Demethylation and Re-Expression of Tumor Suppressor Genes: A Novel Approach for Cancer Therapy.

Genevieve Housman[1*], Megan A. Mataga[1*], Amrita Devalapalli[1], Sarah Heerboth[1], Leah R. Evans[1], Sibaji Sarkar[1,2]

[1]Cancer Center, [2]Genome Science Institute
Boston University School of Medicine, Boston, USA

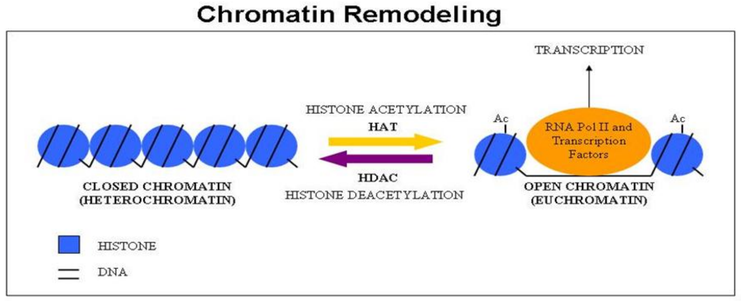
Abstract

Epigenetic regulation is an established mechanism of gene regulation. Gene transcription can be silenced by CpG motif methylation, in which DNA methyltransferases (DNMTs) methylate CpG islands in the upstream promoter region of a gene. Other accessory proteins such as histone deacetylases (HDACs) and MeCP2 then bind to this region, preventing Pol II binding and thus preventing transcription. In cancer cells tumor suppressor genes (p21, p16, p27), differentiation initiating genes (RARβ2), and pro-apoptotic imprinted genes (ARHI) are silenced by methylation. While the genome-wide profiling of methylated genes is widely studied, little is known about their regulation and involvement in cellular events. We previously demonstrated that HDAC inhibitors (HDACi) inhibit ERK kinase, reducing DNA methylation in cancer cells. We observed that a combination therapy using suboptimal doses of HDACi and calpeptin, an inhibitor of the ubiquitous protease calpain which modulates many regulatory proteins, produced synergistic type growth inhibition. The inhibitors also reduced cancer cell motility, a measure of invasiveness. We hypothesize that the re-expression of tumor suppressor genes by demethylation and other mechanisms sensitize the cells and allows for apoptotic death. Currently, we are investigating ERK and Akt signaling pathway-mediated modulation of methylation-demthylation and regulation of DNMTs.

Background

- Chromosome opening requires acetylation and change in methylation status of histones.
- CpG dinucleotide sites are regions of the DNA that contain repeats of a cytosine nucleotide next to a guanine nucleotide separated by a phosphate moiety. These can be methylated (at the cytosine residue) or unmethylated.
- Silencing of genes by CpG methylation is an interesting phenomenon in stem cells, neurological cells, and cancer cells.
- Tumor suppressor genes are transcriptionally regulated by DNA CpG methylation.
- In addition, methylation and demethylation regulate genes that cause a loss of heterozygocity (LOH). For example, ARHI is expressed monoallelically and is maternally imprinted. LOH of the non-imprinted paternal allele by methylation is observed in 40 percent of breast, ovarian, and pancreatic cancers.
- Our previous work shows that ERK regulates methylation.

Chromatin Remodeling



Objectives

- To investigate the mechanisms of re-expression of silenced tumor suppressor genes by demethylation.
- To utilize histone deacetylase inhibitors (HDACi) in combination with calpain inhibitors (calpeptin) to study growth inhibition and develop a successful combination therapy.
- To assess the metastatic ability of cancer cells with and without our drug treatment by a motility assay.
- To investigate ERK and Akt signaling pathways in mediating gene expression.

Fig 1: Inhibition by HDACi and Calpeptin

- MCF-7 breast cancer cells treated with either 0.25mM SB (HDACi sodium butyrate), 10μM calpeptin (protease calpain inhbitor), or 0.25mM SB+10μM calpeptin.
- Viable cells counted (trypan blue negative) on fourth day.
- Results expressed as percent of cells present in the untreated controls.
- Combination of SB+calpeptin produced a synergistic type of growth inhibition.
- Similar growth inhibition observed in ovarian cancer cells (not shown).

