Romidepsin and azacitidine synergize in their epigenetic modulatory effects to induce apoptosis in CTCL

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Running Title

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Conflict of interest

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Translation Relevance

Potential synergistic effects between two important epigenetic regulators are under investigation in phase I/II clinical trials in refractory hematologic malignancies as well as solid tumors. Nevertheless, studies exploring the potential synergistic interactions and insight to the molecular mechanism of such combination therapy is lacking in CTCL. In addition, there has been no common functional mutation or genetic aberration identified to be responsible for malignant transformation of T cells in CTCL. In this study, we describe, the synergistic effect of romidepsin and azacitidine in CTCL cell lines and tumor cells derived from SS patients as well as demonstrate the potential underlying mechanisms and global methylation profile alterations effecting gene expression with potential predictive value in CTCL.

Finally, the lower concentration of each drug administered in the combination as compared to single agent might translate in a favorable tolerability of this combination that will be explored in future clinical trials.
Abstract

Purpose

Cutaneous T cell lymphomas (CTCL) are a heterogeneous group of malignancies that despite available therapies commonly relapse. The emergences of combination epigenetic therapies in other hematologic malignancies have made investigation of such combinations in CTCL a priority. Here, we explore the synergistic anti-proliferative effects of romidepsin, an HDAC inhibitor, and azacitidine, a demethylating agent, combination in CTCL.

Experimental Design

The growth inhibition under combination treatment and single agent was explored by the MTT cell viability assay and the Annexin V/Propidium Iodide (PI) apoptosis assay in different CTCL cell lines and tumor cells derived from Sézary syndrome (SS) patients. Quantitative analysis of dose-effect relationship of romidepsin and azacitidine was done by the CompuSyn software. Investigation of mechanism of action was performed by flow cytometry, immunoblotting, qRT-PCR arrays and chromatin immunoprecipitation. Global CpG methylation-sequencing was utilized to study genome methylation alteration under the treatment modalities.

Results

The combination of romidepsin and azacitidine exerts synergistic anti-proliferative effects and induction of apoptosis involving activation of the caspase cascade in CTCL cell lines and tumor cells derived from SS patients. We identified genes that were
selectively induced by the combination treatment, such the tumor suppressor gene RhoB that is linked to enhanced histone acetylation at its promoter region in parallel with pronounced expression of p21. Global CpG methylation-sequencing in a CTCL cell line and tumor cells demonstrated a subset of genes with a unique change in methylation profile in the combination treatment.

Conclusions

The synergistic anti-proliferative effects of romidepsin and azacitidine combination treatment justify further exploration in clinical trials for advanced CTCL.
Introduction

CTCL comprise a heterogeneous group of malignancies derived from skin-homing T cells. The more common subtypes are mycosis fungoides (MF), and Sézary syndrome (SS), an aggressive leukemic variant of CTCL(1). These two subtypes account for approximately 70–75% of all cases(2). Therapeutic options include skin directed therapy and/or systemic therapy(3). The success rate of most available therapy regimens ranges from 30 to 50%, but relapse is common and difficult to treat, and curative therapy remains elusive. Therefore, there is an indisputable need for more successful treatment approaches for advanced CTCL.

Romidepsin (F228 or desipeptide) is a potent bicyclic histone deacetylase (HDAC) inhibitor approved by the United States Food and Drug Administration (FDA) for the treatment of relapsed/refractory CTCL patients on the basis of two phase II clinical trials showing an overall response rate of 34-35% (4).

Azacitidine (5-azacytidine or Vidaza®) is a cytotoxic cytidine analog and DNA methyltransferase inhibitor with anti-neoplastic activity(5). At lower concentrations, it is known to relieve the transcriptional repression of various tumor suppressor genes that were silenced via hypermethylation of their promoter region (6). It was approved by the FDA for treatment of refractory myelodysplastic syndromes (MDS)(7).

In cancer, tumor suppressors are often down-regulated by aberrant histone deacetylation and/or DNA methylation (8). HDAC inhibitors can synergize with
demethylating agents to relieve this transcriptional repression (9). Moreover, combined epigenetic therapies have been shown to be effective in hematological malignancies (10-13).

