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HDAC inhibitor suppresses proliferation and tumorigenicity of drug-resistant chronic myeloid leukemia stem cells through regulation of hsa-miR-196a targeting BCR/ABL1

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ABSTRACT

Failure to eradicate hematologic cancer stem cells (hCSCs) associated with resistance to tyrosine kinase inhibitors such as imatinib mesylate (IM) in chronic myeloid leukemia (CML) patients is a clinical challenge that highlights the need for discovering and developing therapeutic strategies that target and eliminate these hCSCs. Herein, we document the essential role of the interplay between histone deacetylases (HDACs), the polycomb group proteins, pluripotency transcription factors and the cell cycle machinery in the viability, oncogenicity and therapy evasion of IM-resistant CD34+/CD38- CML stem cells (CML-SCs). Using the proteotranscriptomic analyses of wild type (WT), CD34+/CD38+ and CD34+/CD38− K562 or KU812 cells, we showed that CD34+/CD38− SC-enriched cells expressed significantly higher levels of CD44, CD133, SOX2, Nanog, OCT4, and c-Myc mRNA and/or protein, compared to the WT or CD34+/CD38+ cells. This overexpression of stemness factors in the CD34+/CD38− cells positively correlates with enhanced expression of HDACs 1–6, cyclins D1/D3, CDK 2, 4 and 6, while inversely correlating with p18, p21 and p27. Enhanced co-expression of MDR1, survivin, and Bcl-2 proteins, supposedly involved in IM-resistance and CML-SC survival, was detected in both CD34+/CD38− and CD34+/CD38+ cells. Importantly, we demonstrate that in synergism with IM, SAHA reverses the tumor-promoting proteotranscriptomic profile noted above and elicits marked inhibition of the CML-SCs by up-regulating hsa-miR-196a expression. This hsa-miR-196a-mediated SC-limiting effect of SAHA is dose-dependent, low-dosed, cell cycle-modulating and accompanied by leukemic SC apoptosis. Interestingly, this anti-SC therapeutic activity of SAHA *in vitro* was reproduced *in vivo* using the NOD-SCID mice models.

1. Introduction

Chronic myeloid leukemia (CML), purportedly arising from transformed hematopoietic stem cells (HSCs), and spanning through the early chronic, accelerated, and terminal blast crisis phases, is a clonal triphasic myeloproliferative neoplastic disorder characterized by the presence of the BCR/ABL+ Philadelphia (Ph) chromosome sequel to a balanced t(9;22), (q34;q11) translocation [1,2]. Dysregulation of the activity and/or expression of the 210 kDa constitutively active BCR/ABL tyrosine kinase fusion-gene, is inherently essential and sufficient for the proliferation, growth, evasion of apoptosis, therapy-resistance and survival of leukemic cells, in part because BCR/ABL modulates several oncogenic effector genes, such as the MAPK, RAS, PI3K, and STAT proteins [3].

As a paradigm for stem cell-derived neoplasms, CML in keeping with the hierarchical cancer stem cell (CSC) model of tumor heterogeneity,

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harbors a bio-distinct cellular sub-population with unrestricted self-renewal and proliferative potential. This may relate to the clinically-relevant precarious response to current therapeutic agents, relatively high disease recurrence rate, and poor clinical outcome among CML patients [1,2,4]. The administration of tyrosine kinase inhibitors (TKIs), including imatinib mesylate (IM) which competitively inhibit adenosine triphosphate (ATP) interaction with tyrosine kinase, as first-line agent in CML therapy has been shown to elicit curative (hematologic and cytogenetic) response and improve survival, however, remission is short-term, and minimal residual disease or relapse is almost always inevitable, secondary to the probable development of overt resistance to IM and re-population of malignant leukemic cells [4,5]. This is consistent with the documented innate insensitivity of CML stem cells (CML-SCs) to BCR/ABL -targeting TKIs. These among many reasons highlight the need for the identification of novel CML- relevant molecular targets, as well as discovery and development of novel therapeutic agents with higher efficacy and long-term effectiveness in IM-resistant CML.

In the last two decades, the role of epigenetic modifications in oncogenicity and tumor progression has become a subject of increased interest and investigation [4,6]. Like most epigenetic changes, histone acetylation is reversible, regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs), plays a critical role in the modulation of several gene transcription, and is increasingly implicated in many malignancies, including blood cancers (Reviewed in 6). Currently, HDACs known to 'erase the acetyl mark' from the lysine residues in the histone amino terminal tails, are currently classified into four groups. While HDAC classes I, II, and IV which are Zn^{2+}-dependent metalloproteins are inhibited by suberoylanilide hydroxamic acid (SAHA, vorinostat) and other acid-based HDACi, class III HDACs are not [6]. The post-approval era of this first pan-HDACi, SAHA by the United State food and drugs administration (US-FDA), has been characterized by considerable research centered around the discovery and/or development of HDAC class-selective or isoform small molecule inhibitors for tumor therapy, with several candidates at different preclinical or clinical trial stages [7,8]. Based on its broad-spectrum activity and pluri-mechanistic modulation of oncogenicity in both solid and hematologic malignancies [9,10], we hypothesized that the hydroxamate-based polar SAHA alone or as component of a dual-agent therapy, through the epigenetic modulation of oncogenic signals, may actively suppress the hCSC-like phenotypes of CML-SCs, with limited or no clinical toxicities, unlike conventional chemotherapeutic agents [11].

