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Pharmacological inhibition of arginine and lysine methyltransferases induces nuclear abnormalities and suppresses angiogenesis in human endothelial cells

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ABSTRACT

Posttranslational modifications of histone tails can alter chromatin structure and regulate gene transcription. While recent studies implicate the lysine/arginine protein methyltransferases in the regulation of genes for endothelial metabolism, the role of AMI-1 and AMI-5 compounds in angiogenesis remains unknown. Here, we show that global inhibition of arginine and lysine histone methyltransferases (HMTs) by AMI-5 induced an angiostatic profile in human microvascular endothelial cells and human umbilical vein endothelial cells. Based on FACS analysis, we found that inhibition of HMTs significantly affects proliferation of endothelial cells, by suppressing cell cycle progression in the G_0/G_1 phase. Immunofluorescent studies of the endothelial cells replication pattern by 5-ethynyl-2′-deoxyuridine incorporation disclosed that AMI-5, and the arginine methyltransferase inhibitor AMI-1, induced heterochromatin formation and a number of nuclear abnormalities, such as formation of micronuclei (MNs) and nucleoplasmic bridges (NPBs), which are markers of chromosomal instability. In addition to the modification of the cell cycle machinery in response to AMIs treatment, also endothelial cells migration and capillary-like tube formation processes were significantly inhibited, implicating a stimulatory role of HMTs in angiogenesis.

1. Introduction

Angiogenesis is a multistep process that is regulated by distinct gene expression programs. The recruitment of new capillary blood vessels is essential in physiological processes such as wound healing, but also a critical component of metastatic pathways and tumor growth. Angiogenesis regulation is a major research area for the development of therapeutics for the inhibition of tumor growth [1]. Thrombospondin-1 was the first identified inhibitor of angiogenesis [2], and other endogenous molecules preventing uncontrolled capillary formation have been found among extracellular matrix molecules, including angiostatin and endostatin [3].

In the search for more effective pharmacological compounds to inhibit cancer growth, the attention in recent years has been devoted to epigenetic targets, which regulate gene pathways implicated in metabolism and disease progression [4]. It has been shown that DNA methyltransferases (DNMTs), as well as enzymes involved in histone post-translational modifications, regulate gene expression [5–7]. Studies on histone deacetylases (HDACs) inhibitors show the participation of HDAC enzymes in the regulation of key pathways of endothelial cell biology and tumor angiogenesis [8,9]. The angiostatic effect of DNMTs inhibitors has also been described [10]. Several compounds inhibiting DNMTs or HDACs

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AMI-1, arginine N-methyltransferase inhibitor-1; AMI-5, arginine(lysine) N-methyltransferase inhibitor-5; DAPI, 4′,6-diamidino-2-phenylindole; DNMTs, DNA methyltransferases; FDA, Food and Drug Administration; HDACs, histone deacetylases; HMEC-1, immortalized human microvascular endothelial cells; HKTMs, histone lysine methyltransferases; HUVECs, human umbilical vein endothelial cells; EdU, 5-ethyl-2′-deoxyuridine; MIF, mean intensity fluorescence; PI, propidium iodide; PRMTs, protein arginine methyltransferases.

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have been approved by the FDA for use as anticancer therapies, including Zolinza™ (Vorinostat), Istodax™ (Romidepsin), Vidaza™ (5-azacitidine), Dacogen™ (Decitabine) [11–13]. Little is known, however, about the role of histone methyltransferases in the regulation of angiogenesis. Gene expression is regulated by histone methylation via transcriptionally permissive modifications (H3K4me, H3K36me, H4K20me) or repressive modifications that include (H4K20me/H3K9me, H3K27me, H3K79me) [14]. Several studies have now shown the importance of histone H3 lysine 27 methylation, mediated by the polycomb repressive complex 2 and EZH2 in the regulation of angiogenesis, as well as in the gene silencing of tumor-suppressor genes [15–17]. Arginine methylation of both histone and non-histone proteins has also emerged as an important biochemical modification regulating chromatin organization and function [18]. Arginine methylation is mediated by a large family of protein arginine methyltransferases (PRMTs), which comprises nine isoforms in the mammalian genome (PRMT1-9) [19]. Overexpression of PRMTs has been demonstrated in cancer, and inhibition of PRMTs is currently considered as a promising therapeutic target [20].

In this study, we investigate inhibition of protein and histone methyltransferases by AMI-1 and AMI-5 on the angiogenic potential of human microvascular endothelial cells (HMEC-1) and human umbilical vein endothelial cells (HUVECs), with particular emphasis on the cell's proliferation process and chromatin structure. AMI-1 and AMI-5 are protein arginine methyltransferases (PRMTs) and histone lysine methyltransferases inhibitors (HKMTs) [21–23]. AMI-1 specifically inhibits PRMTs *in vitro*, in an AdoMet-independent manner and *via* prevention of arginine methylation of cellular proteins *in vivo*. It was found that AMI-1 can modulate nuclear receptor-regulated transcription from estrogen and androgen response elements [24]. AMI-5, inhibits both PRMTs and HKMTs in an AdoMet/SAM-competitive manner [24,22].

