Published in final edited form as:

Exp Hematol. 2019 April ; 72: 36–46. doi:10.1016/j.exphem.2018.12.007.

Compounds targeting Class II histone deacetylases do not cause panHDACi associated impairment of megakaryocyte differentiation

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Abstract

Histone deacetylase inhibitors (HDACi) have demonstrated effectiveness against lymphomas and myelomas in clinical practice. However, common to all currently approved broad acting HDACi (panHDACi) is dose limiting thrombocytopenia, which has prevented wider use in cancer therapy. Using CD34⁺ hematopoietic stem cells (HSC), here we show megakaryocyte (MK) cell maturation/differentiation is impaired by broad acting (pan) HDACi, correlating to clinical thrombocytopenia. Importantly, we demonstrate that inhibitors of class II HDACs including LMK235 and tubacin at clinically relevant concentrations do not affect megakaryocyte maturation. Furthermore, we show that HDACi-induced impairment of MK differentiation is associated with reduction of protein levels of the transcription factor GATA-1, but not tubulin hyperacetylation. Finally, we report that panHDACi trigger a rapid loss of GATA-1 protein via a proteasome-dependent pathway. Taken together, our data support the notion that specifically targeting class II HDAC in cancer treatment is a potential strategy that would offer a safer alternative than current panHDAC inhibitors.

Graphical Abstract

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Authors Declare no conflict of interest.

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Keywords

HDAC; HDAC inhibitor; LMK235; Tubacin; thrombocytopenia

Introduction

The first generation of HDAC inhibitors (HDACi) has showed significant efficacy in inhibition of cancer cell proliferation both in *in vitro* and in xenograft *in vivo* models. These inhibitors are generally broad acting (pan), inhibiting a number of HDACs with increasing class preference depending on the concentration. Currently four panHDACi, i.e., SAHA, Panobinostat, Romidepsin and Belinostat, have been approved by the FDA as epigenetic therapies, mainly for treatment of T-cell lymphomas and multiple myelomas. However, clinical panHDACi are limited by serious adverse effects, most notably thrombocytopenia, which is often dose limiting.[1, 2] Limiting dosing, limits the effectiveness and broader use of panHDACi as anti-cancer agents. Identifying specific members of HDACs as cancer therapeutic targets would allow for the development of new generation of HDACi to minimize the risk of thrombocytopenia.

Several reports suggest that the impairment of MK maturation is the cause of panHDACi-induced thrombocytopenia. Preclinical models showed that panHDACi causes no change in serum TPO, or platelet/bone marrow toxicity, but led to elevated immature MKs in spleen and bone marrow.[3] The panHDACi-induced impairment of megakaryopoiesis has been correlated with tubulin hyperacetylation and loss of GATA-1 function.[3–5] However, the underlying molecular mechanisms remain unclear.

Using *in vitro* HSC cell differentiation to MK cells as a model of megakaryopoiesis, we compared the effects of current panHDACi versus specific inhibitors targeting HDAC4, 5 and 6 on HSC (CD34⁺). In addition, using both primary and cell line models, we further explored the role of GATA-1 loss in MK cell maturation, as well as the underlying mechanisms. We provide convincing data to demonstrate LMK235, an HDAC5 (IC₅₀=4 nM) and 4 (IC₅₀=12 nM) inhibitor, does not affect MK maturation, or protein levels of GATA-1 at biologically relevant concentrations. We also show that tubacin, an established HDAC6 specific inhibitor (IC₅₀=4 nM) which causes profound tubulin hyperacetylation, had no

effect on GATA-1 levels or MK maturation. Taken together, these data support the notion that specifically targeting members of class II HDACs, such as HDAC4,5,6, as cancer therapy, may potentially avoid thrombocytopenia caused by panHDACi.