Here, we demonstrate that the combination of romidepsin and azacitidine exerts synergistic anti-proliferative effects by inducing apoptosis in CTCL cell lines as well as CD4+ T cells derived from SS patients with high tumor burden. We show the tumor suppressor gene RhoB as a potential regulator of the synergism, whose anti-neoplastic relevance has been described in other malignancies (14-16). Furthermore, our data suggest that the combination treatment results in a unique global methylation pattern in CTCL, which may contribute to increased efficacy of the anti-proliferative effects of the combination treatment in CTCL.
Material and Methods

Cell lines

MyLa, SeAx and Hut78, well established CTCL cell lines(17, 18), were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamine (2 mM) and streptomycin (100 μg/ml) at 37°C, 5% CO2 and 95% humidity.

Cell viability assay

The MTT (Sigma-Aldrich, St Louis, MO, USA) assay measures the activity of cellular enzymes that reduce the tetrazolium dye, to its insoluble form, formazan, which under defined conditions can reflect the number of viable cells. This assay was utilized to measure cell proliferation in untreated and treated CTCL cell lines, SS patients and healthy donors PBL samples. The absorbance was measured by spectrophotometry, using a 550 nm wavelength ELISA reader.

Flow cytometry

FITC Annexin V Apoptosis Detection Kit II (BD Biosciences, Franklin Lakes, NJ, USA) was utilized for flow cytometry to determine the percentage of cells that are actively undergoing apoptosis within the untreated and treated CTCL cell lines, SS patients and healthy donors PBL samples. After designated treatment schedule, cells were harvested and stained as instructed in manufacturer’s protocol.

Propidium iodide (PI) (Sigma-Aldrich, St Louis, MO, USA) was utilized as DNA stain for flow cytometry. DNA content in cell cycle analysis was evaluated to differentiate
necrotic, apoptotic and normal cells in the above described populations. The proportion of cells that exhibited sub-G1 phase of the cell cycle, indicate DNA degradation and apoptotic cell death. After the designated treatment time cells were harvested in 0.3 mL of cold PBS and then fixed with cold 70% ethanol, left incubating on ice for 1 hour, and washed twice with cold PBS. RNase A (10 mg/mL) was added to each sample and incubated at 37°C for 1 hour. Cells were acquired using a BD FACS Canto flow cytometry using the FL2-A channel and analyzed with the FlowJo software 8.5.2.

**Western blot analysis**

Cells were washed twice with cold phosphate-buffered saline and lysed at 4°C in RIPA protein lysis buffer. The protein concentration of each sample was determined by Bradford assay (Bio-Rad protein assay, Bio-Rad, Hercules, CA, USA). Proteins were separated by SDS-PAGE using the NuPAGE SDS-PAGE Gel System on 4–12% or 10% NuPage Bis-Tris gels (Life Technologies, Carlsbad, CA, USA) under reducing conditions according to the manufacturer’s instructions and transferred onto nitrocellulose membranes (Invitrogen). The following primary antibodies were used: anti-acetylated H3 (#9649), anti-cleaved caspase 3 (#9664, rabbit monoclonal), anti-cleaved caspase 9 (#7237, rabbit monoclonal, anti-cleaved PARP (#5625, rabbit monoclonal), p21 waf1/Cip1 (#2946, mouse monoclonal), and anti–alpha tubulin (#2125, rabbit monoclonal)(Cell Signaling Technology, Beverly, MA, USA). Bound antibodies were detected using ECL Western Blotting Detection Reagent (GE Healthcare, Chalfont, UK).
Immunohistochemistry

After incubation of CTCL cell lines with romidepsin and azacitidine simultaneously for 48 hours, the samples were placed on the slides using the cytospin procedure, as previously published(19). After fixation in 10% acetone the slides were incubated with the RhoB primary antibody (C-5, mouse monoclonal, Santa Cruz Biotechnology, Dallas, TX, USA), and then secondary antibody as suggested by manufactures’ protocol.

Primary Sézary cells and healthy donors CD4+ T-cells

Sample collection and laboratory studies were in compliance with institutional review board, ethics committee and conducted according to the Declaration of Helsinki Principles. All patients signed informed consent, approved by the USZ ethical committee (EK647).

