Anti-breast Cancer Effects of Histone Deacetylase Inhibitors and Calpain Inhibitor

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Abstract. *Development of new breast cancer therapies is needed, particularly as cells become refractory or develop increased drug resistance. In an effort to develop such treatments, class I and II histone deacetylases (HDACs), alone and in combination with other cytotoxic agents, are currently in clinical trial. Herein, we discuss the effects of histone deacetylase inhibitors (HDACi) when used in combination with calpeptin, an inhibitor of the regulatory protease, calpain. We present results of study in two breast cancer cells lines with distinct characteristics: MDA-MB-231 and MCF-7. When used in combination with calpeptin, two chemically distinct HDACi significantly inhibited growth and increased cell death by inducing cell-cycle arrest and apoptosis. MCF-7 cells exhibited a greater proportion of arrest at the G_1 phase, whereas triple-negative MDA-MB-231 cells exhibited increased cell cycle arrest at the S phase. Methylation of the imprinted and silenced proapoptoic tumor suppressor gene aplasia Ras homolog member I (ARHI) was reduced in both cell lines after treatment with HDACi. However, it was only re-expressed on such treatment in MDA-MB-231 cells, suggesting that re-expression operates under differential mechanisms in these two cell lines. Collectively, these results showed that the combination of HDACi and calpeptin inhibited the growth of two distinctly different types of breast cancer cells and could have wide clinical applications, though the mechanisms of inhibition are possibly different.*

Due to various characteristics, many breast carcinomas are notoriously difficult to treat (1). Those with estrogen and progesterone receptor-positive cells respond well to hormone treatments (2). About 30% of breast carcinomas are human epidermal growth factor receptor 2 (HER2)-positive, which is characteristic of more aggressive breast cancers. Such cells respond positively to the HER-2 inhibitor Herceptin (2). Another 30% of breast carcinomas are triple negative, lacking both hormone receptors and expression of HER2 (3). Treating triple-negative breast cancer is a challenge, as target-specific drugs are often ineffective on these tumors. Furthermore, some patients develop resistance to the employed treatment. The current paradigm suggests that combination therapies could be more effective because they are generally less toxic, combat drug resistance, and may demonstrate synergistic efficacy.

Histone deacetylase inhibitors (HDACi) have shown promise in the treatment of certain types of leukemia; suberoylanilide hydroxamic acid (SAHA), commercially referred to as Vorinostat, is an FDA-approved HDACi for T-cell lymphoma (4). Although HDACi themselves are less effective against solid tumors, recent studies have shown that combination therapies using HDACi have the potential to be more effective against solid tumors such as breast cancer (5) and ovarian cancer (6). HDACi are currently in use in clinical trials in combination with other cytotoxic agents. Furthermore, new HDACi have been generated that are effective at much lower doses (6).

HDACi are known to work through the acetylation of histones and the resultant unfolding of chromatin. This facilitates the re-expression and transcription of many genes, including various silenced tumor suppressor genes. We recently observed that HDACi also demethylate upstream CpG islands in the promoter region of cell cycle inhibitors, such as *p16* and *p21*, and differentiation inducers, such as retinoic acid receptor beta 2 *(RARB2)* (11). DNA CpG methylation is a type of epigenetic regulation independent of histone modification, which may silence gene expression, even in euchromatin. Tumor suppressor genes, including *p16*, *p21*, and *RARB2*, are silenced by this mechanism in many types of cancer cells, although the extent of methylation and

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demethylation varies among cancer cell types (7). *ARHI* is another propaptotic tumor suppressor gene, and is silenced in approximately 40% of breast cancer cells. *ARHI* is maternally imprinted; the paternal allele is silenced, causing loss of heterozygosity (8). Forced re-expression of *ARHI* causes apoptosis in many types of cancers (8).