Methods

Cell Culture, Differentiation and Proliferation Assays

Human CD34⁺ hematopoietic stem cells (mixed donor) were purchased from Stem Cell Technologies (cat#70008, Vancouver, Canada) and expanded in StemSpan SFEMII media (cat# 09605) supplemented with StemSpan megakaryocytes (MK) expansion supplement (cat# 02696; both from Stem Cell Technologies) to induce differentiation. To differentiate MK cells; 3,000 CD34⁺ cells were plated in 100 μ L (per well of a 96-well dish) of the complete culture medium (StemSpan® SFEMII medium supplemented with StemSpan® CC220). Cells were incubated in humidified 5%CO₂/95% air atmosphere at 37°C for 7 days. After 7 days of culture, each well was topped up with 100 μ L of fresh complete culture medium. At day 14, cells were processed for flow cytometry analysis. Imaging was conducted using a Zeiss Axiovert 25 and Axiocam 503 mono camera. CD34⁺ cells were imaged in 96-well plates using a 40x objective. Total cells were counted using a hemocytometer.

Meg-01 cells were purchased from ATCC (Manassas, VA) and were grown in RPMI 1640 containing 2 mM glutamine, 10mM HEPES, 1 mM sodium pyruvate, and 4500 mg/L sodium bicarbonate supplemented with 10% FBS and 1% penicillin/streptomycin. All cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

The human ovarian carcinoma cell line A2780 was purchased from Sigma-Aldrich and grown in Eagle's Minimum Essential Medium (EMEM) containing 2 mM glutamine, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate supplemented with 10% FBS and 1% penicillin/streptomycin. Proliferation was assessed using a CyQUANT cell proliferation kit (Thermo Fisher Scientific, Waltham, MA).

Special Compounds, Agents and Cell Treatment

LMK235 (cat#4830), SAHA (cat#4652) and Tubacin (cat#3402) were purchased from Tocris Biosciences (Minneapolis, MN). Panobinostat (cat#A8178) was purchased from ApexBio (Houston, TX). MG132 (cat# 101760–492) was purchased from Selleck Chemicals (Houston, TX).

All cell DMSO control treatments contained 0.1% DMSO.

Western Blotting Assays

Western blotting reagents were purchased from Thermo Fisher Scientific unless otherwise stated. Cells were lysed in M-Per mammalian protein extraction reagent (cat#78501) supplemented with Halt® protease and phosphatase inhibitor cocktail, following manufacturer's instructions. Quantification of total protein was achieved by using a bicinchoninic acid (BCA) assay. For each sample, 20–40 μ g of protein were separated by

electrophoresis using 12% Tris-Glycine gels. Protein transfers were performed using the iBlot2 system (20V for 1 minute, 23V for 3 minutes, and 25V for 2 minutes setting). PVDF membranes were blocked in 4% milk- Tris Buffered Saline with Tween 20 (TBST) buffer for 1 h, incubated in primary antibody overnight followed by the HRP secondary for 1 h.

The sources of primary antibodies used were: GATA-1 (Abcam; cat#89505, mouse monoclonal), acetylated tubulin (Santa Cruz, Dallas, TX; cat#sc-23950, mouse monoclonal), alpha tubulin (Abcam; cat#4074, rabbit polyclonal), histone H3 (ProSci, Poway, CA; cat#31007), and acetylated histone H3 (Thermo Fisher Scientific; Lys6, cat#MA5-11195 and Lys36, cat#MA-24672). Secondary HRP labeled, anti-mouse (cat#6728) and anti-rabbit (cat#6721) antibodies were purchase from Abcam. Membranes were incubated for 1 min in ECL reagent and captured using ChemiDoc MP (BioRad, Hercules, CA).

Flow Cytometry

Differentiated MK cells were centrifuged (5 wells of a 96-well dish were pooled) for 10 minutes at $800 \times g$ and washed $2 \times$ in PBS. The cells were then resuspended in 100 μ L staining buffer (cat# 420201; Biolegend, San Diego, CA) to which 5 μ L of 7-amino-actinomycin D (7-AAD; Thermo Fisher Scientific), 20 μ L of fluorescein isothiocyanate (FITC) -conjugated anti-CD41 (Becton-Dickinson, Mountain View, CA), and 20 μ L of phycoerythrin (PE)-conjugated anti-CD42b (GP1BA) antibodies (Becton-Dickinson) were added and incubated for 20 minutes at room temperature. The fraction of 7-AAD positive cells (non-viable cells) were excluded from the analysis. Anti-CD41 and anti-CD42b were used to label total differentiating MK and mature MK cells, respectively. Scatter plots and MK maturity dot plots represent data generated from the same well/sample. Ploidy was generated from the adjacent duplicate wells to avoid PI and PE (anti-CD42b) interference. Ploidy evaluation studies were modified from previously published protocols.[6, 7] Briefly, cells were fixed overnight at 4°C in 100% ethanol, followed by the DNA staining with 0.05 mg/mL of propidium iodide (PI) for 20 minutes in the dark. Data was acquired using a BD LSR Fortessa flow cytometer and analyzed using a BD FACSDiva software (Becton-Dickinson) and FlowJo software (FlowJo, LLC, Ashland, OR). All experiments were repeated at least three times.

