Exploration of a Combination Therapy on Breast Cancer Cells by HDAC Inhibitors and Calpain Inhibitor

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Abstract

Epigenetic regulation is an established mechanism of gene regulation. Acetylated histones allow an open chromatin structure, which facilitates RNA Pol II and other transcription factors to bind, thereby initiating transcription. Another epigenetic regulation of gene silencing is methylation of upstream CpG islands, which does not allow binding of Pol II for the initiation of transcription. This phenomenon is prevalent in stem cells as well as in many types of cancers, in which numerous genes, including tumor suppressor genes, are silenced by this mechanism. Histone deacetylases inhibitors (HDACi), in combination with other drugs, are in clinical trials for several types of cancers, but with limited success. We initiated a combination therapy with a protease inhibitor, calpain, which inhibits a ubiquitous protease calpain. Calpain regulates various regulatory proteins by proteolytic cleavage and regulates phosphorylation in signaling pathways as well. Theoretically, calpain inhibition could have anti-tumor effects because it inhibits phosphorylation.

We are using two structurally distinct HDAC inhibitors – sodium butyrate (SB) and suberoylanilide hydroxamic acid (SAHA), which inhibit the growth of cancer cells. Interestingly, we found that calpain also inhibits growth of many types of cancer cells. We observed that these drugs, when used in combination at suboptimal concentrations, produced synergistic-type growth inhibition in different types of cancer cells including breast and ovarian cancer cells. We found that these drugs caused cell cycle arrest and induced apoptosis, possibly by expression of several tumor suppressor genes. In particular, protease-inhibited gene ARHI induction of differentiation gene RARB2 were demethylated by the aforementioned drug treatment and were observed to be re-expressed. Furthermore, we determined the inhibition capacity of these drugs on the metastatic properties of cancer cells using a wound-healing assay (motility assay). Currently, we are investigating a hypothesis based on our results that cancer cells are sensitized followed by apoptotic death from the combination therapy.

Background

- Chromosome opening requires acetylation and methylation status change 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 repeats are methylated or unmethylated (at the cytosine residues).
- Silencing of genes by CpG methylation is an interesting phenomenon in stem cells, hematopoietic cells, and cancer cells.
- Tumor suppressor genes such as RARβ2, p21, p53 are transcriptionally regulated by DNA CpG methylation.
- In addition, methylation and demethylation regulate genes that cause a loss of heterozygosity (LOH) (Fig 1).
- For example, ARHI is expressed mononucleotically and is a maternally imprinted. LOH of the non-imprinted allele by methylation is observed in 40 percent of breast, ovarian, and pancreatic cancers.
- Forced re-expression of one or more these tumor suppressor genes causes cell cycle arrest and/or apoptosis of cancer cells.

Chromatin Remodeling

-To investigate the mechanisms of re-expression of silenced tumor suppressor genes by demethylation.

- To utilize histone deacetylase inhibitors in combination with calpain inhibitors to study growth inhibition and develop a successful combination therapy.

- To assess the metastatic ability of breast cancer cells with and without our drug treatment by a motility assay.

Fig 1. MCF-7 Breast Cancer Cells: Inhibition by HDACi and Calpeptin

- Cells were treated with either 0.25mM SB, 10μM calpeptin, 0.25mM SB+ 10μM calpeptin.
- Visible cells counted (Trypan blue negative) on fourth day.
- Results expressed as percent of cells present compared to control
- Combination of SB+calpeptin produced a synergistic type of growth inhibition.

Fig 2. Photographs of MCF-7 and SKOV-3 cells after treatment

Breast Cancer MCF-7 Cells

Control
SB 1mM
SAHA 10μM
SAHA + Calp

Ovarian Cancer SKOV-3 Cells

Control SB 1mM SAHA 10μM

Fig 3. Cell Cycle and Apoptosis Analysis

Fig 4: ARHI & RARB2 CpG Demethylation by HDACi

- Breast cancer MCF-7 cells were treated with 1 mM SB or 10 μM SAHA for 48 hours
- Genomic DNA was isolated
- Treated with bisulfite and purified
- Methylation-specific PCR was performed using methylation-specific primers of ARHI & RARB2
- ARHI & RARB2 promoters were methylated
- ARHI & RARB2 methylation was inhibited by HDACi
- Lowest panel shows leading as Actin

Fig 5: ARHI & p21 Re-expression levels in Breast & Ovarian Tissue

Breast Cancer MCF-7 – ARHI

SAHA 10μM
S A H A

Ovarian Cancer CAOV-3 – ARHI

SAHA 10μM
S A H A

Summary and Further Studies

- HDACi and calpeptin at suboptimal doses produced synergistic type growth inhibition in breast cancer cells (Fig 1). Each inhibitor at higher doses inhibited growth of breast cancer cells when used separately (not shown).
- HDACi showed cell cycle inhibition and calpeptin induced apoptosis (Fig 2). We hypothesize that breast cancer cells are sensitized followed by apoptotic death. Fig 2 indicates alteration of cell morphology characteristic of apoptotic cells. Effects on ovarian cancer cells indicate that this combination works in other types of cancers as well.
- ARHI and RARB2 were demethylated by HDACi treatment suggesting possible re-expression (Fig 4).
- ARHI, p21 and RARB2 (not shown) were re-expressed by drug treatments (Fig 5).
- HDACi and calpeptin inhibited wound healing in breast cancer cells (by decreasing motility of cells which is a measure of metastatic potential). Combination did not enhance inhibition, suggesting that HDACi and calpeptin were possibly used at optimal levels (Fig 6).

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