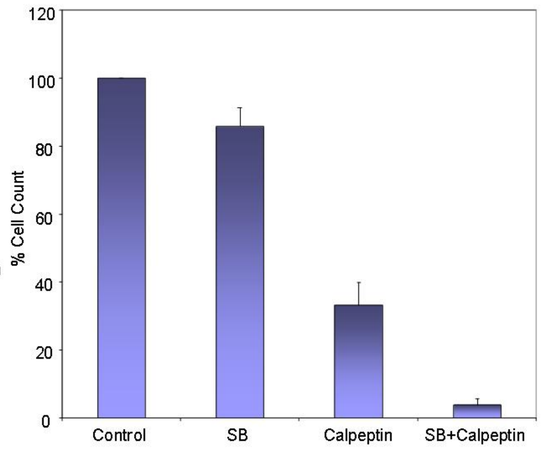


Fig 2: Morphology of Cancer Cells after Treatment

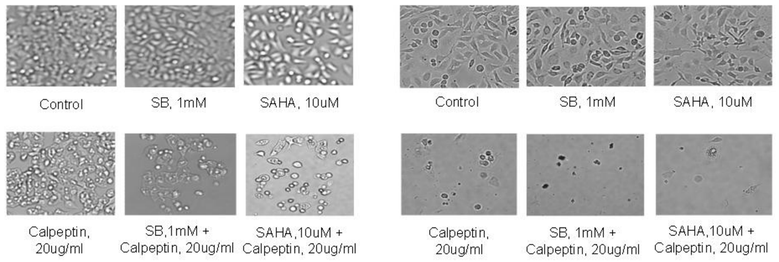


Fig 3: Cell Cycle and Apoptosis Analysis

- MCF-7 breast cancer cells treated with 1mM SB or 10μM SAHA (HDACi suberoylanilide hydroxamic acid) for 48 hrs.
- Cells stained with Propidium Iodide (PI).
- Cell cycle profile determined.

SB, SAHA arrested at G1.

- MCF-7 breast cancer cells treated with 20μg/ml calpeptin for 48 hrs.
- Cells stained with annexin PI.
- Calpeptin showed increased apoptosis.

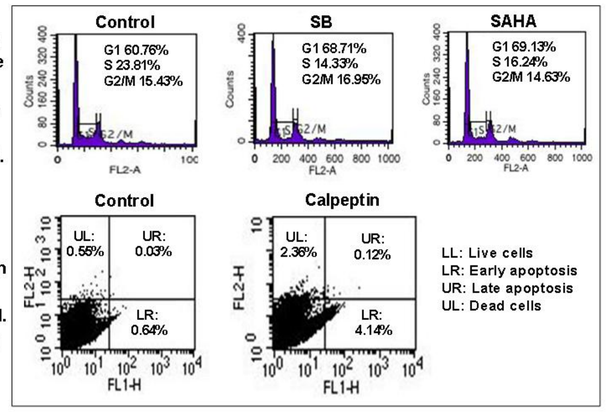
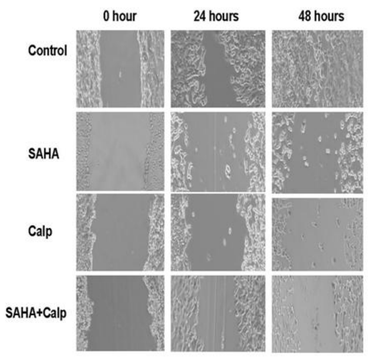
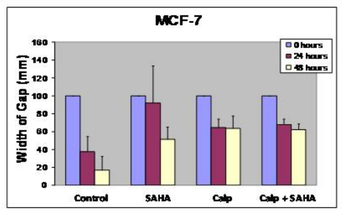


Fig 4: Wound Healing Assay for Motility





- MCF-7 breast cancer cells were treated for 48 hrs with 10μM SAHA and/or 10μg/mL calpeptin. Cells were washed, and conditioned media from untreated cells was added.
- Cells then scratched with a toothpick to create a lane devoid of cells.
- Photographs taken at 0 hr, 24 hrs, and 48 hrs.
- Width at various positions of individual samples from Figure 5 were expressed assuming 0 hr width for each sample as 100%.

Fig 5: Effects of HDACi & Calpeptin on ERK Phosphorylation

- MCF-7 breast cancer cells were lysed after 48 hrs treatment.
- Equal amount proteins were run in an SDS-PAGE.
- Western Analysis was performed for p-ERK. Blot was stripped. A western blot for ERK followed.

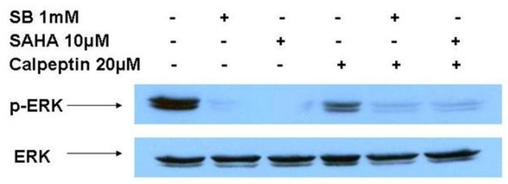


Fig 6: ARHI & RARβ2 CpG Demethylation by HDACi & Calpeptin

- MCF-7 breast cancer cells were treated with 1mM SB or 10μM SAHA for 48 hrs.
- Genomic DNA was isolated, treated with bisulfite, and purified.
- Methylation-specific PCR was performed using methylation-specific primers of ARHI (pro-apoptotic imprinted gene) & RARβ2 (differentiation initiating gene).
- ARHI & RARβ2 promoters are methylated.
- HDACi inhibited ARHI & RARβ2 methylation.
- Lowest panel shows loading as actin.