qRT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Germantown, MD). RT reaction was performed using SuperScript VILO master mix (Thermo Fisher) to generate cDNA. PCR was performed using TaqMan Universal PCR master mix with a ViiA 7 real-time PCR system (Thermo Fisher). All primer/probes were purchased from Thermo Fisher.

Data Analysis

Graphs and statistical analyses were generated using GraphPad Prism software v7 (La Jolla, CA). Mean values were considered statistically significant with a *p* value <0.05.

Results

Targeted inhibition of HDAC4,5 does not impair megakaryocyte differentiation

To address if specifically inhibiting HDAC4,5 has an effect on MK maturation, we induced human HSC (CD34⁺ primary cells) to differentiate to mature MK by following established procedures in the presence or absence of pharmacologically equivalent concentrations of LMK235, SAHA and Panobinostat.[5, 8] Relevant testing concentrations of panHDACi (SAHA and Panobinostat), were established using respective clinical C_{max} (maximum clinical serum concentration) values as reference. LMK235 was tested at concentrations sufficient to increase α -tubulin acetylation, a known HDAC5 substrate.[9] In addition, the concentrations tested were 25–40 times that of enzymatic IC₅₀. After 14 days of directional induction, we characterized and quantitated the cell population using FACS analyses and light microscopy. Treating HSC cells with LMK235 (at 200 nM) had no apparent negative effects on MK differentiation and maturation, as noted by the cell morphology dot plots and numbers of mature MK cells (Figure 1A; top and middle rows, respectively). Two distinct populations (P1 and P2) of high scatter cells were identified and P2 was further characterized as mature MK (CD42b⁺/CD41⁺) while P1 was mainly CD42b^{low}/CD41^{low}, thus representing undifferentiated cells. In contrast, Panobinostat had significant impact on MK maturation, with most of the HSC cells remaining undifferentiated (CD42b^{low}/CD41^{low}). Consistent with previous reports, Panobinostat as low as 5 nM, significantly decreased the numbers of mature MK cells (CD42b⁺/CD41⁺), i.e., from 44% (control) to 12% (Figure 1B). Treatment with SAHA at 500 nM also caused a reduction in MK maturation (from 44% to 20%). When tested at, or slightly below their clinical C_{max} concentrations, both SAHA and Panobinostat resulted in more than a 50% reduction in mature MK, consistent with their high frequency of thrombocytopenias reported in clinical cancer treatment.[10, 11] Interestingly, despite the low number of MK mature cells, Panobinostat and SAHA treatments still resulted in a robust population of high scatter cells. Further characterization identifier two high scatter populations, labeled P1 and P2, with P2 having slightly higher FSC (Figure 1A; top row). Control or cells treated with LMK contained predominantly P2 population, while Panobinostat and SAHA treatments resulted in mostly P1 high scatter cells. In parallel, we show that P1 high scatter population lacked MK mature markers, while P2 cells represented mature MK cells (Figure 1A; middle row). Taken together, these data indicate that inhibition of HDAC4,5 does not impair MK differentiation *in vitro*, suggesting that specifically targeting HDAC4,5 may decrease likelihood of clinical thrombocytopenia.