In our combination therapy, we employed two different inhibitors: HDACi and calpeptin. The HDACi employed included SAHA and sodium butyrate (SB), which are structurally distinct, although both are inhibitors of class I and II HDACs. Calpeptin is a calpain inhibitor. Calpain is a ubiquitous protease that regulates phosphorylation (9). Many phosphorylated proteins are implicated in growth and survival pathways, including v-ask murine thyomoma viral oncogene homolog 1 (AKT) pathways (10). In addition, our laboratory previously showed that HDACi inhibit Mitogen-activated protein kinase 1 (ERK), which down-regulates DNA methyltransferase 1 (DNMT1) (11). Inhibition of DNMT1 leads to demethylation, resulting in the re-expression of certain tumor suppressor genes (11). Therefore, the goal of this study was to investigate how the combination of HDACi and calpeptin affects the growth and survival of breast cancer cells. To do this, we used two breast cancer cell lines: MCF-7 and MDA-MB-231. MCF-7 is estrogen receptor-positive (ER+), progesterone receptor-positive (PR+) and HER2-positive (HER2+, but not overexpressed). MDA-MB-231 is a triple-negative cancer cell line (ER–, PR–, HER2–).

Materials and Methods

Reagents. SB and SAHA were purchased from Sigma (St. Louis, MO, USA). Calpeptin was purchased from Nova Chemicals (Moon Township, PA, USA). Dulbecco's Modified Eagle Medium (DMEM) and RPMI-1640 media were from Invitrogen (Carlsbad, CA, USA). Other reagents were purchased from Invitrogen, Sigma, or Pierce (Rockford, IL, USA). The Annexin/PI apoptosis analysis kit was from BDR (San Diego, CA, USA). The lactate dehydrogenase (LDH) cytotoxicity detection kit was from Roche Applied Science (Indianapolis, IN, USA). The cDNA preparation kit came from Qiagen (SA Biosciences) (Valencia, CA, USA). The SYBR green RT-PCR mix was from Applied Biosciences (Foster City, CA, USA).

Cell culture. MCF-7 breast cancer cells were cultured in DMEM, and MDA-MB-231 breast cancer cells were cultured in RPMI-1640, containing penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS). Cells at 70-80% confluence were exposed to Dimethyl sulfoxide (DMSO) (control) or different concentrations of drugs. Cells were incubated for different times before harvesting.

Cell survival assays. Viable cell counts were performed using a trypan blue exclusion assay in six-well plates. Cells were trypsinized and 20 μl cell suspensions were mixed with 20 μl trypan blue. Viable cells (dye-excluding) were counted. The viable cell percentage was plotted against time. The change in viable cell counts over time provides a measure of growth.

Methylation-specific polymerase chain reaction (PCR). Methylation-specific PCR (MS-PCR) was performed with bisulfite-treated genomic DNA. Genomic DNA was isolated from cells grown in 10-cm plates using reagents from Stratagene (La Jolla, CA, USA), according to the manufacturer's protocol, and treated with bisulfite, according to a published protocol (11). MS-PCR was performed with primers specific for either methylated or bisulfite-modified unmethylated DNA. Methylation-specific primers used for *ARHI* were GTAAGGGAGAAAGAAGTTAGA for the forward reaction and TACTATCCTAACAAAACCCTC for the reverse. β-actin primers used were CTGGCACCCAGCACAATG for the forward reaction and GGACAGCGAGGCC for the reverse. PCR reactions were run using a Hybaid PCR Sprint Thermal Cycler (Cole-Parmer) (Vernon Hills, IL, USA). PCR amplifications without genomic DNA were performed as controls. Each PCR product was run on a 2% agarose gel, stained with ethidium bromide, and either visualized under UV illumination or by Image Quant LAS 4000 LE (General Electric) (Fairfield, CT, USA).

Real-time quantitative PCR (qPCR). Total RNA was prepared from cells using the Trizol reagent (Invitrogen), according to the manufacturer's protocol. Real-time PCR analysis was performed with the cDNA prepared for assessment of *ARHI* transcript expression. Primers used for *ARHI* were TGGGTAACGCCAGC TTTGGCT for the forward reaction and TAACGTGGCGCGTG CAGAGCG for the reverse. The results were normalized to the expression level of β-actin in each sample with the same primers, as described for MS-PCR. The PCR was performed in triplicate for each sample. The means are presented with the standard deviations.

