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Research Paper

Metabolomic Profiling Reveals Cellular Reprogramming of B-Cell Lymphoma by a Lysine Deacetylase Inhibitor through the Choline Pathway

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ABSTRACT

Despite the proven clinical antineoplastic activity of histone deacetylase inhibitors (HDACI), their effect has been reported to be lower than expected in B-cell lymphomas. Traditionally considered as "epigenetic drugs", HDACI modify the acetylation status of an extensive proteome, acting as general lysine deacetylase inhibitors (KDACI), and thus potentially impacting various branches of cellular metabolism. Here, we demonstrate through metabolomic profiling of patient plasma and cell lines that the KDACI panobinostat alters lipid metabolism and downstream survival signaling in diffuse large B-cell lymphomas (DLBCL). Specifically, panobinostat induces metabolic adaptations resulting in newly acquired dependency on the choline pathway and activation of PI3K signaling. This metabolic reprogramming decreased the antineoplastic effect of panobinostat. Conversely, inhibition of these metabolic adaptations resulted in superior anti-lymphoma effect as demonstrated by the combination of panobinostat with a choline pathway inhibitor. In conclusion, our study demonstrates the power of metabolomics in identifying unknown effects of KDACI, and emphasizes the need for a better understanding of these drugs in order to achieve successful clinical implementation.

1. Introduction

Despite the promising activity of histone deacetylase inhibitors (HDACI) in the management of T-cell lymphoma subtypes (Apuri and Sokol, 2016), their relatively low efficacy in unselected patients with diffuse large B-cell lymphoma (DLBCL) (Crump et al., 2008) uncovers the need for a better understanding of their biological effects to successfully implement this class of drugs in clinics.

Traditionally considered as "epigenetic drugs" due to the impact of histone acetylation on the modulation of chromatin structure and subsequent transcription regulation (Hebbes et al., 1988), HDACI affect the acetylation of a plethora of non-histone proteins (Choudhary et al., 2009), and can therefore be more appropriately reffered to as lysine deacetylase inhibitors (KDACI). Acetylation of non-histone substrates can have various effects on function, stability and localization of proteins. For example, the administration of KDACI to DLBCL cells can simultaneously result in increased acetylation and inhibition of Hsp90 and BCL6, and furthermore in acetylation and activation of p53 (Bereshchenko et al., 2002; Cerchietti et al., 2010; Rao et al., 2009).

Acetylation state is tightly balanced through reactions catalyzed by lysine acetyltransferases and lysine deacetylases (KDAC). The interplay between acetylation and deacetylation of proteins is crucial for various important mechanisms that maintain cellular and metabolic homeostasis (Choudhary et al., 2014). However, the precise metabolic consequences of changes in the acetylation state of non-histone proteins are still understudied in patients. Given the interconnection between protein acetylation and metabolism, we hypothesized that the characterization of metabolic effects of KDACI in DLBCL patients will uncover pathways relevant for lymphoma survival and potentially identify novel targets for combination therapies.

Metabolomics is a powerful tool to profile small molecules in biological specimens. The metabolome has been regarded as the link between genotype and phenotype (Fiehn, 2002), and represents a readout of the current state of an organism (Kaddurah-Daouk et al., 2008).

Here, by performing metabolomic profiling, we revealed that the KDACI panobinostat administered to DLBCL patients alters lipid metabolism impacting, in particular, choline metabolism. In DLBCL cell lines, panobinostat induces metabolic adaptations that lead to activation of the PI3K pathway inducing a newly acquired dependency on choline metabolism for survival. In a xenograft DLBCL model, we then prove

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that inhibition of choline or PI3K pathways results in increased anti-lymphoma effect of panobinostat.

2. Materials and Methods

2.1. Metabolomics

We analyzed plasma from a sub-set of 24 DLBCL patients enrolled in the phase 2 trial NCT01238692 with the approval of the Weill Cornell Medicine IRB. Untargeted metabolomics measurements on plasma were performed by Metabolon Inc., NC, USA. Briefly, measurements were performed by ultra-high-performance liquid-phase chromatography and gas-chromatography separation, coupled with tandem mass spectrometry. Each metabolite was annotated with a) one of seven major biochemical super-pathways ("amino acid", "peptide", "lipid", "energy", "carbohydrate", "nucleotide" and "cofactors & vitamins"), and b) one sub-pathway (Table S1). Metabolites classified as "xenobiotic" and those with unknown identity were omitted for this study. Treatment effects for each metabolite were assessed using paired *t*-tests. False discovery rate (FDR) control according to Benjamini and Yekutieli (Benjamini and Yekutieli, 2001) was used to correct for multiple hypothesis testing. Volcano plots were generated by plotting the $\log_2$ fold change of metabolite levels against FDR values for the differences.

For cell metabolomics, the DLBCL cell line OCI-Ly1 was treated with 1 μM of panobinostat for 12 h. Metabolomics analysis was performed on the same LC/GC–MS platform as the plasma data. Each sample was normalized by dividing raw values by cell protein contents, assessed via Bradford assays performed by Metabolonc Inc. Statistical analysis was performed analogously to the plasma analysis, with two-sample *t*-tests to compare treated and untreated groups (Table S2).