Megakaryopoiesis is hyperplastic, hallmarked by an increase in cell size, shape, and DNA content (ploidy). Increasing MK ploidy coincides with an eventual increase in platelet size; with higher ploidy MKs (16N, 32N, and 64N) making most of the platelets in humans.[6] We analyzed MK polyploidization following Panobinostat, SAHA, and LMK235 treatments (Figure 1A bottom row). Following differentiation, higher ploidy MK are clearly present in the vehicle control and LMK235 treated samples. Coinciding with the lack of mature MKs, both Panobinostat and SAHA treatments significantly reduced cell ploidy, with higher ploidy MKs (>8N) low/not present with either panHDACi (Figure 1C). These observations further substantiate that both SAHA and Panobinostat impair MK differentiation.

Specific inhibition of HDAC4,5 does not inhibit CD34⁺ cell expansion

Next, we examined whether HDACi affects CD34⁺ cell expansion and morphology during the directed differentiation processes. To address this question, we cultured CD34⁺ cells in the presence of HDACi (500 μ M of SAHA, 5 nM of Panobinostat, or 75 nM, 200 nM and 400 nM of LMK235) for 8 days (which represents the middle point of the directed differentiation process) and captured images under bright field microscopy (Figure 2A). In addition, cell numbers were quantitated by hemacytometer (Figure 2B). Initially, 30,000 cells/mL were plated and 7h following plating cells were imaged as 'Day 0'. After 8 days of directed differentiation, control cells expanded to approximately 271,000 cells/mL. SAHA significantly reduced the number of expanded cells to approximately 145,000 cells/mL. Furthermore, using 5 nM Panobinostat, a concentration shown to be inhibitory but not cytotoxic,[4, 5] dramatically reduced cell numbers to approximately 68,000 cells/mL. The reduction in cell number expansion following SAHA or Panobinostat treatments coincided with an enrichment of cells with smaller sizes. Importantly, LMK235 up to 400 nM (100 $\times$ of enzymatic IC₅₀) had no significant effect on cell numbers. These data suggest that specifically inhibiting HDAC4,5 does not disrupt HSC expansion during the MK differentiation process.

Inhibition HDAC6 by tubacin has no effect on MK cell maturation

HDAC6 is a class II HDAC that specifically deacetylates acetylated tubulin. Inhibition of HDAC6 by tubacin, increases tubulin acetylation.[12, 13] To evaluate if hyperacetylation of α -tubulin (by inhibiting HDAC6 with tubacin) impairs MK maturation, we treated differentiating CD34⁺ cells for 14 days with tubacin. Tubacin at 300 nM, showed no adverse effect on MK maturation (Figure 3A). The percent of mature MK cells was 47% and 48% in control or tubacin treated cultures, respectively. In addition, P1 high scatter population, shown in Figure 1A to represent majority of mature MK cells, was present in the Tubacin treated cells as well. The experiment was repeated at least three times and confirmed no statistically significant difference in mature MK cells following tubacin treatment. In addition, there was no difference in cell ploidy (Figure 3B), confirming that tubacin also does not affect megakaryopoiesis.

LMK235 attenuates proliferation of ovarian cancer cells

Chemotherapy is an important component of the management of ovarian cancers. To evaluate the effects of LMK235 at given doses on cancer proliferation, we treated ovarian cancer cell line A2780 for 72h with LMK235, tubacin or panHDACi (Panobinostat and SAHA), and compared their effects on cancer cell proliferation (Figure 4). After 72h, all tested HDACi inhibited cancer cell proliferation rates, with potency of 200 nM LMK235 > 500 nM SAHA > 5 nM Panobinostat > 75 nM LMK235 > 300 nM tubacin. These data suggest that the concentrations of LMK235 used to evaluate MK maturation were biologically relevant.

