RSC Advances

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Journal:	*RSC Advances*
Manuscript ID:	RA-COM-07-2014-007154.R1
Article Type:	Communication
Date Submitted by the Author:	15-Aug-2014
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Journal Name

RSCPublishing

COMMUNICATION

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Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Histone methylation has been highlighted in the regulation of gene expression. To explore the role of histone methylation in angiogenesis, methylstat, a Jumonji C domain containing histone demethylase inhibitor, was used as a chemical probe. Methylstat inhibited the cell cycle of human umbilical vascular endothelial cells (HUVECs) at lower concentrations than other cell lines tested. Furthermore, methylstat blocked *in vitro* and *in vivo* angiogenesis at nontoxic dose. These results provide new insights into the role of histone demethylase in angiogenesis. Collectively, methylstat could be a promising chemical probe for addressing its role in angiogenesis.

Histone modifications, such as acetylation and methylation, play an important role in the regulation of gene expression. Histone demethylases are emerging as important players in developmental processes and have been linked to neurological disorders and human diseases like cancer. They regulate epigenetic modification mechanisms by modifying various lysine residues of substrate proteins.[1] Histone demethylases contain a variety of domains that target different substrates, mediating different roles in cellular function. Histone lysine demethylases, such as LSD1, contain flavin-dependent amine oxidase domains, which act exclusively on mono- and di-methylated lysines.[2] In relation to cancer, LSD1 was shown to repress p53 function by inhibiting its interaction with p53 binding protein 1 (53BP1).[3] Jumonji C domain-containing proteins have been classified into different groups, several of which have been found to possess histone demethylase activity.[4] Recent reports demonstrated that inhibition of the Jumonji C domain-containing histone demethylase JMJD1A suppresses tumor growth by regulating angiogenesis.[5, 6] Interestingly, a small molecule named JIB-04, a specific inhibitor of the Jumonji C domain-containing family of histone demethylases, selectively inhibits cancer cell growth but not normal cell growth.[7]

Herein, we investigated the possible effects of a new histone demethylase inhibitor, methylstat, on histone methylation dynamics in cell growth and angiogenesis. Methylstat is a compound that selectively inhibits Jumonji C domain-containing histone demethylases in cells.[8] Methylstat promotes the methylation of histone lysine residues including H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20.[8] However, the anti-angiogenic properties of methylstat have not been investigated. Efficient inhibition of angiogenesis has been considered as a promising strategy for the treatment of angiogenesis-related diseases including cancer.[9, 10] In particular, angiogenesis is essential for the growth, progression, and metastasis of solid tumors and is thus a promising pharmacological target for anticancer therapy.[11] In addition, the tumor suppressor protein p53 plays a key anti-cancer role by regulating cell cycle,

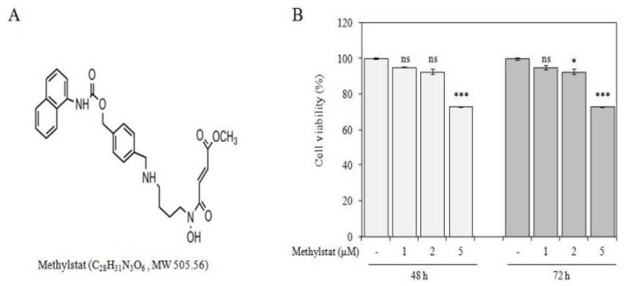

Fig. 1 The chemical structure and effect of methylstat on the viability of HUVECs. (A) The chemical structure of methylstat ($C_{28}H_{31}N_3O_6$, MW 505.56) (B) Effect of methylstat on the viability of HUVECs. Cells were treated with methylstat (0–5 μM) and incubated for 48 or 72 h. Cell viability was measured by trypan blue assay from three independent experiments (mean ±SE, ns (not significant) *p<0.1, **p<0.05, ***p<0.01).

apoptosis, and expression of p21, which mediates p53-dependent G_1 phase cell cycle arrest.[12, 13] To investigate the anti-angiogenic properties of methylstat (Fig.1A), the effect of methylstat on the proliferation of HUVECs was examined using a viability assay.[14]

Various concentrations of methylstat (1–5 μM) were applied to HUVECs for up to 72 h (Fig. 1B). Methylstat did not exhibit cytotoxicity on HUVECs at 1-2 μM. Accordingly, the following studies were performed using a concentration range of 1 to 2 μM, which shows no toxicity in cells

Further examination was performed on the effects of methylstat on HUVEC cell growth. As shown in figure 2A and Table S1, the IC_{50} of HUVECs was 4 μM. HepG2, HeLa, and CHANG cells were treated with various concentrations of methylstat for up to three days and cell growth was assessed by the MTT colorimetric assay. Notably, among the cell types tested, methylstat showed the most potent cell growth inhibitory activity in HUVECs (Table S1). Methylstat arrested cell cycle at the G_0/G_1 phase in a dose-dependent manner at 48 h (Fig. 2B). After exposure to 2 μM methylstat, G0/G1 phase increased 16.8% compared to non-treated cells, whereas S and G2/M decreased 5.5% and 6.1% respectively. Accordingly, methylstat arrested cell cycle at G0/G1 phase.