Cell cycle and apoptosis assays. For cell cycle analyses, cells were treated with different inhibitors for the indicated times and stained with propidium iodide (PI) as described in (11), and then analyzed for DNA content by flow cytometry (11). Briefly, cells were washed with phosphate-buffered saline (PBS), fixed in a medium containing 35% ethanol for 5 min at room temperature, and stained for 30 min in the dark with 25 μg/ml PI and 50 μg/ml RNAse in PBS before FACS analysis.

Apoptosis assays were performed by annexin/PI assay, as previously described (12). In short, cells were harvested and suspended in 100 μl 1× binding buffer, then 5 μl annexin reagent conjugated to fluorescein isothiocyanate (FITC) and 5 μl PI were added to each sample, mixed well, and kept in the dark for 30 min at room temperature. Subsequently, 900 μl 1x- binding buffer was added and the dual fluorescence of FITC and PI was measured by flow cytometry.

LDH cell cytotoxicity assay. Cell death was measured by a cytotoxicity detection kit (LDH) from Roche according to the manufacturer's protocol. In brief, inhibitor-treated and untreated cells were cultured in six-well plates for 96 h. Then 100 μl supernatant from each well was collected and mixed with 100 μl freshly mixed reaction mixture. Following incubation in the dark at room temperature for 30 min, the absorbance was measured at 490 nm.

Results

Growth inhibition and cell death. We previously noted that HDACi allowed for re-expression of silenced tumor suppressor genes *via* demethylation of CpG islands in the upstream promoter regions of tumor suppressor genes (11).