GATA-1 loss, but not hyperacetylation of α -tubulin, is correlated to panHDACi-mediated impairment of MK differentiation

The mechanisms underlying clinical thrombocytopenia induced by panHDACi remains unclear. A reduction in MK maturation caused by impairment of the GATA-1 transcriptional function [3] has been proposed, but underlying mechanism has not been clearly delineated. To explore how inhibition of class II HDACs avoids MK differentiation impairment associated with panHDACi, we treated Meg-01 cells for 48h with LMK235, Panobinostat or SAHA, and investigated effects on the acetylation status of α -tubulin and histone H3, and GATA-1 protein and mRNA levels (Figure 5A and B). We observed that both Panobinostat and SAHA resulted in a significant increase in acetylated α -tubulin, and a profound loss of GATA-1 protein (Figure 5A). LMK235 increased α -tubulin acetylation but did not lead to loss of GATA-1 protein levels. Similarly, tubacin strongly induced α -tubulin acetylation without a detectable decrease in GATA-1 levels. We also noted that all HDACi tested had no significant effects on GATA-1 mRNA levels (Figure 5B), in line with previously reported findings.[5, 11] Both panHDACi (SAHA and Panobinostat) significantly induced histone 3 (H3) acetylation (Figure 5C). Although HDAC4, 5 and 6 mainly affect acetylation of non-histone proteins, slight increase in histone H3 acetylation have been reported.[14] In line with published data, a slight increase in H3 acetylation caused by LMK235 and tubacin was also observed.

To further investigate the mechanism of GATA-1 protein loss, we evaluated if the proteasome system is required for this degradation. We tested if proteasome inhibitor MG132 would prevent the loss of GATA-1 caused by panHDACi (Figure 5D). We treated cells with Panobinostat or SAHA for 6h and observed reduced levels of GATA-1 proteins; however, when cells were co-treated with MG132, GATA-1 levels remained the same or slightly increased. These data establish for the first time that GATA-1 loss caused by panHDACi is mediated by a proteasome-dependent degradation.

Discussion

Our findings show that specifically targeting HDAC4, 5 and 6 may potentially avoid thrombocytopenia, a detrimental adverse effect often caused by panHDACi. We further outline that panHDACi, Panobinostat and SAHA at concentrations relevant to their clinical C_{max} impaired MK cell maturation as noted by reduced number of mature MKs, paralleled by reduced cell ploidy. This effect appears to be in part mediated by the reduction in level of the GATA-1 transcription factor. We also show that GATA-1 loss, but not tubulin hyperacetylation, is critically correlated to the impairment of MK maturation. The HDAC6 specific inhibitor tubacin, which causes α -tubulin hyperacetylation, but does not affect GATA-1 protein levels, had no effect on MK maturation, further supporting the notion that loss of GATA-1 protein is the underlying mechanism of panHDACi-associated thrombocytopenia. Finally, for the first time we show that the GATA-1 protein is rapidly degraded by a proteasome-dependent pathway following panHDACi treatment.

A previous study demonstrated that although panHDACi reduces platelet numbers, it also increases bone marrow MKs in wild type mice[4], suggesting that panHDACi did not affect MK differentiation. However, the increase in bone marrow MK cells following panHDACi

treatment are likely accumulation of immature MKs, cells that do not produce platelets. Follow-up studies using a more definitive CD34⁺ maturation assay showed a clear effect of Panobinostat on MK maturation,[5] which is consistent with our current findings. Importantly, we show that this maturation disruption is correlated with the loss of the transcription factor GATA-1, but not α -tubulin acetylation. GATA-1 is a critical myeloid transcription factor, regulating the differentiation of the MK and erythroid cell lineages. Mice and humans with GATA-1 mutations accumulate immature MK and/or MK-erythroid progenitors.[15–17] Earlier studies using panHDACi noted no change in GATA-1 mRNA levels with a coinciding tubulin hyperacetylation[5], leading to the proposal that GATA-1 expression was not affected by panHDACi treatment. Our qRT-PCR-based gene expression analysis confirmed no change in mRNA levels of GATA-1. However, the protein levels of GATA-1 rapidly decreased following Panobinostat or SAHA treatment, indicating that the reduction in GATA-1 levels is more likely caused by posttranscriptional mechanisms. Proteasome inhibitor MG132 blocks panHDACi-mediated loss of GATA-1 protein, suggesting that panHDACi triggers a proteasome-dependent degradation of GATA-1. Our finding that panHDACi causes GATA-1 degradation may explain another common adverse effect of panHDACi, namely anemia.

Interestingly, a previous study reported that CREB-binding protein (CBP) catalyzed acetylation of GATA-1,[18] which destabilizes GATA-1.[19] Moreover, it has been reported that panHDACi can synergize CBP/p300 catalyzed acetylation.[20] Whether the GATA-1 destabilization caused by panHDACi is mediated by an enhanced GATA-1 acetylation remains to be investigated.