Patients’ and healthy donors peripheral blood was collected in EDTA tubes. PBMCs were separated from whole blood by Ficoll density gradient, then CD4+ T-cells were selected by MACS CD4+ negative selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany) per manufacture’s protocol. After negative cell selection, CD4+ T-cells were stimulated with CD3/CD28 Dynabeads (Life technologies, Carlsbad, CA, USA) in addition in IL-2 30 Unit/ml and treated with/without romidepsin and/or azacitidine as described.
Quantitative real-time reverse-transcriptase–PCR

Total RNA was extracted after incubation with designated time of treatment or untreated cells using TRIzol according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Concentration was determined by NanoDrop spectrophotometer. A total of 2 mg RNA was used for cDNA synthesis using Promega’s Reverse Transcription System (Promega, Madison, WI) according to the supplied protocols. Gene expression was quantified using the Universal SYBR Green Master (ROX; 04913914001; Roche, Basel, Switzerland) and the Viia7 system from Applied Biosystems (Carlsbad, CA). The primers for were purchased from Qiagen (Venlo, the Netherlands) and Cell Signaling. The qRT-PCR arrays (RT² Profiler™ Cancer Drug Targets PCR Array) were purchased from SA Biosciences (Qiagen, Venlo, the Netherlands). Total RNA from untreated and treated samples were characterized in technical triplicates, and the relative expression levels for each gene in single agent or combination treated samples were plotted against DMSO in the Scatter Plot. All analyses were conducted in relative quantitation using the ΔΔC_t–based method for calculation of relative quantitation values (20).

Chromatin immunoprecipitation-PCR (ChIP-PCR)

DNA was immunoprecipitated with the MAGnify™ Chromatin Immunoprecipitation System (Life Technologies, Carlsbad, CA, USA) as per manufacturer’s instructions. Selective enrichment was performed using an antibody specific to histone H3 acetylated at K9 (Cell Signaling Technology, Beverly, MA, USA) and unspecific IgG (Cell Signaling Technology, Beverly, MA, USA) as control. Then, we quantified the ratio of immunoprecipitated DNA over 10% input with primers specific to the RhoB promoter.
region (Forward primer: GGTTTCCCATTTGGACGGCTA; Reverse primer: GCCTCGCTGAGCATACAAGA) by qRT-PCR, in the MyLa cell line.

**Next generation DNA methylation sequencing**

DNA was isolated using TRIzol, according to manufacturer’s instructions. Total of 3ug of DNA was used as input amount in the Agilent SureSelectXTMethyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing protocol. Quality of the isolated DNA was assessed by an Agilent 2200 Tapestation. DNA was sheared using a S220 Focused-ultrasonicator (Covaris, Woburn, MA, USA). Bisulfite conversion was done using EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to manufacturer’s instructions.

Sequencing was performed by Illumina Hiseq 2000. We sequenced 0.25 lane per sample paired end 100bp. Average coverage was 20x. Data analysis was done using “Bismark” for aligning and “Methylkit” for differential methylation quantification. P-values were adjusted for multiple hypothesis testing according to the false discovery rate (FDR) method. Data has been uploaded to NCBI GEO GSE66732.

**Compounds and Reagents**

Romidepsin and azacitidine were provided by Celgene (Summit, NJ, USA).

**Statistical analysis**

The median dose effect (21) (or half the maximal inhibitory concentration [IC50]) values for each cell line at different time points were determined using CompuSyn software(22) based on the quantitative analysis of dose-effect relationships multiple drugs or enzyme
inhibitors by Chou and Talalay (23). Combinational Index (CI) were calculated to confirm synergy. CI value <1 indicates synergistic effects, CI values equal to 1 indicate the mean additive effect of the drugs, and CI values > 1 represent an antagonistic effects.

