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Off-Target Effects of SAHA May Inhibit HIV Activation

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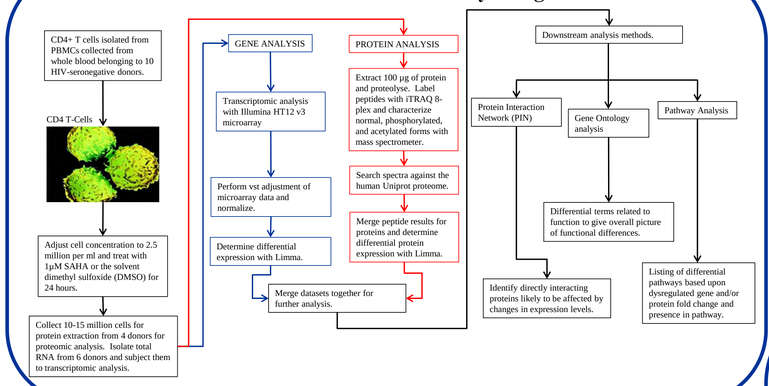
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1. Introduction

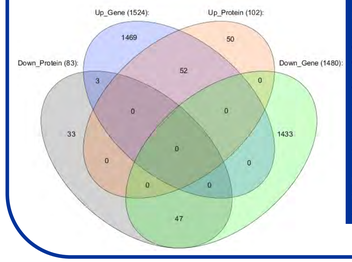
OBJECTIVE:
To assess the genomic and proteomic effects of histone deacetylase inhibitors (HDACis) such as SAHA (Vorinostat) on CD4 T cells.

- **A major hindrance to the eradication of HIV is the persistence of the latent viral reservoir that remains stable despite treatment.**
- **SAHA has been tested in clinical trials as an anti-latency therapy and shown some promise as a treatment to shock a cell out of latency allowing for the termination of the latently infected cell. Unfortunately, treatment with SAHA alone has not resulted in a reduction in size of this reservoir (Elliott, *et al.* 2013).**
- **We hypothesize that SAHA dysregulates the expression of genes and proteins which can interfere in one or multiple steps of the HIV life cycle thereby inhibiting reactivation of HIV and recognition of the infected cell by the immune system.**
- **These off-target effects (in relation to HIV as the selected target) may include protein functions that are altered as a result of transcriptional upregulation induced by SAHA. Furthermore, SAHA binds not only to HDACs, but to other proteins (Bantscheff *et al.* 2011), therefore is it necessary to assess the effect of SAHA at both the gene and protein level.**
- **Preliminary data suggest that the use of SAHA in a "shock and kill strategy" must take into account these off-target effects as they may explain why SAHA treatment has not resulted in a more dramatic reactivation of HIV and a reduction in size of the latently infected cell reservoir.**

2. Methods and Study Design



3. Overlap Among Differentially Expressed Genes (DEGs, FDR p<0.05) and Proteins (DEPs, p<0.05)



Initial testing with Illumina microarrays (~18,000 probes after filtering) produced 3,004 significant genes (DEGs), 1,524 of which were upregulated and 1,480 downregulated. Significance was determined with Limma testing (FDR < 0.05).

Proteins were quantified by iTRAQ proteomics (~6,250 proteins). 1,547 proteins survived ratio testing and 185 were determined to be differentially expressed (DEPs). Of these, 102 were upregulated and 83 were downregulated (p-value < 0.05).

There was high agreement between DEGs and DEPs. At the RNA and protein levels, 52 were upregulated at both while 48 were downregulated at both. There were 3 results upregulated at the RNA level but downregulated at the protein level. However, the fold changes of these 3 in either direction were small (≤1.8).

Due to the increased number of gene results, we applied a 2x fold change filter to DEGs. This produced 368 DEGs, 204 of which were upregulated and 164 downregulated.

4a. Pathway Analysis

Pathway Name	P-Value
Antigen processing and presentation	4.03E-03
MAPK signaling pathway	6.41E-03
Biosynthesis of unsaturated fatty acids	1.26E-02
Influenza A	1.29E-02
GABAergic synapse	1.92E-02
Legionellosis	1.92E-02
Asthma	1.99E-02
HIF-1 signaling pathway	2.19E-02
Rheumatoid arthritis	3.61E-02
Oxytocin signaling pathway	3.79E-02
ErbB signaling pathway	4.47E-02

Table 1. Significantly altered pathways for gene data (Top) and protein data (bottom) P-value <0.05