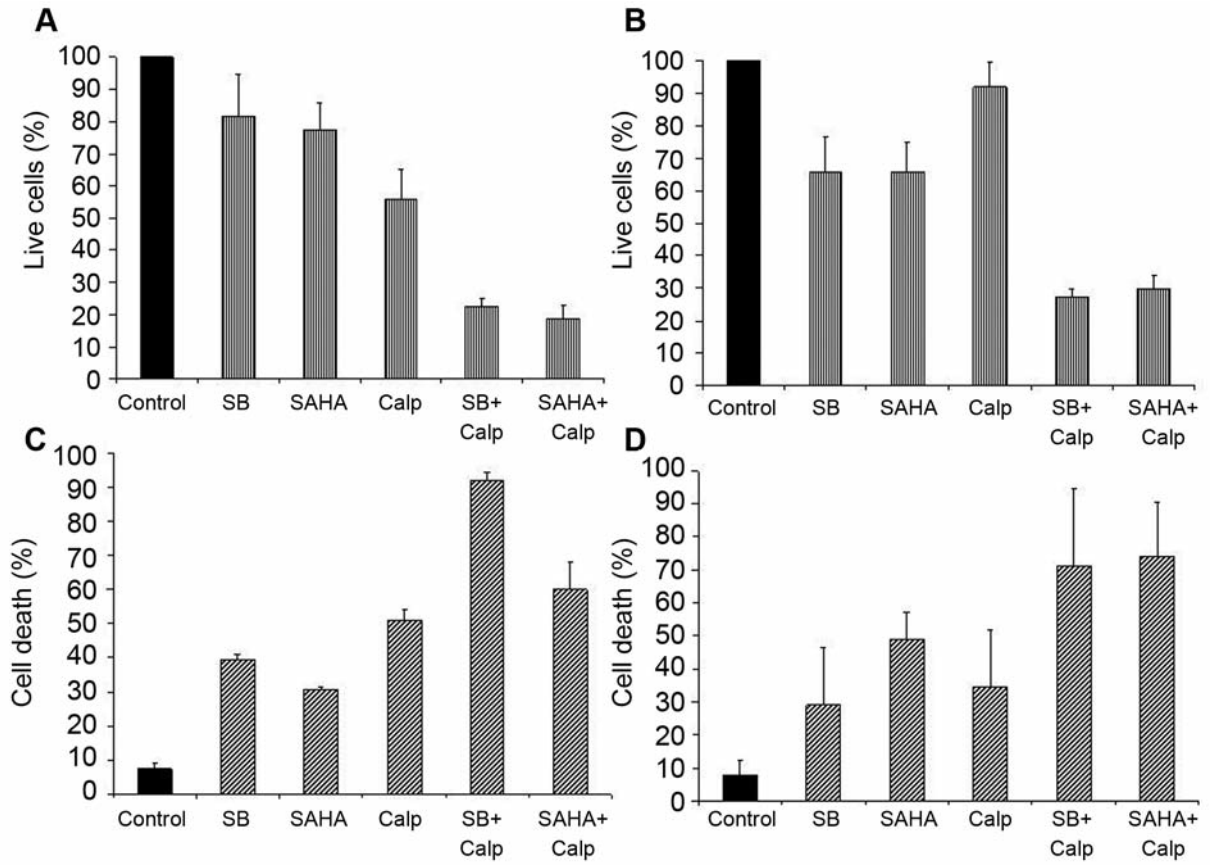

Figure 1. *Effects of HDAC inhibitors and calpeptin on the growth and cell survival of MCF-7 and MDA-MB-231 breast cancer cells. A: MCF-7 cells were exposed to either sodium butyrate SB (0.25 mM), SAHA (7.5 μM), calpeptin (Calp, 10 μg/ml) or combination of SB and calpeptin (p value = 0.0001) or SAHA and calpeptin (p value = 0.0001) at the same concentrations. B: MDA-MB-231 cells were exposed to either SB (0.5 mM), SAHA (7.5 μM), calpeptin (7.5 μg/ml) or combination of SB and calpeptin (p-value = 0.0001) or SAHA and calpeptin (p-value = 0.0001) at the same concentrations. After 96 h, cells were washed, trypsinized, and the viable cells were counted. Results are expressed relative to the numbers of vehicle-treated (control) cells, arbitrarily assigned a value of 100%. The dark columns represent the control cells and the striped columns represent the cells exposed to inhibitors. C: MCF-7 cells were treated with inhibitors as described in A. D: MDA-MB-231 cells were treated as described in B. After 96 h, the supernatants were collected and a lactate dehydrogenase cytotoxic assay was performed. The results are expressed as the percent cell death compared to the amount of total lactate dehydrogenase present in total untreated cells.*

In this work, we investigated the synergistic effect of HDACi in combination with calpeptin to inhibit cell growth of breast cancer cells. We studied two cell lines with different characteristics (MDA-MB-231 and MCF-7), as well as used two chemically distinct HDACi (SB and SAHA). We observed approximately 70-80% growth inhibition, 96 h after the combinatorial treatment in both cell lines by using each HDACi (Figure 1A and B). These findings were statistically significant ($p<0.001$). The optimal inhibitor concentrations differed between the two breast cancer cell lines.

To investigate the effect of growth inhibition *versus* induced cell death, we performed an LDH release assay on the supernatant of the conditioned media produced from the drug-treated cell cultures. As demonstrated in Figure 1C and D, there was a significant increase in cell death resulting from the combinatorial treatment. Furthermore, the results presented in Figure 1 show that HDACi and calpeptin reduced cell growth and induced cell death.

Cell cycle inhibition and induction of apoptosis. MCF-7 and MDA-MB-231 cell lines exhibited different cell-cycle inhibition patterns after 48 h treatment with SB or SAHA. The MCF-7 cells exhibited about 18-19% G_1 arrest (Figure 2A). There was an observed decrease in the proportion of cells in the S phase (from an average of 23% to about 15%). The extent of G_1 arrest observed is typical of cell-cycle inhibition resulting from treatment with HDACi (11). In MDA-MB-231 cells, the proportion of cells arrested in the S