Statistically significant differences within different experiments were determined using Student t-test with P value < 0.05 as minimal significance.
Results

Romidepsin and azacitidine combination treatment synergize in induction of apoptosis in CTCL

We observed a concentration- and time-dependent decrease in cell viability as measured by the MTT colorimetric assay, in all cell lines at 24 and 48 hours after incubation with a range of romidepsin or azacitidine concentrations (Figure 1a). We determined the IC$_{50}$ for romidepsin and azacitidine in all cell lines (MyLa, SeAx and Hut78) at 24-hour and 48-hour time point utilizing the CompuSyn software. IC$_{50}$ of romidepsin was within the range of what was previously reported in preclinical studies (24). The calculated IC$_{50}$ of azacitidine varied between cell lines but remained mostly $>$ 5uM even after 48 hours of treatment (Figure 1b).

Similar pattern in time and dose dependent decrease in cell viability was observed in all 3 cell lines. Therefore, we continued our experiments with MyLa and SeAx cell lines. We next analyzed the dose-dependent growth inhibition under romidepsin and azacitidine combination treatment and single agent, normalized to control, at different concentration levels with a constant dilution ratio of 1:2000 at 48-hours via MTT assay (Figure 2a). Moreover, the combination index (CI) was calculated using the CompuSyn software with CI<1 representing synergy (Figure 2b), as detailed in the methods section. The combination treatments were synergistic at different combination concentrations especially at lower concentrations. The lack of synergy at the highest concentration is likely due the fact that potent drugs, such as romidepsin, can become highly cytotoxic at high concentrations and are not an accurate representation of the combination effects
on cell viability(25). The concentration of azacitidine, as a hypomethylating agent, in our experiments correlates with the concentration used in many preclinical experiments in various cell lines (26, 27).

Further experiments with the CTCL cell lines were performed with romidepsin 1.25nM and/or azacitidine 2.5uM, as at these concentrations, the combination had the lowest CI reflecting a higher level of synergy at 48 hours. MyLa and SeAx cells exhibited a higher percentage of apoptosis under romidepsin 1.25nM and azacitidine 2.5uM combination treatment compared to each single agent added together as analyzed by flow cytometry after Annexin V/PI staining (Figure 2c). Moreover, under the combination treatment a higher percentage of MyLa and SeAx cells demonstrated a shift to the sub-G1 population, as measured by flow cytometry after PI staining, further confirming the anti-proliferative effects (Figure 2d).

**Romidepsin and azacitidine combination treatment synergize in inducing apoptosis in CD4+ T cells derived from SS patients but not on healthy donor CD4+ T cells**

To analyze the growth inhibitory effects of the combination treatment on Sézary cells, we separated CD4+ T cells from the peripheral blood of SS patients and two healthy donors (HD), as described in the methods section. Some studies suggest that treatment with hypomethylating agents should precede treatment with HDAC inhibitors for optimum synergy (12, 28). Therefore, based the in vitro experiments and preliminary experiments on tumor cells derived from Sézary syndrome patients that were treated with different concentration of romidepsin and/or azacitidine (data not shown), optimum
antiproliferative effects were reached with azacitidine 2.5uM for 4 days and romidepsin 2.5nM was added for the last 24 hours. This combination was used in the patient derived samples for further experiments.

A significant synergistic effect on cell viability and induction of apoptosis was evident, measured by the MTT assay (Figure 3a) and by flow cytometry after Annexin V/PI staining (Figure 3b-c), \( p<0.05 \). To analyze the effects of this combination on CD4+ derived from HD peripheral blood, we incubated CD4+ cells derived from healthy donors with the same schedule and noted only low cytotoxicity without increased growth inhibition under the combination treatment (Figure 3c), indicating that the combination has synergistic growth inhibitory effect on the tumor cells beyond increased cytotoxicity. As demonstrated with the CTCL cell lines, a higher percentage of CD4+ T cells derived from SS patients treated with the combination showed a shift to the sub-G1 population, further confirming increased apoptosis (Figure 3d). Additionally, the combination treatment compared to single agent resulted in pronounced cleavage of caspase 9, caspase 3 and PARP analyzed by western blot, as shown in CTCL cell line as well as a representative patient sample (Figure 3e).