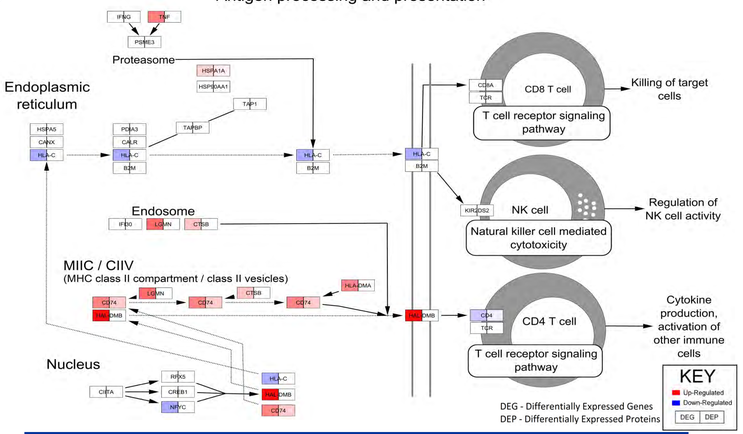
Pathway Name	P-Value
Lysosome	3.65E-03
Regulation of autophagy	1.26E-02
Cell adhesion molecules (CAMs)	4.03E-02
Ras signaling pathway	4.07E-02
Epithelial cell signaling in Helicobacter pylori infection	4.74E-02
FoxO signaling pathway	5.38E-02

Presented are the results when DEGs and DEPs were analyzed separately. When analyzed together, gene hits dominated the top pathways (data not shown) due to the greater number of DEGs compared to DEPs.

MAPK signaling pathway (enriched for DEGs) and Ras signaling pathway (enriched for DEPs) have demonstrated roles in HIV reactivation.

4b. Select Pathway with Gene and Protein Data

Antigen processing and presentation



DEG - Differentially Expressed Genes
DEP - Differentially Expressed Proteins

The Antigen Processing and Presentation pathway is an integral part of the immune system and plays a role in the conversion of antigens from pathogens. This process is critical for mounting a protective T cell response during infection.

While this pathway was not significantly enriched for DEPs, some did map to it. This pathway was chosen as an example of one with both gene and protein hits. Results indicated that several members were dysregulated at RNA or both RNA and protein levels, which may alter the response to HIV transcription.

Red indicates upregulation while blue indicates downregulation. Boxes are divided into two segments for gene expression (left) and protein expression (right).

5. Protein Interaction Network

Network generated from DEGs, DEPs, and differentially expressed phosphorylated and acetylated proteins (DPPs, DAPs). Clockwise from top right, DEGs, DEPs, DPPs, and DAPs.

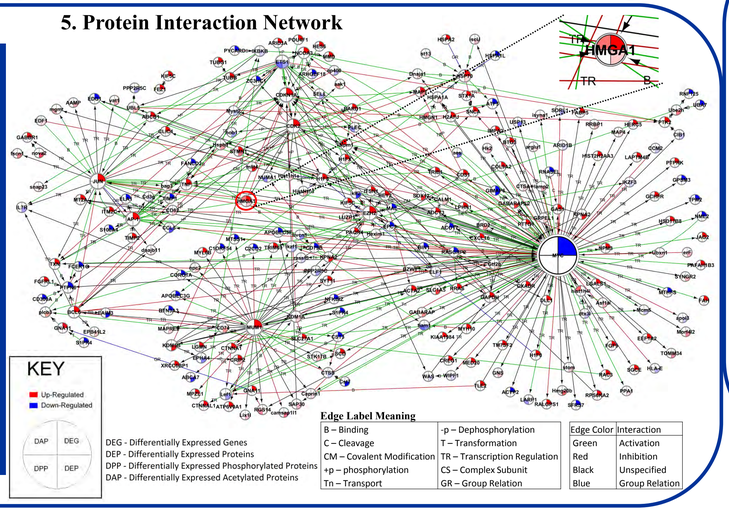
Several differentially expressed factors relevant to HIV reactivation were highly connected: *JUN*, *EZH2*, *HSP70*, *BRD2*, *HMGA1* (upregulated), *MYC*, *ETS1*, *LEF1*, *AES* (downregulated).

Of these, *JUN*, *HSP70*, *ETS1*, *LEF1,* and *MYC* are positive regulators of HIV transcription. Their upregulation may have a beneficial effect on HIV reactivation, while their downregulation may inhibit HIV reactivation.

Conversely, *EZH2*, *HMGA1*, *BRD2,* and *AES* are negative regulators of HIV transcription. Therefore, their upregulation would inhibit, while their downregulation would promote HIV reactivation.

***HMGA1* was upregulated at both RNA and protein levels (both normal and acetylated). *HMGA1* inhibits HIV transcription by competing with Tat for TAR binding and recruiting the inactive p-TEFb complex to the LTR (Eilebrecht et al. 2013). This suggests that upregulation of *HMGA1* may have a negative effect on HIV reactivation.**

These results indicate a complex interplay between SAHA and various genes and proteins which may explain differences in the degree of reactivation induced in HIV-infected individuals. These results may also aid in the selection of compounds to be used in combination with SAHA to more potently reactivate HIV.



