Electronic Supplementary Material (ESI) for RSC Advances.

Electronic Supplementary Information

A histone demethylase inhibitor, methylstat, inhibits angiogenesis *in vitro* and *in vivo*

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Materials and methods

Materials

Endothelial basal media-2 (EBM-2) was purchased from Cambrex Bio Science (Walkersville Inc, MD) and Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY). Transwell plates, recombinant human vascular endothelial cell growth factor (VEGF) and Matrigel were obtained from Corning Stars (Cambridge, MA), KOMA Biotech, Inc. (Seoul, Korea), and BD Bioscience (Bedford, MA) respectively. Methylstat was synthesized and characterized the University of Colorado as described previously[1]. p53 and p21 antibodies were purchased from Santa Cruz (Santa Cruz, CA) and cyclinD1 antibody was purchased from Cell Signaling (Berverly, MA). H3K27me3 antibody was purchased from Abcam (Cambridge, MA) and tubulin antibody from Millipore (Billerica, MA).

Cell culture

Early passage (4–8 passages) HUVECs (human umbilical vascular endothelial cells) were grown in EGM-2 supplemented with 10% FBS. HeLa (human cervical carcinoma, purchased from ATCC Cell Lines) cells and CHANG (human liver) cells were grown in DMEM with 10% FBS and 1% antibiotics. HepG2 (human liver carcinoma) cells were grown in RPMI 1640 containing 10% FBS and 1% antibiotics. All cell lines were purchased from ATCC and maintained at 37°C in a humidified incubator with 5% CO_2.

Cell growth and viability assays

HUVECs, CHANG, HeLa, HepG2, were seeded onto 96-well plates, incubated for 24 h, and treated with various concentrations (0, 1, 2, 5 μM) of Methylstat for 72 h. Cells proliferation was measured using a 3-(4,5-dimehylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, Saint Louis, MO) colorimetric assay. Optical density (OD) values were measured at wave length 540 nm. OD values were converted to cell proliferation (%) with non-treated sample as 100%. IC_{50} value was calculated based on measured data. Viability was assessed using trypan blue staining as described previously.[2] Cell viability (%) = the number of treated viable cells / the number of non-treated viable cells X 100.

Chemoinvasion assay

A Transwell chamber system with 8.0 μm pore sized polycarbonate filter inserts was used to examine the *in vitro* invasiveness of HUVECs. The lower side of the filter was coated with 10 μL of gelatin (1 mg/mL), and the upper side was coated with 10 μL of Matrigel (3 mg/mL). Methylstat was added to the lower chamber in the presence of VEGF (50 ng/mL), and HUVECs (7×10^5 cells/well) were placed in the upper part of the filter. The chamber was incubated at 37°C for 16 h. The invasiveness of cells, fixed with 70% methanol and stained with hematoxylin and eosin, was measured by counting the total number of cells in the lower

side of the filter. Cells were observed under a microscope (IX71, Olympus) and photographed at 100 ×magnification (DP70, Olympus).

Capillary tube formation assay

Capillary tube formation of endothelial cells *in vitro* was assessed as described previously[3]. Matrigel (150 μL, 10 mg/mL) was layered on a 48-well culture plate and allowed to polymerize for 1 h at 37°C. HUVECs (6×10^4 cells/well) were seeded on the Matrigel surface and treated with VEGF (50 ng/mL). Then, methylstat was added for 3–16 h at 37°C. Morphological changes of cells and tube formation were observed under a microscope (IX71, Olympus) and photographed at 100 ×magnification (DP70, Olympus).