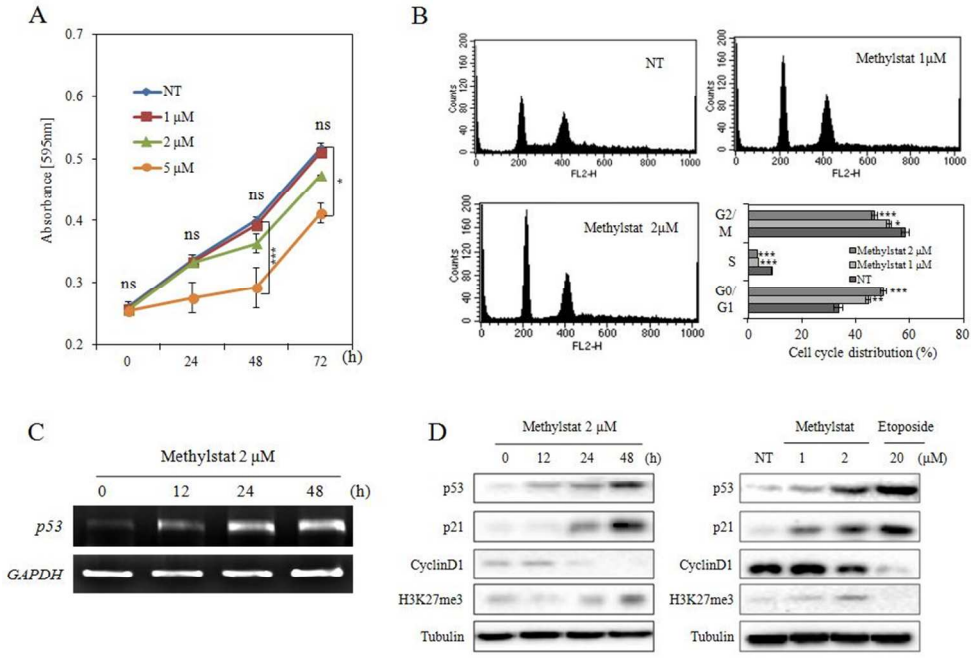

Fig. 2 Methylstat arrests cell cycle by regulating expression levels of p53, p21, and cyclinD1. (A) Effect of methylstat on the growth of HUVECs. Cells were treated with various concentrations of methylstat for 72 h and cell growth was measured using the MTT colorimetric assay from three independent experiments (mean ±SE, ns (not significant),*p<0.1, **p<0.05, ***p<0.01). (B) Effect of methylstat on cell cycle distribution at 48 h. Graph indicates cell cycle distribution rates of each phase from three independent experiments (mean ±SE, ns (not significant)*p<0.1, **p<0.05, ***p<0.01). (C) Effect of methylstat on p53 mRNA levels at 12, 24, and 48 h. GAPDH is used as control. (D) Time and dose-dependent effect of methylstat on p53, p21, and cyclinD1 expression and H3K27 methylation in HUVECs after 48 h.