HDAC1 and HDAC2 knockout mice have shown thrombocytopenia [21] and Romidepsin, which predominantly inhibits class I HDACs, also causes thrombocytopenia,[22, 23] strongly indicating that the panHDACi-associated thrombocytopenia is linked to the inhibition of HDAC1 and HDAC2. In addition, an HDAC6 (class II) inhibitor Ricolinostat was previously shown to have no effects on MK differentiation.[24]

Although class IIa members HDAC4 and 5 are not essential for normal function of most somatic cells (based on mouse knockout data),[25] HDAC5 has been implicated in regulating erythro- and megakaryopoiesis; its function is thought to be limited to maintaining an early progenitor pool as part of the transcriptional inhibitory complex.[26–28] However, it was shown that during early erythropoiesis, HDAC5 represses key hematopoietic differentiation transcription factors and keeps the cells in the progenitor state. Although erythro- and megakaryo-poiesis share some similarities, they differ when it comes to epigenetic transcriptional regulation. A recent report showed that erythroid transcriptional complex NuRSEY containing HDAC5 is present in erythroid but not in megakaryocytic lineage cells.[28] However, it was also shown that inhibition of HDAC5 had no effect on apoptosis or survival of erythroid cells, alluding to the safety of HDAC5 inhibition in hemostasis.[28] On the other hand, it was shown that a reduction in HDAC5 activity was required for GATA-1 mediated hematopoiesis, leading to the hypothesis that targeting HDAC5 may actually improve hematopoiesis by stimulating differentiation of erythrocytes and megakaryocytes.[26]

It has been shown that HDAC5 plays a major role in cancer stress response and survival.[9, 14, 29, 30] Typically HDAC5 is expressed in tissues requiring high levels of ATP (heart, neuronal, and placenta) and during energy inadequate states (hypoxia, low glucose, and amino acid deprivation).[31, 32] HDAC5 overexpression was observed in a number of different tumors, suggesting a role in cancer. In tumors, cytosolic HDAC5 may act to stabilize oncogenic proteins during pervasive metabolic or chemotherapy stress.[33] To this point, we show that LMK235, at concentrations sufficient to inhibit cancer cell proliferation, resulted in no disruption of MK cell maturation. This strongly suggests that targeting HDAC5 would avoid interference with CD34⁺ cell maturation, making HDAC5 an attractive (and safe) target in sensitizing cancer cells to metabolic stress and chemotherapy.

A recent clinical trial of HDAC6 (class II) inhibitor Ricolinostat, in combination with bortezomib and dexamethasone, was shown to have no negative effects on platelet formation. [24] Another study also reported that HDAC6 inhibition-induced tubulin hyperacetylation has no effect on proplatelet formation. However, the latter also reported that HDAC6 inhibition or genetic knockdown led to a strong decrease in human proplatelet formation.[34] It was proposed that the decrease in proplatelet induced by HDAC6 inhibition was attributed to cortactin hyperacetylation, associated with actin disorganization inducing significant changes in the distribution of megakaryocyte (MK) organelles. This report highlights that the effects of HDAC inhibition on the final step of platelet formation also needs further evaluation. It should be noted that although CD34⁺ cell differentiation assay is a well-established approach for evaluating MK maturation *in vitro*, it does not evaluate platelet release from mature MK *in vivo*. Nevertheless, these findings provide a rationale for *in vivo* studies to further address the effects of HDACi on platelet release and clotting function.

Based on our findings and previously published reports, we outline a mechanistic explanation of panHDACi-related hematologic adverse effects and propose new strategies for cancer therapy based on selective targeting of class II HDACs, particularly HDAC4,5 (Figure 6). We propose that HDAC1 and HDAC2 are required for MK maturation and possibly erythropoiesis, whereas HDAC4, 5, 6 are not critical for MK differentiation. Therefore, specifically targeting HDAC4 and 5 as anti-tumor strategies may provide clinical benefit by avoiding thrombocytopenia, a common panHDACi adverse event.