Our previous results showed that changes in the methylation status of proteins, induced by AMIs-treatment, have a significant effect on the transcriptome of human endothelial cells, affecting many crucial genes governing endothelial cells metabolism and whole cardiovascular system functioning [25]. The more detailed analysis shown here concerning the cell biological responses to AMIs shows that AMI-dependent inhibition of both types of methyltransferases, i.e. lysine and arginine methyltransferases, results in the modulation of all investigated steps of angiogenesis '*in vitro*', including cell proliferation, cell migration as well as capillary-like tube formation process.

2. Materials and methods

2.1. Cell culture and chemical inhibitors

HMEC-1 (Human Microvascular Endothelial Cells) were obtained from the Centre for Disease Control and Prevention, Emory University (Atlanta, GA, USA). Cells were cultured in MCDB131 medium (Gibco®, Life technologies, Merelbeke, Belgium) containing 10 ng/ml of epidermal growth factor (Merck Millipore, Darmstadt, Germany), 5 mM Glutamine (Gibco®, Life technologies, Merelbeke, Belgium) and 10% heat-inactivated fetal bovine serum (Gibco®, Life technologies, Merelbeke, Belgium) and antibiotics (penicillin/streptomycin) (Gibco®, Life technologies, Merelbeke, Belgium). The same cell culture conditions were applied to HUVECs (Human Umbilical Vein Endothelial Cells). The cells were isolated from veins of freshly collected umbilical cords, by collagenase type II digestion, according to Jaffe's protocol [26], and used for the experiments at passage 3–4. A permission for HUVEC's isolation was obtained from the Bioethics Commission at Medical University of Lodz (decision No. RNN/264/15/KE).

Arginine N-methyltransferase inhibitor-1 (AMI-1, 7,7′-(carbonylbis(azanediyl))bis(4-oxidonaphthalene-2-sulfonate) sodium salt) and Arginine and lysine N-methyltransferase inhibitor-5 (AMI-5, 2-(2,4,5,7-Tetrabromo-3-oxido-6-oxoxanthen-9-yl)benzoate disodium salt) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Calbiochem (Darmstadt, Germany), respectively. Both inhibitors were dissolved in sterile water, according to the suggestions of the manufacturers, aliquoted (to avoid freeze-thaw cycles) and stored up to 3 months according to the company's leaflet. Final concentration of the solvent, at the highest concentration of drugs, did not exceed 0.6% of the volume sample and did not affect cellular metabolism, which was checked in the viability assay, to exclude osmotic stress effect. The biochemical and biological features of AMI-1 and AMI-5 are summarized in Table 1.

2.2. ECs viability assay

Cells were seeded onto 96-well plates at density 1.5×10^4 cells per well. After 16–24 h cells were treated with the inhibitors at the indicated concentration for 24 h. In the next step, medium containing inhibitors were removed, wells were rinsed twice with PBS Ca^{2+}/Mg^{2+}.

2.2.1. Resazurin reduction assay

HMEC-1 and HUVECs viability after inhibitor treatment was estimated by the ability of live cells to reduce non-fluorescent resazurin to rezorufin, a fluorescent product. After removal of inhibitors-containing medium, cells were incubated in PBS containing Ca^{2+}/Mg^{2+}, 5.5 mM glucose and 0.0125 mg/ml resazurin (Sigma-Aldrich, St. Louis, MO, USA). After a 2 h incubation fluorescence was recorded at λ_{ex} = 530 nm, λ_{em} = 590 nm, using a Fluoroscan Ascent microplate reader (Labsystem Inc.).

2.3. Cell cycle analysis by FACS

2.3.1. Preparation of samples

Preparation of cell nuclei was based on the method described by Kohlmeier et al. [30]. Briefly, HMEC-1 cells were placed on 6-well plates (NUNC™, Thermo Scientific™, Denmark), trypsinized, washed with PBS and centrifuged for 5 min at 200*g*. For DNA content analysis, cells were fixed in 70% ethanol for 24 h at 4 °C, centrifuged (10 min at 200*g*), washed with PBS and centrifuged once again (10 min at 200*g*). Then, cells were resuspended in propidium iodide (PI) buffer containing: RNase A (0.4 mg/ml; Sigma-Aldrich, St. Louis, MO, USA), 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and PI (5 μg/ml; Gibco™ Invitrogen, Merelbeke, Belgium) in PBS and incubated for 30 min.

2.3.2. Measurement parameters

Selection of the population of cells for cell cycle analysis was multilevel, and based on (i) identification of cells by forward (FSC) and side scatter (SSC), where the preliminary selection (removing of debris) for further analysis was performed (SSC-A *vs* FSC-A plot). Then (ii) pulse shape analysis was performed to identify and exclude clumps and doublets (FSC-H *vs* FSC-A plot). Next, (iii) selected population of cells was applied to the scatter plot (SSC-H *vs* SSC-A) to gate out obvious debris and most of the apoptotic cells. In the last step (iv) gated cells were applied to the propidium iodide (PE-A *vs* PE-W) and (v) generation of histogram plot (Count *vs* PE-A). The gating of cells was validated in the control sample (HMEC-1 untreated) and the same parameters of gating were applied to the next one (cells treated with different concentrations of AMI-1 or AMI-5). Samples were measured using a LSRII flow cytometer and FACSDiva Software 6.2 (BD Biosciences). Further analysis of the identification of