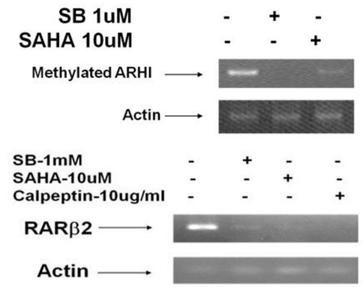
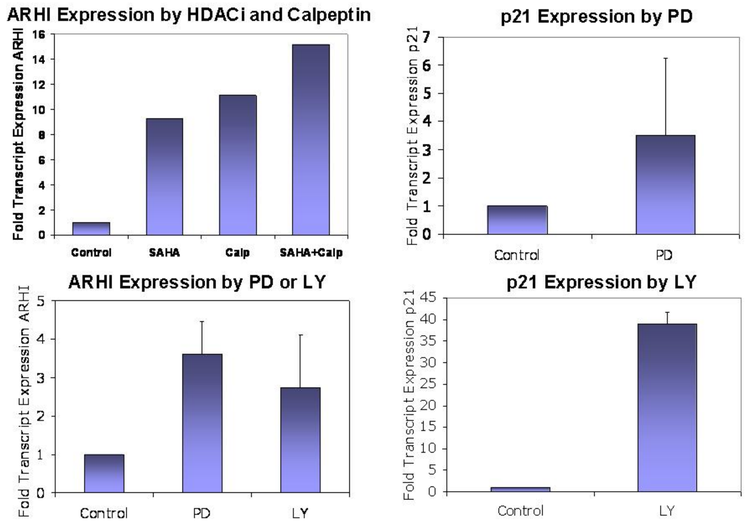


Fig 7: ARHI & p21 Re-expression

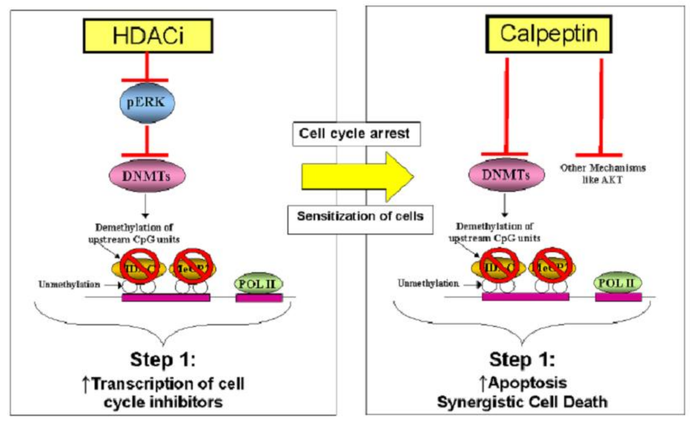


- Total RNA was extracted from CAOV-3 ovarian cancer cells treated with HDACi and/or calpeptin, 50μM PD 98059 (ERK inhibitor), or 50μM LY 294002 (Akt inhibitor) for 48 hrs.
- cDNA was prepared, after genomic DNA digestion.
- Real time PCR was performed for ARHI and p21 (cell cycle inhibitor).

Fig 8A: Model of Methylation and Inhibition of Transcription

DNMT1 maintains methylation status of upstream CpG motifs → HDACs, MeCP2, & other accessory proteins bind methylated CpG motifs → Pol II binding blocked → Transcription inhibited

Fig 8B: Model of Synergistic-Type Inhibition



Summary and Further Studies

- HDACi and calpeptin at suboptimal doses produced synergistic type growth inhibition in breast cancer and ovarian cancer cells. Each inhibitor at higher doses inhibits growth of cancer cells when used separately (not shown).
- HDACi showed cell cycle inhibition, and calpeptin induced apoptosis. We hypothesize that during combination therapy, cancer cells are first sensitized and killed by apoptosis. Fig 2 indicates alteration of cell morphology characteristic of apoptotic cells.
- HDACi and calpeptin inhibited wound healing (motility of cells which is a measure of metastatic potential) in breast cancer cells. Combination did not enhance inhibition, suggesting that HDACi and calpeptin were possibly used at optimal levels.
- HDACi inhibited ERK phosphorylation (a measure of ERK activity). Calpeptin did not show significant inhibition of ERK phosphorylation.
- ARHI and RARβ2 were demethylated by drug treatments.
- HDACi and calpeptin increased the expression of ARHI. PD and LY increased the expression of ARHI and p21.
- HDACi is known to express many genes which are not silenced by methylation. Our's and others' recent findings showed that HDACi also regulate the demethylation of genes.
- This demethylation process most likely is mediated by signaling.
- Our data suggest that the re-expression of many tumor suppressor genes could be achieved by ERK or Akt inhibition.
- We are investigating the regulation of methylation/demethylation processes by ERK and Akt to understand the mechanism of growth inhibition, induction of apoptosis, and inhibition of metastasis.

Genevieve Housman and Megan A. Mataga contributed equally to this work.

• Acknowledgement: The work was supported by a grant to SS from ACS. Genevieve Housman was funded by UROP, BU. AD was funded by the BUMC Evans Medical Foundation. MM was funded by UROP, BU. LRE was funded by Aids in Cancer, Boston. We are thankful to Dr. D. V. Faller, Director, Cancer Center, for the use of facilities. We also thank Lucy Rosenbaum and Shoshana Rosenthal for technical assistance.