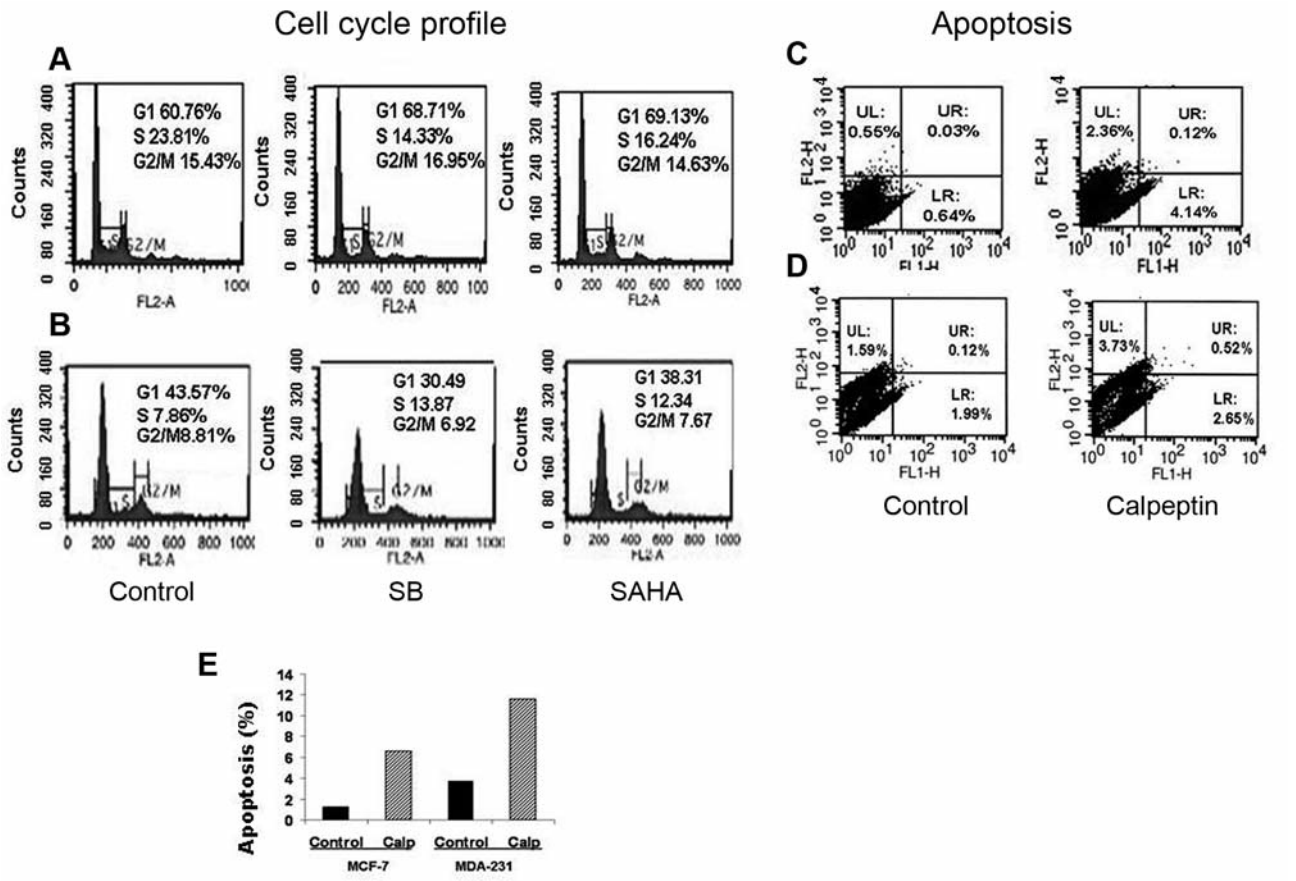

Figure 2. *Effects of HDAC inhibitors and calpeptin on cell cycle profile and apoptosis of MCF-7 and MDA-MB-231 breast cancer cells. A: MCF-7 cells were treated SB (0.25 mM) or SAHA (7.5μM). B: MDA-MB-231 cells were treated SB (0.5 mM) or SAHA (7.5 μM). After 48 h cells were stained with propidium iodide (PI) and cell cycle profiles were analyzed by flow cytometry. Vehicle-treated cells served as a control. Results are expressed as the percentage of cells in a particular phase of the cell cycle. C: MCF-7 cells were treated with 10μg/ml calpeptin. D: MDA-MB-231 cells were treated with 7.5 μg/ml calpeptin. After 48 h cells were stained with FITC-conjugated annexin antibody (FL1 channel) and propidium iodide (PI, FL2 channel). Apoptosis profiles were analyzed by flow cytometry. Vehicle exposed cells served as controls. E: Graphical depiction of apoptotic fractions. The data from panel C and D was expressed as percent of cells undergoing apoptosis (annexin-positive, annexin/PI-positive, PI positive fractions).*

phase was greater by about 5%, and thus the percentage of cells in the G_1 phase was relatively lower (Figure 2B). While calpeptin did not induce cell-cycle inhibition of MCF-7 cells, it inhibited the cell cycle at the S phase in MDA-MB-231 cells (data not shown). It has been demonstrated that this G_1 arrest is dependent upon the induction of specific tumor suppressor genes, including cyclin-dependent kinase inhibitors (CDKI) *p21* and *p16* (11).