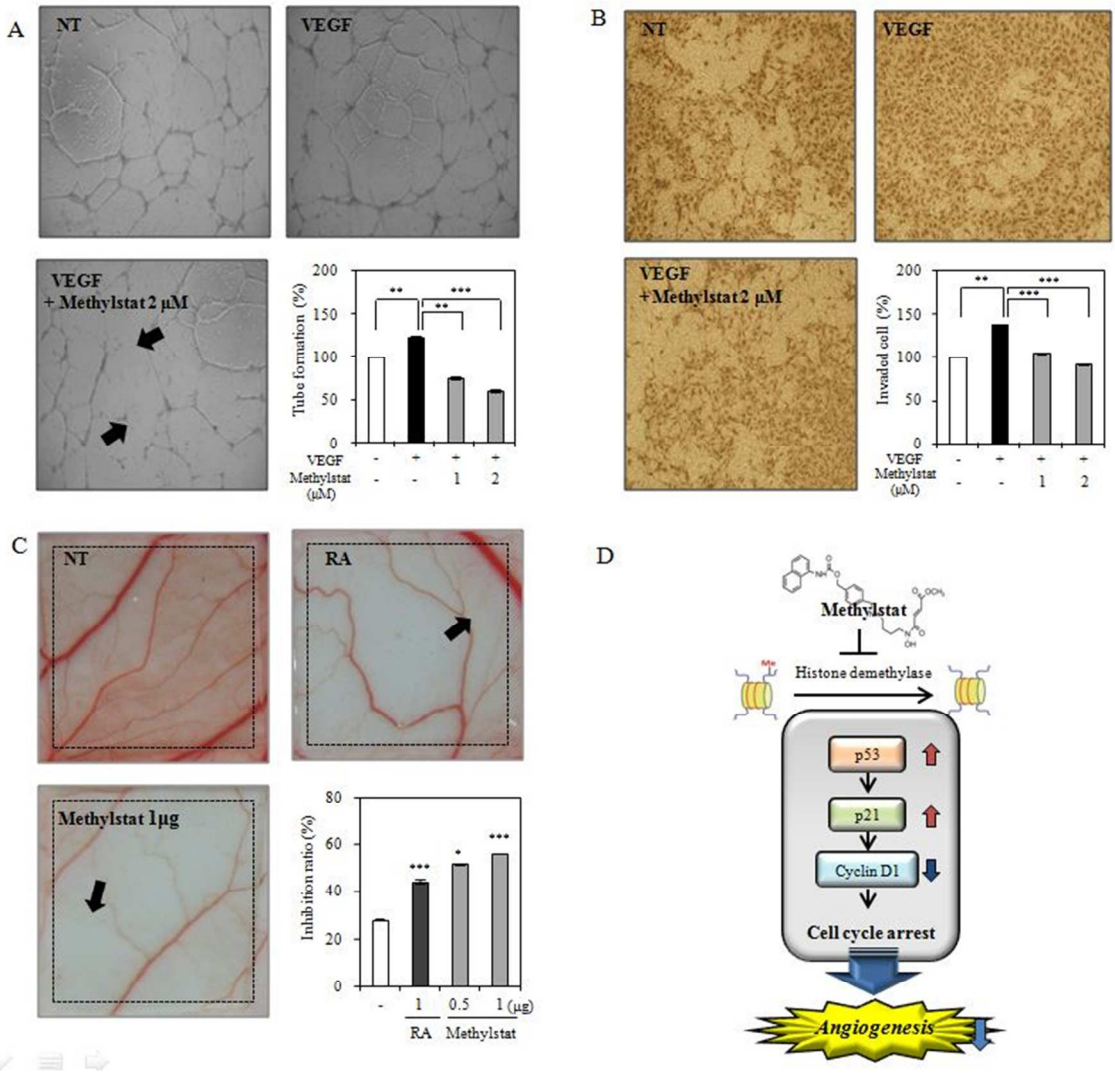

Fig. 3 Anti-angiogenic activity of methylstat *in vitro* and *in vivo*. (A) Effect of methylstat on tube-forming abilities of HUVECs. Arrows indicate the truncated tube formations. (B) Effect of methylstat on VEGF-induced chemo invasion of HUVECs. (C) Anti-angiogenic effect of methylstat *in vivo* chick embryo chorioallantoic membrane (CAM) assay. Arrows represent vascular zones. Calculations are based on the proportion of angiogenesis inhibited eggs to the total number of eggs tested. Graph indicates angiogenesis-inhibition rates from three independent experiments (RA: Retinoic acid, mean ±S.E, *p<0.1, **p<0.05, ***p<0.01). (D) Schematic summary of methylstat-mediated signaling pathway related to anti-angiogenic activity.

We further examined p53 levels and downstream signaling in growth-inhibited HUVECs by methylstat. p53 is one of the most extensively studied proteins in cancer research because of its potent tumor suppressive activity and its roles in apoptosis and angiogenesis inhibition.[13] p53 is directly involved in cell cycle arrest the G_1 phase through the induction of p21.[15] Methylstat treatment in HUVECs induced p53 mRNA level in time-dependent manner (Fig. 2C). Transcriptional activation of p53 by methylstat resulted in accumulation of p53 and p21 protein levels in a time- and dose-dependent manner, whereas methylstat suppressed the protein level of cyclinD1, a G1/S phase specific protein (Fig 2D). These traits of methylstat are similar to those of etoposide, a known anti-cancer drug that inhibits cell proliferation and cell cycle by p53 induction and G_0/G_1 phase arrest.[16] To validate the functional inhibition of histone demethylases by methylstat, H3K27 methylation levels were examined. HUVECs were treated with methylstat up to 48 h, which resulted in time- and dose- dependent increase of H3K27 methylation indicating the inhibition of histone demethylase by metylstat (Fig. 2D). Etoposide did not induce H3K27 methylation. These results suggest that methylstat inhibits proliferation of

HUVECs by inducing p53, p21 levels and inhibiting cyclinD1 level, through the inhibition of histone demethylase activity.

