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Targeting the invasive phenotype of cisplatin-resistant Non-Small Cell Lung Cancer cells by a novel histone deacetylase inhibitor

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ABSTRACT

Non-Small Cell Lung Cancer (NSCLC) remains an aggressive and fatal disease with low responsiveness to chemotherapy, frequent drug resistance development and metastatic behavior. Platinum-based therapy is the standard of care for NSCLC with limited benefits. Since epigenetic alterations have been implicated in the aggressive behavior of lung cancer, the purpose of the present study was to examine the capability of the pan-histone deacetylase inhibitor SAHA and of ST3595, a novel hydroxamate-based compound, to interfere with the proliferative and invasive potential of NSCLC cells. We used two NSCLC cell lines (H460 and A549) and the cisplatin-resistant variants (H460/Pt and A549/Pt), to mimic a frequent clinical condition. The resistant models exhibited increased invasive properties as compared to parental cells, features associated with a wide modulation of the level of angiogenesis- and invasion-related factors in the cell conditioned media. The levels of urokinase-type plasminogen activator, IL-8, and macrophage migration inhibitory factor were increased in the conditioned media from both H460/Pt and A549/Pt cells. SAHA and ST3595 induced a strong inhibition of cell invasive properties, which was more marked after ST3595 exposure. Both HDAC inhibitors up-regulated the metastasis suppressor KiSS1 at the mRNA level. Forced expression of KiSS1 significantly decreased the invasive capability of drug-resistant cells. ST3595 displayed an anti-metastatic effect in tumors associated with decreased of phosphorylation of Src. Our data indicate that HDAC inhibitors are effective in NSCLC cell systems. The ability of ST3595 to counteract the invasive potential of resistant cells through mechanisms involving KiSS1 is an interesting novel finding.

1. Introduction

Non-Small Cell Lung Cancer (NSCLC) is characterized by late diagnosis and poor prognosis in advanced disease. The efficacy of platinum compounds in advanced disease is marginal and disease outcome still remains unsatisfactory because the majority of patients develop drug resistance and metastases [1]. Recent advances in unraveling the molecular origin of lung cancer may provide novel targets for therapy [1]. Several factors have been implicated in the drug resistance development, including gene mutations, genome alterations and epigenetic changes [2]. It has been recently proposed that patients with NSCLC might benefit from treatments including inhibitors of histone deacetylases (HDAC) [3].

Histone acetylation is a reversible event regulated by HDAC which have been implicated in tumor development and progression [4–7]. These enzymes remove acetyl groups from histones at prominent amino-terminal lysine residues, thereby promoting chromatin condensation and generally acting as transcription repressors [6]. In addition, some HDAC isoforms deacetylate non-histone proteins, for instance p53 and tubulin, affecting multiple cellular functions such as transcription, DNA repair and

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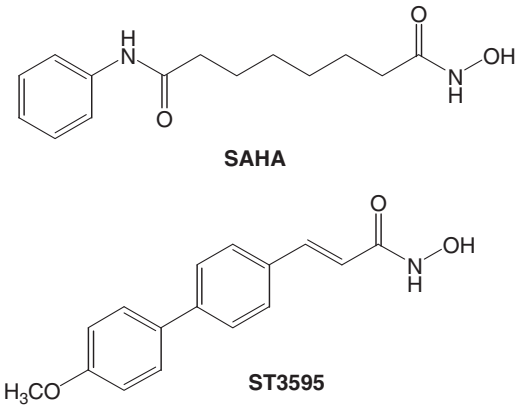

Fig. 1. Chemical structures of the HDAC inhibitors. The structure of SAHA and ST3595 is shown.

metabolism [6]. Human HDACs, which comprise 18 proteins, are grouped into four classes based on phylogenic analysis [6,7]. HDAC 1–11 are Zn-dependent enzymes and are targeted by broad spectrum inhibitors such as the clinically available vorinostat (SAHA, suberoylanilide hydroxamic acid) [6].

HDAC are deregulated in tumors and have been implicated in silencing of growth regulatory and apoptotic pathways [8]. Deregulation of such pathways and, more specifically the failure to undergo apoptosis in response to antitumor agents may result in drug resistance [9]. Over-expression of specific HDAC has been described in many types of cancer [7], and their alteration may modulate cell motility and adhesion [10]. Moreover, the microenvironment of solid tumors and metastases may influence the response to cytotoxic treatments by activating epigenetic mechanisms contributing to drug resistance [11].

A variety of factors has been implicated in the aggressiveness of NSCLC and the identification of a few driver alterations, such as *EGF-R* mutations or *ALK* translocations, has already provided novel therapeutic options [12]. At present, no effective strategies have been defined to target the most frequent mutations present in lung adenocarcinoma, *i.e.*, *KRAS* mutations [13]. Such mutations and the consequent activation of multiple pathways controlling tumor cell growth and survival have been correlated with an unfavorable prognosis [14,15].