To analyze apoptosis, annexin/PI staining was employed. In cells exposed to calpeptin, evidence of an increase in an early apoptotic population (annexin-positive, PI-negative) was evident after 48 h in both cell lines (Figure 2C and D). HDACi did not induce apoptosis of MCF-7 cells (data not shown) at the given doses, although higher concentrations of HDACi are known to induce apoptosis (data not shown). In contrast, HDACi did induce apoptosis of MDA-MB-231 cells at the given concentrations (Figure 2E). Figure 2F is the graphical presentation of the results shown in Figure 2C and D.

These results suggest that at the given concentrations, HDACi induced cell-cycle arrest while calpeptin induced apoptosis, and that these inhibitors together produced the observed decrease in live cells (Figure 1A and B). We also acknowledge the possibility that HDACi may induce apoptosis and *vice versa*, although based on the data we do not believe this to be the predominant mechanism responsible for the observed results.

Demethylation and re-expression of tumor suppressor gene ARHI. To investigate whether tumor suppressor genes are in fact demethylated by HDACi treatments, we determined the promoter methylation status of the maternally imprinted tumor suppressor gene, *ARHI*. MCF-7 and MDA-MB-231 cells were

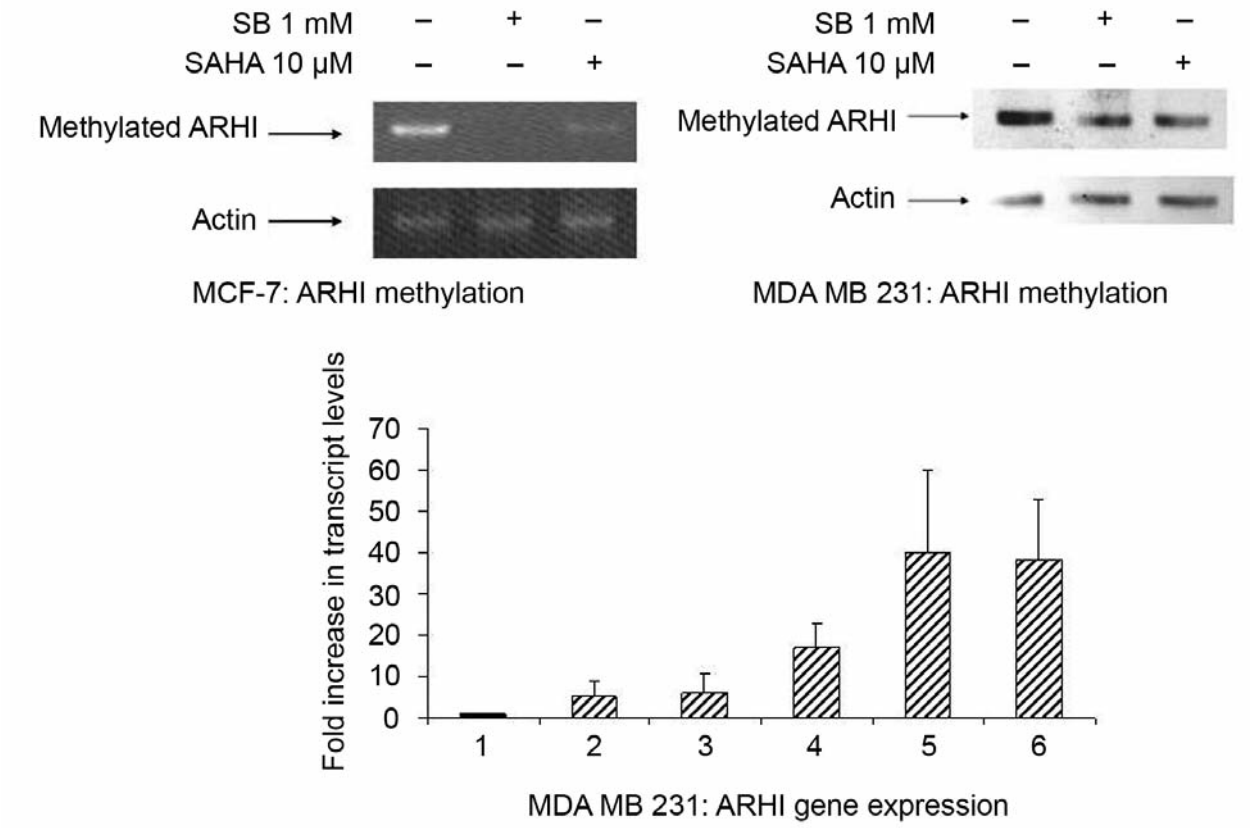

Figure 3. *DNA methylation analysis ARHI promoter and transcript level. (A) MCF-7 and (B) MDA-MB-231 cells were treated with SB (1mM) or SAHA (10μM). Genomic DNA was isolated and treated with bisulfite. After purification, methylation-specific PCR (MS-PCR) was performed for each gene. The lower panel shows the total actin in each sample, as a loading control. (C) MDA-MB-231 cells were treated with SB (0.5 mM) or SAHA (7.5 μM) , calpeptin (7.5 μg/ml) or combination of SB and calpeptin and combination of SAHA and calpeptin at the same concentrations for 48 h. Total RNA was isolated and cDNA was prepared. Real-time PCR (qPCR) for ARHI transcripts was performed. Results for each gene in each sample were normalized to the level of β-actin transcripts. The results are expressed as fold increase over the untreated control. The results are the average of 3 sets of independent experiments. Bars indicate the standard deviation.*

incubated with SB or SAHA for 48 h, genomic DNA was isolated and treated with bisulfite, and methylation-specific PCR (MS-PCR) was performed. Vehicle-treated cells served as a control. As a loading control, amplification of total β-actin was also performed. We show that *ARHI* is methylated in both breast cancer cell lines (Figure 3A and B). Exposure to HDACi reversed or reduced the methylation of the *ARHI* gene.