Acknowledgements

We thank Ms. Marry Malley and Dr. Shannon Dallas for editorial suggestions. Authors declare no financial interest relating to the topic of this article. Research in Dr. Sang's lab is supported in part by grant R01-CA129494 (to NS) from NCI, National Institutes of Health (NIH), a research contract from Lipogen, and a CURE grant from the State of Pennsylvania. DS would like to thank Dr. Monicah Otieno, Dr. Felice Elefant, and Dr. Tali Gidalevitz for their guidance and helpful suggestions.

List of Abbreviations:

GATA-1	GATA-binding factor 1
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor

HSC	Hematopoietic stem cells
MK	Megakaryocyte
Pano	Panobinostat (aka. Farydak)
SAHA	Suberoylanilide hydroxamic acid (aka. Vorinostat)
TPO	Thrombopoietin

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HIGHLIGHTS

- PanHDAC inhibitors cause GATA-1 degradation, which is associated with clinical thrombocytopenia
- Selective inhibition of HDAC4, 5 or 6 avoids impairment of megakaryocyte differentiation
- Tubulin acetylation caused by HDAC inhibitors does not affect megakaryocyte differentiation.

Figure 1. Specifically targeting HDAC4,5 by LMK235 avoids impairing megakaryocyte differentiation.

CD34⁺ HSC cells were cultured and induced to differentiate to MK in the presence of 0.1% DMSO only (control), LMK235 (75 and 200 nM), Panobinostat (5 nM), or SAHA (500 nM) for 14-days followed by FACS analyses.

A. Top row: Dot plot of forward scatter (FSC) versus side scatter (SSC) outlining MK cell size and shape, respectively. Two distinct populations (P1 and P2) of high scatter cells were identified, and P2 was further characterized as mature MK while P1 was mainly undifferentiated MK. Middle row: To evaluate MK cell maturity, cells were labeled with

FITC anti-CD41 (marker present on all MK cells) and PE-anti-CD42b (mature MK cell marker). Analytical gate was set to include only viable cells (7-AAD negative). Dot plot upper right quadrant (Q2) indicates mature, differentiated MK cells. Percentages of cells were given in charts. As part of the high scatter population characterization, P1 cell population was labeled red, higher FSC population P2 was labeled blue color and the rest of the viable cell were black. Bottom row: Cell ploidy was evaluated by staining DNA with propidium iodide (PI) and quantifying PI ploidy classes in CD41⁺ cells. The arrows indicate ploidy classes from 2N to >32N; N=3.

B. Following 14-day induced differentiation of CD34⁺ cells, mature MK cells were quantified in three independent experiments, and presented as means with error bars representing standard deviations. Control samples were set as 100%, and HDACi treated cells were compared to controls. *** p<0.005; NS = not significant; N=3.

C. Cell ploidy was quantified following three independent experiments and presented as means with error bars representing standard deviations. For each ploidy, treated samples were compared to the respective control samples. Ploidy was presented as the absolute number of CD41⁺ cells. Following 14-day maturation, high ploidy MK cells were evident in the control and LMK treated samples. Treatment with Panobinostat at 5nM or SAHA 500nM, significantly decreased cell ploidy. * p<0.05; ** p<0.01; *** p<0.005; NS = not significant; N=3.

Figure 2. Effects of different HDACi on MK cell expansion during maturation.

A. HSC CD34⁺ cells were plated at 30,000 cells/mL and were induced to differentiation for 8 days in the presence of 0.1% DMSO (control), LMK235 or SAHA and Panobinostat as positive controls. Data indicate that LMK235 at 75, 200 and 400 nM resulted in the cell number and morphology of MK similar to those of DMSO treated control cells. Panobinostat treatment resulted in a significant reduction in the cell number and morphologically smaller cells, indicating immature cells. Similarly, SAHA (at 500 nM)

reduced the overall number of cells, however, some large MK were evident. Images were captured using $40\times$ objective.

B. CD34⁺ cells were quantified using hemocytometer. Cells were quantified based on three independent experiments and presented as means with error bars representing standard deviations. *** $p<0.005$.

Figure 3. Tubacin does not impair MK differentiation.