2.2. Reagents

Panobinostat, AZD8186, GDC-0941, CUDC-907 and the compounds from the screened library (Table S3) were from Selleckchem. CK37 was from EMD Millipore. Phosphatidic acid was from Avanti Lipids. Choline chloride was from Sigma.

2.3. Cell Culture

Human diffuse large B-cell lymphoma (DLBCL) cell lines OCI-Ly1 and OCI-Ly7 were grown in Iscove's modified Eagle medium and 10% fetal bovine serum (FBS) (20% for OCI-Ly7) supplemented with 1% penicillin G and streptomycin, 1% L-glutamine, and 1% HEPES. Toledo and SU-DHL-8 were grown in medium containing 90% RPMI-1640 and 10% FCS supplemented with 1% penicillin G and streptomycin, 1% L-glutamine, and 1% HEPES. Cells were maintained in a 37 °C, 5% CO_2, humidified incubator. All cell lines were purchased from the American Type Culture Collection or obtained from the Ontario Cancer Institute, and regular testing for Mycoplasma sp. and other contaminants and cell identification by single nucleotide polymorphism were conducted.

2.4. Cell Viability Assays

Growth inhibition 50 (GI50) was determined by a fluorescence assay using 7-hydroxy-3H-phenoxazin-3-one 10-oxide (CellTiter-Blue, Promega) according to the manufacturer's protocol. Cell viability was determined by employing a fluorescence-based assay that relies in live-cell protease activity (CellTiter-Fluor, Promega) following the manufacturer's protocol. All fluorescence measurements were performed in a Synergy4 microplate reader (BioTek).

Caspase assays. Caspase-3 and -7 activity was assessed using the Apo-ONE caspase 3/7 assay (Promega) following the manufacturer's instructions with measurement of fluoresence emission in a Synergy4 microplate reader (BioTek). Caspase activity was normalized by the cell number determined by CellTiter-Fluor (Promega).

2.5. Transient Transfection

OCI-Ly1 cells (2.5×10^6 cells/well) were transfected by electroporation (Amaxa, Lonza AG) in presence of 100 nM of CHKA siRNA (CHKAHSS140690 and CHKAHSS140691, Invitrogen).

2.6. Real-Time Reverse Transcriptase-PCR

Total RNA was purified using TRIzol Reagent (Thermo Fisher Scientific) following manufacturer's instructions and resuspended in RNAse-free water. cDNA was synthesized using high capacity RNA-to-cDNA kit (Applied Biosystems). SYBR Green FastMix was from Quanta BioSciences. Primer sequences can be found in Table S4.

2.7. Immunoblotting

Protein concentrations were determined using the BCA kit (Pierce Biotechnology) according to the manufacturer's instructions. Protein lysates (15–40 μg) were electrophoretically resolved by SDS/PAGE, transferred to PVDF (polyvinylidene difluoride) membrane, and probed with the indicated primary antibodies: Anti-Choline Kinase α (D5X9W) (1:500, 13,422; Cell Signaling), Anti-Acetyl-Histone H3 (Lys9/Lys14) (1:1000, 9677; Cell Signaling), Anti-Phosphate Cytidylyltransferase 1 (1:1000, 109,263, Abcam). Membranes were then incubated with a 1:5000 dilution of a peroxidase conjugated corresponding secondary antibody (sc-2004 and sc-2005, Santa Cruz Biotechnology). Equal loading of the protein samples was confirmed by α-tubulin (1:25,000, ab4074; Abcam) blotting. We used ECL Western Blotting Substrate (Pierce Biotechnology) according to the manufacturer's instructions and the blots were visualized by autoradiography. Quantitative densitometry analysis of western blot bands was performed employing Image J version 10.2 (NIH). The normalized relative densities were calculated relative to the expression of α-tubulin.

2.8. Screening Analysis

Compound screening was conducted in a 96 well format and the effect with or without panobinostat pretreatment was evaluated against 425 targeted compounds. Viability was assessed after 48 h based on rezazurin reduction by cells using Cell titer blue. The data was linearized, normalized to in-plate controls, and the differential effect was computed as the difference between the observed effect with panobinostat and the effect with vehicle, i.e. positive differential effect denotes compounds that are more effective with panobinostat pretreatment. In order to gauge the degree of the effect, meaning, to determine whether an effective drug becomes more effective (higher effect), or a non-effective drug becoming effective we computed a deviation score. The deviation score is defined as the mean-normalized variance between vehicle and panobinostat treatments, approximating to the CV, computed based on the equation below:

$$D_{AB} = \frac{\sqrt{Dif_{AB}{}^2}}{\mu_{AB}}$$

where D_{AB} is the deviation score of panobinostat vs. vehicle, Dif_{AB} is the defferential effect, and μ_{AB} is the mean of the effects observed in panobinostat vs. vehicle treated.

2.9. Mice Studies

All animal procedures were approved by The Research Animal Resource Center of the Weill Cornell Medicine Adult (6- to 8-week-old,