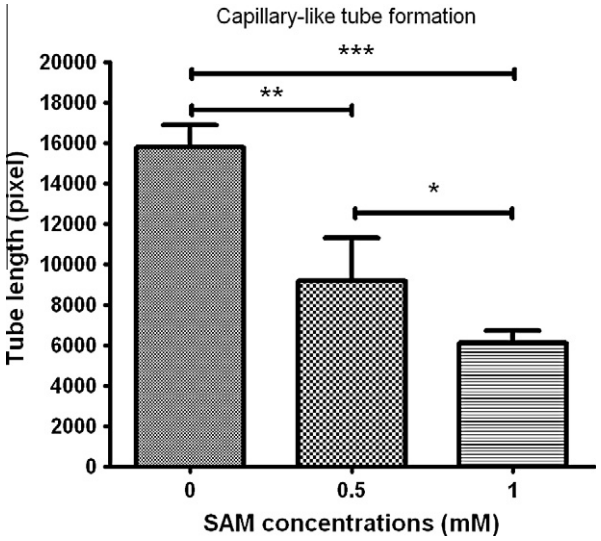


**Fig. 3.** Tube lengths of capillary-like structures. As mentioned in Section 2, lengths in digital photograph were calculated as pixel. Bar graphics of tube lengths represent groups affected by SAM at concentrations 0, 0.5 and 1 mM. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

### 3.3. SAM blocks the endothelial cell migration

In a modified Boyden chamber system SAM at concentrations of 0.5 and 1 mM were found to significantly decrease fluorescence intensity of endothelial cells (15.39 ± 1.92, 7.87 ± 1.72) as compared with controls which were expressed as 100% ($p < 0.001$) (Fig. 4). About 1 mM concentration of SAM was found to be more effective than 0.5 mM in suppression of migration ($p < 0.01$).

## 4. Discussion

Tumor angiogenesis is essential for the growth and metastasis of solid tumors. The angiogenesis cascade starts with the activation of ECs by angiogenic growth factors and continues with extracellular matrix degradation, ECs proliferation, migration and tube formation, and, finally, maturation of the blood vessels [10]. In this

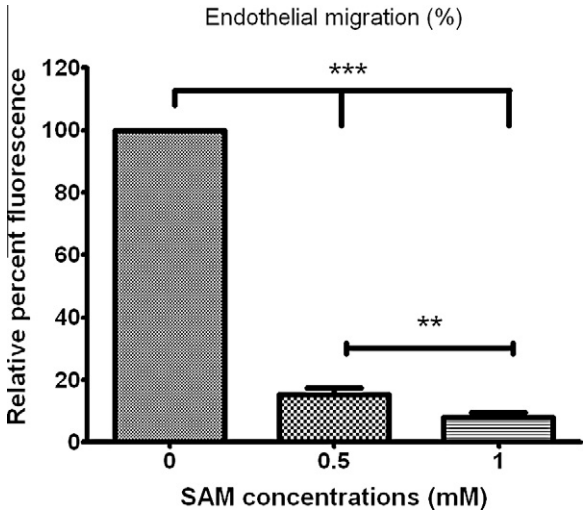


**Fig. 4.** SAM treatment suppresses the migration of endothelial cells in the upper chamber (3 μm) toward the bottom plate containing serum and growth factors. Fluorescent dyed cells were assayed by using fluorescence plate reader as mentioned in Section 2. Data were given as relative percent fluorescence intensity according to controls. ***: $p < 0.001$, **: $p < 0.01$.

study, we have investigated the *in vitro* effects of SAM, which serve as methyl donor in biological systems, on most of these steps.

Angiogenic activation of ECs gives rise to some changes in their gene expression profiles. In fact, very little is known about the role of epigenetic alterations in ECs as compared with that in cancer cells [3].

Exogenous treatment of cancer cells with SAM results in an increase in DNA hypermethylation and inhibits DNA demethylation either by enhancing DNA methyltransferase activity or by inhibiting active demethylation [6,11]. It was reported that uPA gene was silenced in cells treated with SAM because of its promoter hypermethylation, resulting in an inhibition of cancer growth and metastasis [6,12]. In our study, we have observed that SAM inhibited the proliferation of ECs as a dose dependent manner (Fig. 1). Additionally, SAM was demonstrated to dramatically block the endothelial cell migration (Fig. 4). Compared the percent inhibition of migration with that of proliferation, we observed that cell migration was more affected by SAM treatment than cell proliferation. SAM may have stopped one or more migration signaling pathways which are not related with proliferation. Pulukuri et al. [6] reported that SAM-dependent silencing of uPA significantly inhibited *in vitro* tumor cell invasion and *in vivo* tumor growth and metastasis with which our results are consistent.

Several studies have shown that DNMT and HDAC inhibitors are potent angiostatic agents and directly inhibit EC growth and tumor angiogenesis *in vitro* and *in vivo* [7,8,13]. Antiangiogenic therapy of cancers offers the possibility of a low toxicity treatment without acquisition of drug resistance due to the genetic stability and low mutational rate of ECs [13,14]. Thus ECs may be a more suitable target than cancer cells to suppress tumor growth.

Briefly, many studies have focused on the effects of HDAC and DNMT inhibitors on angiogenesis and cancer cells. In the present study, we first demonstrated SAM repressed angiogenesis or capillary formation *in vitro*. SAM significantly decreased both tube lengths and the number of branch points (Figs. 2 and 3). As a conclusion, we have suggested that SAM may be used as a promising antiangiogenic agent in the fight against cancer. However, further studies are needed to explore inhibiting mechanisms of SAM on angiogenesis.

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