**Romidepsin and azacitidine combination treatment results in re-expression of tumor suppressor RhoB in CTCL by enhancing histone acetylation of its promoter region**

To elucidate the synergistic epigenetic modulatory effects of histone deacetylation and DNA methylation on known dysregulated cancer genes, we used the cancer drug target qRT-PCR array to analyze the CTCL cell lines (MyLa, SeAx), treated with romidepsin 1.25nM alone, azacitidine 2.5uM alone, in combination, or DMSO treated cells for 48-
hours. Analysis of this dataset demonstrated that the gene expression profile of the tumor cells treated with the combination treatment is unique in comparison to single agent, which was more pronounced in the MyLa cell line (Figure 4a). Among top 5 genes that demonstrated more than a 4 fold significant re-expression in the combination treatment compared to the single treatments and vehicle, in both cell lines, was the GTP binding protein from the Rho protein family, RhoB. This was also the only one that was consistently differentially expressed in cell lines and in patient-derived primary cells. RhoB is suggested to be induced by genotoxic stress and mediates proapoptotic effects on transformed cells(29). The expression of RhoB is attenuated in many cancer derived cell lines compared to their normal tissue counterparts(30).

We validated the significant upregulation of RhoB in CD4+ cells derived from SS patients upon combination treatment in most patients' samples (Figure 4b), \( p < 0.05 \). Moreover, previous studies have suggested that after genotoxic stress RhoB expression is induced within in the first few hours, and then is attenuated prior to another cycle of higher expression(31). To analyze this pattern, we incubated the MyLa and SeAx cell lines with romidepsin and azacitidine and extracted RNA at 6 hours, 24 hours and 48 hours and measured the fold change in RhoB gene expression. RhoB expression was rapidly upregulated under the combination treatment in both cell lines as early as 6 hours, reaching a lower level at 24 hours prior to another cycle of up-regulation again at 48 hours (Figure 4c). RhoB protein expression is illustrated by immunohistochemistry in MyLa cell line (Figure 4d).

Our methyl-sequencing data showed no methylation in the CpG island of RHOB promoter region of vehicle treated cells. Additionally, it did not demonstrate any
differential methylation of RhoB promoter region under the combination treatment compared to single-agent or untreated cells (data not shown). HDAC inhibitors have been shown to reactivate RhoB expression in lung(32), ovarian(33) and thyroid cancer(34). In Figure 4e, we demonstrated more pronounced histone H3 acetylation in CTCL cell lines and a representative patient sample under the combination treatment by Western blot. We speculated that histone acetylation of the RhoB promoter region is responsible for reactivation of RhoB in CTCL and hypomethylating agent further enhances this acetylation. To test this, we performed ChIP-PCR by selective enrichment using an antibody specific to histone H3 acetylated at K9 and unspecific IgG as control, and then we quantified the ratio of immunoprecipitated DNA over input with primers specific to the RhoB promoter region by qRT-PCR, in the MyLa cell line (see methods). Figure 4f demonstrates the significantly higher histone acetylation of the RhoB promoter region under the combination treatment compared to romidepsin alone correlating with a significant decrease in cell viability by MTT assay (Figure 4g) and more pronounced p21 signal by Western blot under the combination treatment (Figure 4h). There was no change in histone H3 acetylation in single-agent azacitidine treated cells.

The combination of romidepsin and azacitidine results in a specific global (CpG) methylation profile alteration

The marked activity of DNA hypomethylating agents have been attributed to hypomethylation of key regulators of apoptosis and cell cycle arrest (26, 35). To identify the global methylation changes under the combination treatment, MyLa cell lines and CD4+ T cells derived from a SS patient were treated with romidepsin and azacitidine in
combination or each agent alone.

A principal component analysis of the global methylation datasets demonstrated distinct clustering of azacitidine treated tumor cells, alone and in combination, which were distant from romidepsin alone and DMSO treated cells (Figure 5a). The percentage of differentially methylated regions per chromosome analysis reveals a global CpG hypomethylation under azacitidine treatment, alone or in combination, but absent in romidepsin single-agent treated cells (Figure 5b). Genome-wide CpG island methylation profiling identified 1956 genes shared between the azacitidine and romidepsin with azacitidine treatments. 285 genes were exclusive to the combination treatment (Figure 5C). Interestingly, pathway analysis by Metacore of the genes that are differentially methylated in the azacitidine and combination treatment revealed many pathways involved in apoptosis, inflammation and immune response.
Discussion

In many other malignancies, characteristic chromosomal abnormalities have been identified as therapeutic targets, such as bcr-abl fusion gene in chronic myelocytic leukemia (CML) and BRAF status in melanoma. In CTCL, various cytogenetic studies found multiple genetic alterations that recur in a subset of MF and/or SS patients (36-38). But few common genetic variants including targetable mutations have been identified (39).