A. Primary CD34⁺ cells were induced to differentiate to MK cells for 14 days in the presence of 0.1% DMSO (control) or tubacin at 300 nM. Flow cytometric analysis was carried out subsequently. Top row: Dot plot of forward scatter (FSC) versus side scatter (SSC) outlining MK cell size and shape, respectively. Two distinct populations (P1 and P2) of high scatter cells, were identified and further characterized for MK cell maturity. Middle Row: To evaluate MK cell maturity, cells were labeled with FITC anti-CD41 (marker present on all MK cells) and PE-anti-CD42b (mature MK cell marker). Analytical gate was

set to include only viable cells (7-AAD negative). Dot plot upper right quadrant (Q2) indicates mature, differentiated MK cells. As part of the high scatter population characterization, P1 cell population was labeled red, higher FSC population P2 a blue color and the rest of the viable cell were black. Bottom row: Cell ploidy was evaluated by staining DNA with propidium iodide (PI) and quantifying PI ploidy classes in CD41⁺ cells. The arrows indicate ploidy classes from 2N to >32N. All experiments were performed three times and representative results were shown.

B. Cell ploidy was quantified following three independent experiments and presented as means with error bars representing standard deviations. For each ploidy, tubacin treated samples were compared to the respective control samples. NS=not significant; N=3.

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Figure 4. HDACi inhibit ovarian cancer cell proliferation.

Ovarian adenocarcinoma cells A2780 was treated for up to 72h with LMK235 (75 and 200 nM), tubacin (300 nM), SAHA (500 nM) or Panobinostat (5 nM). Cell proliferation was quantified using CyQUANT DNA dye and presented as absolute cell numbers based on the standard curve. Experiments were carried out with 4 biological repeats. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Figure 5. panHDACi trigger proteasome-dependent GATA-1 degradation.

Meg-01 cells were treated with either 0.1% DMSO or HDACi at indicated concentrations for 48h, total cell lysates were prepared and examined by Western blotting analysis.

A. Panobinostat and SAHA reduced GATA-1 levels and increased α -tubulin acetylation. LMK235 also increased α -tubulin acetylation but did not decrease GATA-1 levels. Similarly, Tubacin increased α -tubulin acetylation but did not suppress GATA-1 levels. Total α -tubulin levels were detected as loading control.

B. Treatment with HDACi resulted in no significant change in GATA-1 mRNA levels. Meg-01 cells were treated for 48h with indicated compounds, and total RNA were extracted. GATA-1 mRNA expression levels were evaluated by qRT-PCR, Ct was calculated by normalized GATA-1 gene Ct values to 18S RNA. Bar graph represents means of fold expression (compared to control) with error bars as standard deviations from three qRT-PCR reactions.

C. Inhibition of HDACs induces histone H3 acetylation. SAHA and Panobinostat significantly induced histone acetylation. HDAC Class II inhibitors LMK235 and Tubacin induced histone H3 acetylation but at much lower intensity, consistent with the roles of class II HDACs as non-histone deacetylases.

D. PanHDACi mediated GATA-1 protein degradation is proteasome-dependent. Meg-01 cells were treated for 6h with Panobinostat (50 nM) and SAHA (500 nM) alone or in combination with proteasomal inhibitor MG132 (5 μ M). Cell lysates were prepared, and GATA-1 protein levels determined by Western blotting analysis.

Figure 6. Effects and proposed mechanisms of cancer therapeutic HDACi on MK differentiation. Based on current and published literature, we conclude that panHDACi cause impairment of megakaryopoiesis mainly through the inhibition of HDAC1 and HDAC2. Since HDAC1,2 function is required for GATA-1 stabilization, the inhibition of HDAC1,2 leads to GATA-1 degradation, which may be mediated by GATA-1 acetylation. Compounds specifically targeting Class II HDACs such as 4, 5 and 6 avoid disrupting HDAC1,2 function, thus avoiding GATA-1 degradation and subsequent impairment of differentiation of hematopoietic progenitor cells. Therefore, specifically targeting class II HDACs as part of cancer therapy, may emerge as a safer approach than non-selective HDAC inhibition.