Finally, we investigated whether demethylation allowed re-expression of silenced tumor suppressor genes in our experimental cell lines. Cells were subjected to the combination treatments for 48 h, and the transcript levels of *ARHI* and β-actin were examined by quantitative RT-PCR. The transcript levels of *ARHI* tumor suppressor gene were significantly increased after exposure to HDACi and calpeptin in MDA-MB-231 breast cancer cells. Results were normalized against β-actin controls (Figure 3C). Interestingly, *ARHI* was not re-expressed in the MCF-7 cells after 48 h.

Collectively, the above findings demonstrate that combinatorial treatment of HDACi and calpeptin caused a significant decrease in the number of viable cells *via* cell-cycle inhibition and induction of apoptosis and cell death. The data also suggest that the mechanism of cell-cycle arrest and apoptosis may be different in MCF-7 and MDA-MB-231 breast cancer cell lines, as HDACi arrested the cell cycle at the G_1 phase in MCF-7 cells and at the S phase in MDA-MB-231 cells.

Discussion

In the present study, we investigated the efficacy of a combination therapy for breast cancer cells using HDACi and calpeptin, a calpain inhibitor. Many current approaches in cancer therapy employ combinations of cytotoxic agents with distinct modes of action. In this study, two breast cancer cell

lines with different characteristics were used: MCF-7 and MDA-MB-231. Unlike MDA-MB-231, which is a triple-negative line, MCF-7 is responsive to hormone therapy. About 30% of breast cancers are triple negative and are therefore extremely difficult to treat. HDACi lead to re-expression of many genes, including tumor suppressor genes, by acetylation of chromatin and by demethylation of CpG islands around promoter regions of silenced genes (11). We hypothesized that HDACi lead to re-expression of silenced tumor suppressor genes, while concurrently sensitizing cells to other cytotoxic drugs. Evidence for this hypothesis has been found in connection with TNF-related apoptosis-inducing ligand (TRAIL) (5). Our previously published work indicated that in ovarian cancer cells, HDACi in combination with DNA oligonucleotides homologous to the telomere 3' overhang region (GT oligo) produced significant growth inhibition (6). In this work, we chose to employ calpeptin because inhibition of calpain inhibits phosphorylation of regulatory signaling pathways, including AKT (10, 13-15). Furthermore, recent studies have shown that inhibition of calpain induced cell death in many types of cancer cells (10, 13-15).