Hence, over the past decade there has been an emergence of studies exploring the involvement of epigenetic mechanisms that contribute to the pathogenesis of CTCL (40-42). These data are limited to only molecular assessments or identification of epigenetic changes as prognostic markers.

Potential synergistic effects between two important epigenetic regulators: histone acetylation and DNA hypomethylation is under investigation in phase I/II clinical trials in refractory lymphoid and myeloid malignancies as well as solid tumors(43).

Nevertheless, studies exploring the potential synergistic interactions and insight to the molecular mechanism of such combination therapy is lacking in CTCL. In this study, we describe the synergistic effect of romidepsin and azacitidine in CTCL cell lines and CD4+ T cells derived from SS patients as well as demonstrate the potential underlying mechanisms and global methylation profile alterations affecting gene expression. Our data suggests combination of romidepsin and azacitidine in CTCL synergistically activates the caspase cascade inducing apoptosis, as compared to single-agent treatments at the same concentrations. Moreover, the concentration of each drug...
administered in the combination was almost fifty percent less than the IC50 of each agent. This might translate in a favorable tolerability of this combination that will be explored in future clinical trials.

Intriguingly, the combination induced the re-expression of the tumor suppressor gene, RhoB, as early as 6 hours post-treatment. This could be explained by the rapid induction of RhoB in response to early stress to trigger pathways involved in DNA damage response and cell death. RhoB is believed to be a central factor in growth inhibition of transformed cells (15, 29, 44). Investigations in multiple cancers have found that RhoB is rarely mutated in cancer genomes (45). Additionally, there is no known aberrant methylation at its promoter region that could be responsible for the loss of RhoB expression, as others (32) and we have shown. Therefore, histone modifications or other transcriptional regulatory mechanism may be responsible for the loss of RhoB expression. Pre-clinical data suggests increased histone acetylation at the RhoB promoter region results in its re-expression in lung carcinoma and anaplastic thyroid cancer(30, 34). In our study, the combination treatment resulted in significant histone H3 acetylation at the promoter region of RhoB gene compared to romidepsin alone suggesting histone acetylation is responsible for transcriptional activity of RhoB, which is further enhanced by the hypomethylating agent azacitidine. This might be due to the fact that DNA hypomethylation results in a more accessible chromatin for histone acetylation or due to DNA-hypomethylation independent effects of azacitidine that could contribute to the synergistic effects of the combination. It is suggested that RhoB promotes cell cycle arrest by controlling the expression of cell cycle regulators such as p21(34). We observed an up-regulation of p21 in cell lines and tumor cells derived from
SS patients. This might be RhoB dependent or due to RhoB playing a crucial role in the sensitization of the CTCL cells to DNA damage and subsequent apoptosis. RhoB had a high expression under the combination treatment in the cancer drug target qRT-PCR array screen but there are other potential genes in this cancer drug target screening array that showed significant change in their expression under the combination. For example ErbB-3 was significantly expressed under the combination treatment. This gene has been shown to play a role in sensitizing NSCLC cells to HDAC inhibitors (21). Additional detailed mechanistic experiments are required to better understand the role of these genes and their targets in CTCL.