Next, we explored the activity of methylstat on *in vitro* angiogenic phenotypes, such as tube formation and chemoinvasion. The majority of anti-angiogenic agents target, in particular, the VEGF pathway since VEGF expression increases throughout the process of neovascularization of solid and hematological tumors.[16.17]Therefore tumor vasculature can be inhibited through endothelial cell proliferation inhibition or activation of endothelial cell apoptosis. In this way, the source of new blood vessels is destroyed, preventing further tumor growth by starving tumor cells.[17] Likewise, the effects of methylstat on VEGF induced angiogenesis were examined. Serum-starved HUVECs were stimulated by VEGF prior to methylstat treatment. Methylstat inhibited VEGF-induced tube formation in a dose-dependent manner without cell toxicity (Fig. 3A). The effect of methylstat on the invasive activity of VEGF-stimulated HUVECs was also investigated. While VEGF enhanced the chemoinvasion of HUVECs, methylstat inhibited VEGF-induced invasion in a dose-dependent manner (Fig. 3B). Furthermore, methylstat inhibited HUVEC chemoinvasion induced by other growth factors bFGF and TNF-α (Table S2). These results demonstrate that methylstat inhibits angiogenesis induced by various cytokines.

The anti-angiogenic activity of methylstat was further validated *in vivo* with the chick embryo chorioallantoic membrane (CAM) assay. Normal chick embryo CAM displayed extensive networks of capillaries. However, methylstat-treated CAM exhibited an inhibition of capillary formation during CAM development without any sign of thrombosis and hemorrhage (Fig. 3C). Altogether, these data demonstrate that methylstat potently inhibits angiogenesis both *in vitro* and *in vivo*. The anti-angiogenic activity of methylstat results from the activation of p53 expression. p53 transcription induced by methylstat resulted in p21 activation and cyclinD1 inhibition, which resulted in cell cycle arrest at G0/G1 phase, followed by angiogenesis inhibition in VEGF stimulated HUVECs (Fig. 3D).

Conclusions

In this study, we explored the possible role of histone demethylase in angiogenesis using methylstat, an inhibitor of Jumonji C domain-containing histone demethylases as a chemical probe. Methylstat exhibited notable anti-proliferative effects in HUVECs. Results showed that methylstat arrested the G_0/G_1 phase of HUVECs in a dose- and time-dependent manner. There are various types of endothelial cells, but HUVECs could be used as a representative of all types of endothelial cells because of their inherently stable characteristics and low mutation rates, and the methylstat response shown in HUVECs may represent the response in other endothelial cells. Previous reports on the histone demethylase inhibitor JIB-04 raised the possibility that histone demethylase inhibitors display anti-cancer activity. However, only tumor cells were examined and the effect of JIB-04 on angiogenesis has not been investigated. Herein, we have demonstrated that methylstat is a potent angiogenesis inhibitor and may provide a new molecular scaffold for the development of histone demethylase inhibitors as anti-angiogenic and anti-cancer agents. There have been reports that p53 transcriptional expression can be regulated by histone lysine residue hypermethylation in p53 promoter.[18, 19] Accordingly, we speculate that methylstat could regulate p53 expression by induction of hypermethylation of diverse lysine residues of histones.[8] Although methylstat was reported as a specific histone demethylase inhibitor, there remains a possibility that methylstat induced p53 expression results from p53 lysine methylation.[3, 20] Whether methylstat affects non-histone methylation including p53 should be examined in following studies.

Collectively, this is the first study indicating the activity of histone demethylase inhibitor, methylstat, in terms of its potency as an anti-angiogenic agent by inhibiting HUVECs proliferation. Methylstat could be a basis for developing new drug therapies treating angiogenesis-related diseases by targeting histone demethylases.

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† Electronic Supplementary Information (ESI) available: Experimental and spectroscopic data. See DOI: 10.1039/c000000x/

Acknowledgments

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP; 2009-0092964, 2009-0083522, 2010-0017984, 2012M3A9D1054520), the Center for Food and Drug Materials of Agriculture Science & Technology Development (PJ0079772012), Rural Development Administration, National R&D Program, Ministry of Health &Welfare (0620360-1), and the Brain Korea 21 Project, Republic of Korea.

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