When used in combination with calpeptin, both SB and SAHA produced significant growth inhibition in both MCF-7 and MDA-MB-231 breast cancer cells. The required concentrations differed between the two cell lines (Figure 1A and B). HDACi alone have shown only modest clinical efficacy, as well as little effect when used in combination with conventional chemotherapeutic agents (8). Therefore, it is of particular interest that the combination of calpeptin with two HDACi of different chemical classes produced more than additive efficacy. These results are congruent with other findings on the combinatory effects of HDACi and GT oligos in ovarian cancer cells (6).

The results shown in Figure 1C and D demonstrate that in both breast cancer cell lines, the HDACi and calpeptin combinatorial therapies induced significant cell death at 96 h. Early induction of apoptosis was detected in both MCF-7 and MDA-MB-231 breast cancer cells after treatment with calpeptin for 48 h (Figure 2C and D). HDACi are well-known cell-cycle inhibitors (11). In addition, recent results have shown that calpeptin causes cell death by inducing apoptosis and autophagy (10). Most drug-induced apoptotic events in cancer cells are mediated by intrinsic or extrinsic signaling pathways (5). Therefore, we hypothesize that inhibition of growth could be the result of inhibition of cell division (and thus cytostatic in nature) as well as cell death. It remains unclear as to what extent the observed growth inhibition is a result of cessation of cell division and/or cell death.

To understand the mechanism of tumor suppressor gene re-expression, we investigated cell-cycle inhibition and apoptosis after combined HDACi and calpeptin treatment. After 48 h, cell-cycle progression was inhibited in both MCF-7 and MDA-MB-231 breast cancer cells by the HDACi treatments. MCF-7 cells exhibited a greater proportion of G_1 arrest and thus a lower proportion of cells in the S phase, whereas MDA-MB-231 cells exhibited a greater proportion of cells arrested in the S phase in comparison to the G_1 phase (Figure 2A and B). This result is not surprising given the different characteristics of these two cell lines. It is also significant that the structurally distinct HDACi, SB and SAHA, yielded similar results. This suggests differential mechanisms of cell-cycle arrest. Interestingly, one of the important regulators of the G_1 phase, *p16*, is null in both MCF-7 and MDA-MB-231 breast cancer cell lines. *p27*, *p21* and other factors may also be involved, underlining a need for further investigation.

Several tumor suppressor genes are often silenced in breast cancer cells, including the maternally imprinted, pro-apoptotic gene *ARHI* (7). In this study, we showed that *ARHI* is methylated in MCF-7 and MDA-MB-231 breast cancer cells (Figure 3A and B). Treatment with SB and SAHA differentially reduced the methylation level of this silenced tumor suppressor gene in both cell lines (Figure 3A and B). Initially, demethylation was expected to lead to re-expression of *ARHI*. To our surprise, we observed a re-expression of *ARHI* in MDA-MB-231 cells after treatment (Figure 3C), but not in MCF-7 breast cancer cells (data not shown). This result indicates involvement of *cis*/*trans* acting elements, which act differently in these two cell lines with respect to *ARHI*. The combinatorial therapy induced significant re-expression of *ARHI* in the MDA-MB-231 cells, thus it may be involved in cell-death induction. At present, we do not know which apoptotic markers regulate cell death in MCF-7 cells; other apoptotic regulators may also contribute to the observed cell death in the MDA-MB-231 cell line. As HDACi inhibits ERK (11) and calpeptin inhibits AKT (13, Sarkar *et al.* unpublished), these two pathways may contribute to cell growth inhibition and induction of apoptosis. The differential behavior of MCF-7 and MDA-MB-231 cell lines poses an interesting query concerning the mechanism of silencing and re-expression of tumor suppressor genes and warrants further investigation. In addition, this study raises the possibility of using this type of combination therapy for diverse types of breast cancers including those that are hormone-refractory and develop drug resistance to conventional chemotherapy.

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