Genome-wide methylation analysis of the MyLa cell line and tumor cells derived from an SS patient revealed distinct clustering of azacitidine and combination treated cells as compared to romidepsin treated or untreated cells. This is not surprising as the majority of genes differentially methylated between azacitidine and combination treatment are shared. Romidepsin has a small effect on hypomethylation and this is in line with reports demonstrating the role of HDAC inhibition increasing global DNA hypomethylation (46, 47). Interestingly, the combination treatment had an exclusive set of differentially methylated genes, which suggests specific pathways are involved in the synergistic action of romidepsin and azacitidine. The 285 genes were involved in many signaling pathways including apoptosis, immune response, and inflammation. Overlap with the RT-PCR array for cancer target genes was not prominent, as this array is focused on mostly cancer associated kinases. Furthermore, there could be upstream factors that when demethylated activate the genes seen in the RT-PCR array. For instance, our methylation data demonstrated SOX5 was demethylated in the
combination treatment, which is known to regulate RhoB in the neural tube (48). As genes regulated by histone acetylation also contribute to the synergistic effect, genome wide histone acetylation analysis would be necessary to fully understand the mechanisms of action of the combination treatment. Nonetheless, the differentially methylated genes specific in the combination treatment underline the important contribution of demethylation to the synergistic effect. This profile might serve as the basis for the identification of a methylation signature that is predictive of clinical response as shown in myelodysplastic syndrome (49) or to a limited extent suggested recently in CTCL (50).

In summary, there has been no common functional mutation or genetic aberration identified to be responsible for malignant transformation of T cells in CTCL. Our data provide insight to unique methylation profile alterations with potential predictive value due to the synergistic anti-proliferative effects of combined romidepsin and azacitidine treatment in CTCL, and paves the road for combining these agents in clinical trials for advanced CTCL.
Conflict of interest

Celgene provided financial support for the project. Authors do not have any other conflict of interest.

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References


Figure 1. Change in cell viability measured via MTT colorimetric assay. (A) Dose–response curves were explored for each CTCL cell line (Hut78, MyLa and SeAx,) treated with romidepsin or azacitidine for 24 and 48 h. Values are defined as means converted to percentages after normalization to untreated control; error bars represent standard deviation. (B) Median dose effect (analog of IC50) was calculated for romidepsin and azacitidine at 24 and 48hrs for each CTCL cell line (HUT78, MyLa, SeAx).

Figure 2. Synergistic decrease in cell viability and induction of apoptosis. (A) Constant-ratio analysis of combination treatment demonstrated synergistic decrease in cell viability as measured by MTT colorimetric assay after 48hrs incubation of MyLa and SeAx cell lines with romidepsin and/or azacitidine combination at different concentration at a constant dilution ratio of 1:2000. (B) Fraction-affected (Fa) and combination index (CI) are explored after 48hrs incubation with romidepsin and azacytidine combination, CI<1 represents synergy. (C) MyLa and SeAx cells were 48hrs treated with the romidepsin (1.25nM) and/or azacitidine (2.5uM) or DMSO only treated cells for 48hrs. Then, induction of apoptosis was measured by flow cytometry after FITC Annexin V/ PI staining (Values in the right upper quadrants represent the percentages of cells that were Annexin V+/ PI- plus Annexin V+/PI+) and (D) Cell cycle analysis, sub-G1 population measured by flow cytometry after PI staining.
Figure 3. Assessment of cell viability and apoptosis in Sézary Syndrome (SS) patients, healthy donor (HD) derived cells and caspase cascade activation. (A) Significant decrease in cell viability in the combination treatment compared to single agents \((p<0.05)\) shown by MTT assay in 4 SS derived tumor cells. (B) Significant increase in apoptosis in the combination treatment compared to single agents \((p<0.05)\) shown by flow cytometry after FITC Annexin V/PI staining (Values in the right upper quadrants represent the percentages of cells that were Annexin V+/ PI- plus Annexin V+/PI+). (C) Grouped graph depiction of induction of apoptosis in combination treatment vs single agent in 3 SS patients derived cells compared to 2 HD derived cells. Treatments in HD derived cells only resulted in low growth inhibition without additive toxicity by the combination. (D) Cell cycle analysis, sub-G1 population measured by flow cytometry after Propidium Iodine (PI) staining in patient samples (E) Increase in cleavage of caspase 9, caspase 3 and PARP in the combination compared to single-drug or DMSO treated cells in MyLa cell line and representative SS derived patient cells.