Thus, in this present study, we explored the anti-CML-SCs potential of the SAHA and its ability to synergize with first-line CML chemotherapeutic, IM, by pharmacologically targeting posttranslational histone deacetylation, as well as interacting with and positively modulating the expression and/or activity of the small non-coding RNA, hsa-miR-196a. Data presented herein validate our hypotheses that SAHA potentiates IM and sensitizes CML-SCs to the therapeutic effect of IM, thus positioning SAHA as an efficacious small molecule anticancer therapeutic in both solid and hematologic malignancies, as well as a putative adjuvant in overcoming IM – resistance in CML cells.

2. Materials and methods

2.1. Reagents and drugs

SAHA (SML0061 SIGMA, ≥ 98% HPLC) and IM (SML1027 SIGMA, ≥ 98% HPLC) were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). Stock solutions of 100 mM in dimethyl sulfoxide (DMSO, Sigma-Aldrich) or sterile ddH_2O for SAHA or IM, were stored at −20 °C or 4 °C, respectively, until use. Antibodies against c-Myc, c-Met, Nanog, CD44, CD133, ERK, p-ERK, SUZ12, Ezh2, MDR, HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, Ring 1A, Ring 1B, α-tubulin and GAPDH were purchased from Cell Signaling Technology (CST, Beverly, MA, USA). Anti- Bad, p-Bad, Bax, Bim, Bcl-2, Survivin, CDK2, CDK4, CDK6 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Histone H3 (acetyl K9 + K14 + K18 + K23 + K27; Ac-H3; ab47915), pan-acetylated lysine (Ac-Lys; ab61257) and α-tubulin (acetyl K40; 6–11B-1; ab24610) were purchased from Abcam (Abcam Trading Co. Ltd). Alexa Fluor 647 donkey anti-rabbit IgG and Alexa Fluor 488 donkey anti-rabbit IgG were purchased from Invitrogen (Grand Island, NY, USA).

2.2. Cells and cell culture

Human BCR/ABL+ CML cell lines K562 (CCL-243) and KU812 (CRL-2099) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in Iscove's modified Dulbecco medium (IMDM) and RPMI-1640 medium, respectively. Both media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C, in 5% humidified CO_2 incubator. Cells were sub-cultured at 90% confluence and the media changed every 48 h.

2.3. Western blot analysis

Cultured CML cells were collected and lysates prepared. Protein lysates were heated for 5 min, then subjected to immunoblotting. Blots were blocked with 5% non-fat milk in TBST for 1 h, incubated at 4 °C overnight with specific primary antibodies against BCR/ABL (1:1000), Bax (1:1000), Bad (1:1000), p-Bad (1:1000), Bcl-2 (1:1000), Bim (1:1000), β-catenin (1: 1000), Survivin (1: 1000), c-Myc (1: 1000), MDR (1:2000), CD44 (1:2000), CD133 (1:1000), ERK (1:1000), p-ERK (1:1000), C-Met (1:1000), Nanog (1:1000), Ring 1A (1: 1000), Ring 1B (1: 1000), SUZ12 (1: 1000), Ezh2 (1: 1000), HDAC 1–6 (1:1000), Cyclin D1 (1: 1000), Cyclin D3 (1: 1000), CDK2 (1: 1000), CDK4 (1: 1000), CDK6 (1: 1000), p18 (1: 1000), p21 (1: 1000), p27 (1: 1000), Ac-H3 (1:1000), Ac-Lys (1:1000), Ac-α-tubulin (1: 1000), α-tubulin (1: 500), and GAPDH (1:1000). The polyvinylidene difluoride (PVDF) membranes were washed trice with TBST after incubation with the primary antibodies, then incubated with horseradish peroxidise (HRP)-labeled secondary antibody at room temperature for 1 h and washed with TBST again before band detection using the enhanced chemiluminescence (ECL) Western blotting reagents and imaging with the BioSpectrum Imaging System (UVP, Upland, CA).

2.4. Cell viability and drug combination assays

CML cells were seeded in triplicates in 96-well microtitre plates at a density of 4×10^3 cells/well in supplemented medium, incubated in humidified 5% CO_2 at 37 °C for 24 h before exposure to different concentrations of the therapeutic agents, SAHA and/or IM for 48 h. Drug cytotoxicity and cell proliferation were assessed by sulforhodamine B (SRB) colorimetric assay as previously described [12]. Untreated wild-type cells served as control. Assay was performed two times in triplicates. Optical density (OD) was measured at 495 nm wavelength, using SpectraMax microplate reader (Molecular devices, Kim Forest Enterprises Co., Ltd, Taiwan). The SAHA + IM combination effect in CML cells were analyzed using the Chou-Talalay multi-drug effect analysis method [13]. Combination index (CI) was estimated with the CompuSyn software (CompuSyn, Inc., Paramus, NJ, USA); herein, CI $<<$ 1.0, CI = 1.0 and CI $>$ 1.0 defined synergism, additivity and antagonism, respectively.

2.5. Flow cytometry cell cycle analysis of CML cells

CD34+/CD38− CML cells in exponential phase growth were treated with indicated concentrations of SAHA and/or IM for 24 h, while wild-type cells treated with DMSO served as control. Cells were collected, fixed in ice-cold 70% ethanol, phosphate-buffered saline (PBS)-washed, treated with RNase A (50 μg/ml) for 30 min at 37 °C,