Chorioallantoic membrane (CAM) assay

The CAM assay was performed as described previously[4]. Fertilized chicken eggs were kept in a humidified incubator at 37°C for 4 days. Approximately 4-5 mL of egg albumin was removed with a hypodermic needle, allowing the CAM and yolk sac to drop away from the shell membrane. On day 5, the shell membrane was peeled away, and then compound-loaded Thermanox coverslips (NUNC, Rochester, NY) were applied to the CAM surfaces. Two days later, 1 mL of Intralipose (Greencross Company, Korea) was injected beneath the CAM, and the membrane was observed under a microscope. Inhibition ratio was calculated as follows: Inhibition ratio (%) =the number of angiogenesis inhibited eggs by the compound / the number of total eggs X 100 (%). Retinoic acid (RA), an anti-angiogenic compound, was used as a positive control.

Western blot analysis

The cell lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) using standard electroblotting procedures. Blots were then blocked and immunolabeled overnight at 4°C with primary antibodies, including anti-p53 (1:2000), anti-p21 (1:2000) and anti-cyclinD1 (1:1000), anti-H3K27me3 (1:1000), anti-tubulin (1:2000) antibodies. Immunolabeling was detected with an enhanced chemiluminescence (ECL) kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

Cell cycle analysis by fluorescence-activated cell sorting (FACS)

HUVECs (2.5×10^5 cells) were seeded in 60-mm dishes, grown for 48 h, and exposed to serum-restricted conditions (0.5% serum) for 16 h to induce synchronization. The cells were treated with Methylstat for 12, 24, or 48 h in the presence of 10% serum[5]. Cells were harvested, fixed in 70% ethanol, resuspended in PBS (pH 7.4), and treated with RNase (80 μg/mL) and propidium iodide (50 μg/mL) for 1 h at 37°C. DNA histograms were then obtained using a Beckton-Dickenson FACS Vantage flow cytometer system (Becton-Dickenson, San Jose, CA). The cell cycle distribution was analyzed using Cell Quest software (Version 3.2, Becton-Dickenson).

RNA isolation, reverse transcription polymerase chain reaction(RT-PCR) analysis, and primer construction

Cells were collected with Trizol (Invitrogen, Carlsbad, CA), and total RNA was extracted according to the manufacturer's protocol. This procedure was followed by precipitation with isopropanol, washing with 70% ethanol, and elution with diethylpyrocarbonate (DEPC)-treated water. Total RNA (5 μg) was reverse transcribed with Molony murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) using Oligo-d(T)15 primer. A cDNA mixture (2 μL) was used for PCR amplification of each gene with exTaq (Takara Bio Inc., Japan). Primers used for p53 (Accession number: NM_000546) and GAPDH (Accession number: J02642) are as follows: p53 forward: TGACACGCTTCCCTGGAT, p53 reverse: CTCCGTCATGTGCTGTGACT, GAPDH forward: AACAGCGACACCCACTCCTC, and GAPDH reverse: GGAGGGGAGATTCAGTGTGGT**.**

Statistical analysis

Statistical analysis all statistical analyses were calculated with Graph pad Prism (ver.5.00 for Windows, Graph Pad Soft ware, San Diego, CA, USA, www. graph pad. com). Results are expressed as mean ± standard error (±SE). Student's t-testwas used to determine statistical significance between control and test groups. A *p*-value less than 0.05 was considered statistically significant (indicated ns: not significant, indicates $p< 0.05$, indicates $p< 0.001$, indicates $p< 0.0001$).

Table S1. IC_{50} values of methylstat on various cell lines

	Normal cells		Cancer cells	
Cell line	HUVEC	CHANG	HeLa	HepG2
IC_{50} (μM)	4	10	5	7.5

Table S2. Inhibitory activity of methylstat on cytokine-induced chemoinvasion

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	Methylstat (μM)		
	NT	1	2
VEGF (+)	137±1	104±1	91±1.2
bFGF (+)	131±0.3	113±0.4	96±0.2
TNF-α (+)	134±1.8	110±0.4	100±0.2

* Values represent the % of invaded cells compared to no cytokine stimulation (mean±SE from three independent experiments). Numbers of invaded cells with no cytokine stimulation were converted to 100%.

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