Figure 4. Combination treatment results in re-expression of the tumor suppressor gene RhoB by enhanced histone acetylation of its promoter region. (A) Cancer Drug Targets qRT-PCR array demonstrated a unique expression profile with the combination treatment in the MyLa cell line (top) and SeAx cell line (bottom) after 48 hours. (B) RhoB mRNA expression was analyzed by RT-PCR in CD4+ T-cells derived from four SS patients (C) and in the CTCL cell lines at 6h, 24h, 48h time points demonstrating the cyclic expression of RhoB. (D) RhoB expression by
immunohistochemistry. (E) H3 acetylation after treatment with romidepsin and/or azacitidine or DMSO analyzed by western blot. (F) H3 acetylation at the RhoB promoter region is significantly increased in the combination treatment vs single agent, analyzed by ChIP-PCR. (G) Parallel cell viability analysis by MTT assay. (H) Effect of combination therapy on p21 protein analyzed by western blot.

Figure 5. Combination of romidepsin and azacitidine results in specific global methylation changes. (A) Principal component analysis demonstrated clustering of azacitidine treated cells, alone or in combination away from romidepsin alone or DMSO treated cells. (B) Percentage of differentially methylated regions per chromosome showed azacitidine alone or in combination causes a global CpG hypomethylation in the CTCL genome. (C) A Venn diagram illustrated CpG methylation changes of overlapping genes in different treatment groups with a subset of genes that are differentially methylated only in the combination treatment (adjusted \( p < 0.05 \)).
Figure 1

A

B

<table>
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<td>48h</td>
<td>1.1</td>
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<td>15.8</td>
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Figure 2. 

A. Graph showing cell viability for MyLa cells treated with Romidepsin and/or Azacitidine. 

B. Table showing IC50 values for MyLa cells treated with Romidepsin and/or Azacitidine.

C. Flow cytometry analysis for MyLa and SeAx cells treated with DMSO, Romidepsin, Azacitidine, and combinations thereof.

D. Histogram showing cell count distribution for MyLa and SeAx cells treated with DMSO, Romidepsin, Azacitidine, and combinations thereof.
Figure 3

A

% cell viability

\[
\begin{array}{c}
\text{Patient 1} \quad \text{Patient 2} \quad \text{Patient 3} \quad \text{Patient 4} \\
\text{R 2.5nM+A 2.5uM} \\
\text{Romidepsin 2.5nM} \\
\text{Azacitidine 2.5uM}
\end{array}
\]

B

Annexin V FITC

DMSO
Romidepsin
Romidepsin+Azacitidine
Azacitidine

B

% Apoptosis

\[
\begin{array}{c}
\text{Patient 1} \quad \text{Patient 2} \quad \text{Patient 3} \quad \text{HD 1} \quad \text{HD 2} \\
\text{R 2.5nM+A 2.5uM} \\
\text{Romidepsin 2.5nM} \\
\text{Azacitidine 2.5uM}
\end{array}
\]

D

Cell count

\[
\begin{array}{c}
\text{Patient 1} \quad \text{Patient 2} \quad \text{Patient 3} \\
\text{DMSO} \\
\text{Romidepsin} \\
\text{Romidepsin+Azacitidine} \\
\text{Azacitidine}
\end{array}
\]
Figure 4

**A**

![Image of expression levels comparison](image)

**B**

![Graph showing RhoB expression fold change](image)

**C**

![Graph showing RhoB expression fold change over time](image)

**D**

![Images of tissue samples](image)

**E**

![Western blot images of Acetyl-H3 and a-Tubulin](image)

**F**

![Bar graph of RhoB gene fold change normalized to IgG](image)

**G**

![Bar graph of viability normalized to control](image)

**H**

![Western blot images of p21 and a-Tubulin](image)

2. DMSO, 3. Romidepsin + Azacitidine, 4. Romidepsin, 5. Azacitidine
Figure 5

A

B

C

DMSO Red
Romidepsin+Azacitidine Blue
Romidepsin Green
Azacitidine Purple

Romidepsin+Azacitidine
% of hyper & hypo methylated regions per chromosome
qvalue<0.01 & methylation diff. >=25%

DMSO Red
Romidepsin+Azacitidine Blue
Romidepsin Green
Azacitidine Purple

Romidepsin

Romidepsin+Azacitidine

Azacitidine

Romidepsin
Romidepsin and azacitidine synergize in their epigenetic modulatory effects to induce apoptosis in CTCL

Sima Rozati, Phil F Cheng, Daniel S Widmer, et al.

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