DEG - Differentially Expressed Genes
DEP - Differentially Expressed Proteins
DPP - Differentially Expressed Phosphorylated Proteins
DAP - Differentially Expressed Acetylated Proteins

Edge Label Meaning

B – Binding	-p – Dephosphorylation
C – Cleavage	T – Transformation
CM – Covalent Modification	TR – Transcription Regulation
+p – phosphorylation	CS – Complex Subunit
Tn – Transport	GR – Group Relation

Edge Color	Interaction
Green	Activation
Red	Inhibition
Black	Unspecified
Blue	Group Relation

6. Gene Ontology Analysis

Terms from Upregulated Genes	FDR
regulation of multicellular organismal process	8.77E-04
system development	8.77E-04
anatomical structure development	8.77E-04
multicellular organismal development	2.94E-03
multicellular organismal process	2.94E-03
regulation of developmental process	6.19E-03
nervous system development	6.19E-03
developmental process	6.19E-03
regulation of cell differentiation	7.67E-03
regulation of neurogenesis	1.18E-02
regulation of cell development	1.18E-02
regulation of neuron differentiation	1.18E-02
regulation of synaptic transmission	1.53E-02
regulation of nervous system development	1.67E-02
neurogenesis	1.67E-02
regulation of transmission of nerve impulse	1.67E-02
regulation of cell projection organization	1.67E-02
generation of neurons	1.81E-02
regulation of neurological system process	1.81E-02
regulation of organ growth	1.81E-02

Terms from Upregulated Proteins	FDR
protein folding	3.38E-03
loss of chromatin silencing	1.37E-02
negative regulation of chromatin silencing	1.37E-02
organelle organization	1.37E-02
nucleosome organization	1.37E-02
chromatin organization	3.15E-02
histone H3-K4 demethylation	3.15E-02
negative regulation of gene silencing	3.15E-02
regulation of chromatin silencing	3.15E-02
microtubule-based process	3.15E-02
chromatin assembly or disassembly	3.15E-02
nucleosome assembly	3.47E-02
regulation of gene silencing	3.66E-02
chromatin assembly	3.66E-02
DNA conformation change	3.71E-02
microtubule cytoskeleton organization	3.75E-02
protein-DNA complex assembly	3.85E-02
negative regulation of proteasomal ubiquitin-dependent protein catabolic process	3.92E-02
nucleosome positioning	3.92E-02
intracellular transport	4.08E-02

Table 2. Top 20 Terms for GO term enrichment (Biological Processes)

Top 20 terms for GO term enrichment for genes (left) and proteins (right). All results are from upregulated genes or proteins only. The Cytoscape plug-in BINGO was used to generate these lists.

GO term analysis for DEGs indicated widespread effects of SAHA on multiple different functional categories. The analysis of DEPs revealed more modest, but also more targeted effects. As expected, several terms related to chromatin modification were enriched for upregulated DEPs (red).

Interestingly, the term *Protein folding* was enriched for upregulated proteins. HSP70 (gene symbol *HSPA1A*) was upregulated at the protein level. This protein is important for proper folding and stabilization of cyclin-dependent kinase 9 (CDK9) and assembly of p-TEFb (O'Keeffe *et al.* 2000), which are required for HIV transactivation.

7. Conclusions

- **This study is the first, to our knowledge, to indentify the effects of SAHA at both the RNA (gene expression) and protein levels.**
- **Chromatin-related effects of SAHA were detected, both at protein and RNA levels, consistent with expectations based on SAHA's primary mechanism of action.**
- **Pathways relevant to HIV reactivation (MAPK signaling and RAS signaling) and to the response of the immune system to infection (Antigen processing and presentation) contain both dysregulated genes and proteins. This dysregulation may alter the immune system's ability to recognize and remove the infected cells after HIV reactivation.**
- **Both HIV transcriptional activators (*JUN*↑, *HSPA1A* (HSP70)↑, *ETS1*↓, *LEF1*↓, *MYC*↓) and repressors (*EZH2*↑, *BRD2*↑, *HMGA1*↑, AES↓) were dysregulated by SAHA. Based on the directionality of the effect (up- or downregulated), SAHA may have both positive and negative effects on HIV reactivation from latency.**
- **Balance between positive and negative effects induced by SAHA may partly explain differences in the degree of HIV reactivation achieved in HIV-infected individuals on anti-retroviral therapy.**
- **Further functional studies, using SAHA and other latency reversing agents, are required to confirm and elucidate the roles of indentified factors in HIV reactivation and the immune system response.**
- **This systems biology approach of combining gene and protein expression data will aid in the selection of combination therapies to be used with SAHA to counteract its possible negative effects on HIV reactivation.**

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