In the search for novel therapeutic options, HDAC inhibitors may represent promising tools in the treatment of *KRAS* mutant NSCLC tumors. In the present study, we used cell lines characterized by *KRAS* mutations and cisplatin-resistant variants found to display increased invasive capability to investigate the mechanisms by which the pan-HDAC inhibitor SAHA and the novel hydroxamate-based HDAC inhibitor ST3595 [16] (Fig. 1) interfere with the invasive potential of these NSCLC cells. We found that the anti-metastatic effect of these inhibitors, which up-regulate the metastasis suppressor KiSS1, is associated with down-modulation of Src signaling.

2. Materials and methods

2.1. Cell lines, cell sensitivity to drugs, and apoptosis assays

The human NSCLC cell lines H460 and A549 and the cisplatin-resistant variants H460/Pt and A549/Pt were cultured in RPMI-1640 medium plus 10% FBS. The cisplatin-resistant sublines were obtained by exposure of parental cell lines to increasing concentrations of cisplatin [17]. All cell lines were authenticated using the Stem Elite ID System (Promega Italia, Milan, Italy) between June 2012 and April 2014. Cell sensitivity to drugs was measured by growth-inhibition assays. Twenty-four hours after seeding, cells were exposed to drugs for 1 or 72 h. After 1 h treatments the drug-containing medium was removed and after adding fresh medium cells were cultured for 72 h. Cells were counted with a cell counter. At least three independent experiments were performed for treatment type. ST3595 (Sigma-Tau, Pomezia, Italia) and vorinostat (SAHA, Selleck Chemicals, Aurogene, Rome, Italy) were dissolved in DMSO (final concentration 0.25%) and diluted in culture medium. Cisplatin (Teva Pharma Italia, Assago, Italy) was diluted in saline.

For analysis of apoptotic cell death, 24 h after seeding, cells were exposed to cisplatin, ST3595 or SAHA for 72 h. After treatment floating and adherent cells were harvested and processed for apoptosis evaluation by TUNEL assay (Roche, Mannheim, Germany).

2.2. Cell invasion assays

Cells were seeded in complete medium and treated with HDAC inhibitors for 24 h. Then, control and treated cells were transferred (8×10^5 per well) to 24-well transwell chambers (Costar, Corning, Inc., Corning, NY) in serum-free medium. The transwell membranes were previously coated with 12.5 μg Matrigel per well (BD Biosciences, San Jose, CA) and dried for 1 h. After 24 h of incubation at 37 °C, cells that invaded the Matrigel and migrated to the lower chamber were fixed in 95% ethanol, stained with a solution of 0.4% sulforhodamine B in 0.1% acetic acid, and counted under an inverted microscope.

2.3. Antibody Arrays and ELISA

Cells were seeded at 2×10^4 cells/dish (19.6 cm^2) in complete medium and cultured for 1 day before serum starvation and treatment with HDAC inhibitors (3 μM or as indicated) for 24 h. Then, conditioned media were harvested and clarified by centrifugation at 13,000 rpm for 15 min. Cells were trypsinized, counted and lysed for assaying protein content. Supernatant aliquots were used for analyses of angiogenesis-related protein, cytokine and chemokine content by Antibody Arrays (R&D System, SPACE Import Export, Milan, Italy) following the manufacturer's instructions. ELISA kits for vascular endothelial growth factor (VEGF, human VEGF immunoassay kit, Biosource International, Camarillo, CA) MIF (R&D System), IL-8 (Abcam, Cambridge, UK) were used according to the manufacturer's instructions for quantitative analysis.

2.4. Gain of function studies

To generate KiSS1 over-expressing cells, the H460/Pt cells were transfected with the pCMV6-XL5 vector containing the full-length cDNA of KiSS1 (NM_002256.2) or empty vector (Tema Ricerca, Castenaso, Italy) using Lipofectamine2000 (Life Technologies Italia, Monza, Italy), according to the manufacturer's protocol. Stably transfected cell populations were selected using 400 μg/ml G418 (Calbiochem Inalco, Milan, Italy). The expression vector was checked by sequencing the full-length insert.

2.5. Western blot analysis and antibodies

Western blot analysis was carried out according to standard procedures [18]. In *in vitro* studies, cells were harvested using a scraper and lysed in a buffer composed of 0.125 M Tris–HCl pH 6.8 (Sigma–Aldrich, St. Louis, MO), 5% sodium dodecyl sulfate (SDS, Lonza, Verviers, Belgium) and protease/phosphatase inhibitors (25 mM sodium fluoride, 10 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride,