Decitabine Priming Enhances Mucin 1 Inhibition Mediated Disruption of Redox Homeostasis in Cutaneous T-Cell Lymphoma

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Abstract

Cutaneous T-cell lymphoma (CTCL) is a heterogeneous neoplasm and patients with relapsed/refractory disease exhibit resistance to standard therapies. We have previously demonstrated that the Mucin 1 C-terminal subunit (MUC1-C) plays a critical role in protection from oxidative stress in CTCL cells. Targeting of MUC1-C with a pharmacologic inhibitor, GO-203, was associated with apoptosis in CTCL. However, disease responses were incomplete underscoring the need for combinatorial strategies that could exploit the vulnerability of CTCL cells to oxidative signals. Cell lines, primary samples, and xenograft models of CTCL were used to assess synergy of GO-203 with decitabine, a hypomethylating agent. Present studies demonstrate that exposure of CTCL cells to decitabine in combination with GO-203, increased the generation of reactive oxygen species (ROS) levels and decreased levels of scavenger molecules, NADP, NADPH, glutathione, and TIGAR, critical to intracellular redox homeostasis. Dual exposure to GO-203 and decitabine resulted in marked downregulation of DNA methyl transferases demonstrating significant synergy of these agents in inducing global and gene specific hypomethylation. Accordingly, treatment with decitabine and GO-203 upregulated the ROS generating enzymes, NADPH oxidase 4 and dual oxidase 2 potentially due to their effect on epigenomic regulation of these proteins. In concert with these findings, exposure to decitabine and GO-203 resulted in heightened apoptotic death in CTCL cell lines, patient-derived primary samples and in a murine xenograft model. These findings indicate that decitabine intensifies MUC1-C inhibition induced redox imbalance and provides a novel combination of targeted and epigenetic agents for patients with CTCL. Mol Cancer Ther; 16(10); 2304–14. ©2017 AACR.

Introduction

Cutaneous T-cell lymphoma (CTCL) is a hematologic malignancy trophic to the skin with diverse patterns of disease presentation and clinical outcome. Although therapy of localized disease is highly effective, therapeutic options for patients with advanced disease remains limited. Patients with advanced stages of mycosis fungoides (MF) and Sezary syndrome (SS), the two most common subtypes of CTCL, have estimated 5-year survival rates of 20% to 40% (1, 2). Even more challenging has been the treatment of patients who experience relapsed or refractory (R/R) disease. Better understanding of the tumor biology of CTCL has yielded an opportunity to change the treatment paradigm with the potential for cure.

The Mucin 1 C-terminal subunit (MUC1-C) oncoprotein plays a pivotal role in the survival of malignant cells including self-renewal, proliferation, and resistance to apoptosis (3). A primary mediator of these effects is the capacity of MUC1-C to regulate cellular levels of reactive oxygen species (ROS; ref. 4). Notably, maintenance of redox balance appears to be a critical factor in protecting CTCL cells from apoptosis in comparison with normal T cells (5, 6). We recently demonstrated that MUC1-C is over-expressed in CTCL cells (7). We have developed a clinical grade cell-penetrating peptide that disrupts homodimerization of the MUC1-C subunit necessary for nuclear translocation and downstream signaling (ref. 8; Supplementary Fig. S1E and S1F). Treatment of CTCL cells with the MUC1-C inhibitor (GO-203) was associated with increased oxidative stress resulting in cell death in the context of late apoptosis and necrosis. These findings indicated that MUC1-C contributes to redox balance in CTCL and thereby is a novel target for its treatment. However, disease response in in vivo models was incomplete highlighting the need to explore synergistic combinations of the MUC1-C inhibitor with other agents that can enhance redox disruption mediated cytotoxicity of the CTCL cells.

Histone deacetylase and hypomethylating agents (HMA) were recently shown to exhibit efficacy in preclinical T-cell lymphoma models (9, 10). Of note, CTCL demonstrates abnormal patterns of
methylation and expression of tumor suppressor genes correlating with the clinical disease presentation (11, 12). In addition, decitabine has also been shown to induce ROS accumulation in acute myeloid leukemia models (13). As such, we hypothesized that CTCL would demonstrate enhanced sensitivity to combination therapy with agents that increase oxidative stress by modulating the epigenome.

In this study, we demonstrate that exposure of CTCL cells to decitabine and the MUC1-C inhibitor at minimal cytotoxic concentrations results in a marked increase in ROS levels and depletion of NADP, NADPH, and glutathione (GSH). The combination also induced greater downregulation of the TP53-induced glycolysis and apoptosis regulator (TIGAR), critical to protection from oxidative stress. Further, we demonstrate that GO-203 exerts an independent effect on hypomethylation via its suppression of oxidative stress. Further, we demonstrate that GO-203 exerts an independent effect on hypomethylation via its suppression of oxidative stress. Further, we demonstrate that GO-203 exerts an independent effect on hypomethylation via its suppression of oxidative stress. Further, we demonstrate that GO-203 exerts an independent effect on hypomethylation via its suppression of oxidative stress.

Materials and Methods

Cell culture

The CTCL cell lines, H9 (HTB-176), HuT-78 (TIB-161), and HH (CRL-2105) were obtained from ATCC 1 month before start of experiments in July 2015, and authentication was performed using short tandem repeat (STR) profiling. CTCL cell lines, Myla, and SeAx was obtained from Dr Robert Gniadecki (University of Copenhagen, Denmark) and authenticated by STR before use. All cell lines were grown as previously described (7). All cell lines were tested for mycoplasma detection with the MycoSEQ Mycoplasma Detection Assay from Applied Biosystems prior to use. Cell lines were in passage for three months between thawing and use in described experiments.

Materials

Decitabine was purchased from Selleckchem (S1200). Genus Oncology LLC provided GO-203. N-acetyl cysteine (NAC) was purchased from Sigma.

Measurement of ROS levels

Briefly, H9, HuT-78, Myla and primary CTCL cells were incubated with 5 μmol/L carboxy-H2DCFDA (2’,7’-dichlorodihydrofluorescein diacetate) for 30 minutes at 37°C to assess ROS-mediated oxidation to the fluorescent compound 2’,7’-dichlorofluorescein (DCF), which was measured by excitation at 480 nm and emission at 590 nm. The experiment was done in triplicate for the cell lines and repeated thrice.

Flow cytometric analysis of cell death

H9 and HuT-78 cells were incubated with propidium iodide (PI)/annexin V-fluorescein isothiocyanate (BD Biosciences) for 15 minutes at room temperature and then analyzed by flow cytometry. Each experiment was done in triplicate and repeated thrice.

Determination of NADP, NADPH, and GSH levels

Intracellular NADP concentrations were measured using the NADP/NADPH-Glo Assay Kit (Promega G9081). Intracellular GSH concentrations were measured using GSH-Glo Glutathione Assay Kit (Promega V6911) as per manufacturer’s instructions. Each experiment was done in triplicate and repeated thrice.

Immunoblot analysis

Cell lysates were prepared as described (7). Soluble proteins were analyzed by immunoblotting with anti-DNMT1, 3b (Abcam), anti-Histone 3 (tri methyl K27; Abcam), anti-TIGAR (Abcam), anti-phospho-p38 (Abcam), anti-Total p38 (Abcam), anti-phospho JNK (Abcam), anti-Total JNK (Abcam), anti-Nrf2 (Abcam), anti-phospho Smad2, and Smad3 (Cell Signaling Technology), anti-Total Smad2 and Smad3 (Cell Signaling Technology), anti-c-Myc (Abcam), and anti-GAPDH (Cell Signaling Technology).

DNA methylation analysis

Untreated and treated H9 cells were harvested. Following bisulfite conversion of DNA, direct pyrosequencing was performed to quantify methylation levels in 4 CpG positions of the LINE-1 element as a global measure of DNA methylation. Using the same technique promoter specific methylation of 8 CpG positions in the human N0x3 gene and 7 CpG positions in the human Duox2 gene was performed and quantified. Experiment was done in triplicate in conjunction with EpigenDx, Inc.

RNA extraction and RT-qPCR

RNA was extracted from H9 and HuT-78 cells under described experimental conditions using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RT-PCR primers for human N0x3 and Duox2 were purchased from Sigma-Aldrich. Real-time quantitative PCR analysis was performed using Bio-Rad’s iQ5 Universal One-step RT-qPCR Kit per manufacturer’s instructions.

Cytotoxicity assays

H9, HuT-78, Myla, SeAx, and HH lines were resuspended at a concentration of 1.0 to 1.5 × 10⁶ cells per well in a 96-well plate (CoStar) and incubated at 37°C in a 5% CO₂ humidified incubator for 96 hours. Decitabine was added at concentrations from 10 to 40 nmol in 100 μL as a single dose, selected to approximate the IC₅₀–IC₁₀ (concentration at which 10% to 20% cells are dead), GO-203 was added daily for 3 days at concentrations ranging from 1.5 to 3 μmol in 100 μL selected to approximate the IC₅₀–IC₂₅ (concentration at which 10–25% cells are dead). When combining decitabine and GO-203, identical drug concentrations and schedule was used. At 96 hours, the cells were evaluated for viability using the CellTiter-Glo reagent from Promega (G7572) as previously described (7). Each experiment was done in triplicate and repeated thrice and viability compared with untreated cells.

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FACS sorting of primary CTCL cells and cytotoxicity assay
Blood samples were obtained from three patients with leukemic CTCL (L-CTCL) seen at the Cutaneous Lymphoma Program at the Dana-Farber/Brigham and Women’s Cancer Center under an IRB approved protocol for sample acquisition as previously described (7, 14). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll centrifugation and incubated with mAb DF3 (anti-MUC1-N) followed by secondary labeling of the cells with FITC-conjugated goat anti-mouse IgG and malignant clone specific PE-conjugated anti-TCR Vβ mAbs as previously described (7, 14). The MUC1-N and TCR Vβ double positive population was FACS sorted on a MoFlo Legacy cell sorter (Beckman Coulter). Cells were counted and re-suspended at an approximate concentration of 1.0 x 10^6 per well in a 96-well plate (Becton Dickinson Labware) and incubated at 37°C in a 5% CO2 humidified incubator for up to 96 hours. Cells were left untreated, or a single dose of decitabine was added at a concentration of 40 nmol, or GO-203 was added daily for 3 days at 3 μmol or the combination. At 96 hours, the cells were evaluated for viability using the CellTiter-Glo reagent.

Therapy with decitabine and/or GO-203 in a murine xenograft model
Five- to seven-week-old female NSG mice were injected with 2 million H9 cells in the flank via a subcutaneous route. Three-dimensional ultrasound imaging data sets were collected for each xenograft using a Vevo 2100 ultrasound microimaging system (VisualSonics Inc.) designed for small animal imaging. For analysis of ultrasound data, images were imported into Amira 5.2 (VisualSonics) for volumetric analysis. Representative flow cytometry histograms are presented in Supplementary Fig. S1A and S1B. Similar results were confirmed in Myla, another MUC1-C expressing CTCL cell line (Supplementary Fig. S2A and S2B). We subsequently investigated the impact of GO-203 in combination with decitabine on pro-apoptotic proteins such as p38 MAPK and JNK characteristically induced by increased oxidative stress. A marked increase in the ratio of phospho-p38 and phospho-JNK to Total-p38 and Total-JNK, respectively, was observed in H9 cells with dual exposure to decitabine and GO-203 (Figs. 1C and D).

Results

Combination of decitabine and MUC1-C inhibitor increases ROS in CTCL cells
CTCL cell lines H9 and HuT-78 overexpress MUC1-C in comparison to other cell lines SeAx and HH and hence these were chosen to perform in vitro and in vivo experiments (7). Treatment of H9 and HuT-78 cells with minimal inhibitory concentrations of decitabine in combination with the MUC1-C inhibitor, GO-203, was associated with a marked increase in ROS levels (Fig. 1A and B) as compared to either agent alone. This increase was at least partially reversed by concurrent exposure of the cells to the antioxidant NAC that promotes the scavenging of ROS underpinning greater disruption of redox balance with the combination. Representative flow cytometry histograms are presented in Supplementary Fig. S1A and S1B. Similar results were confirmed in Myla, another MUC1-C expressing CTCL cell line (Supplementary Fig. S2A and S2B).

Combination of decitabine and MUC1-C inhibitor decreases NADPH, GSH, and TIGAR in CTCL cells
Consistent with above findings, exposure to decitabine and GO-203 resulted in a marked reduction of the ROS scavenging molecules, NADP, NADPH, and GSH in H9 cells as compared to cells treated with either agent alone (Fig. 2A and B). Similar findings were observed in HuT-78 cells (Fig. 2C and D). TIGAR is a potent regulator of glycolysis, redox potential, and apoptosis whose expression is modulated by p53 expression. We have previously demonstrated that MUC1-C inhibition was associated with decrease in the expression of TIGAR that escalates ROS expression. In this study, we demonstrate that combination of GO-203 and MUC1-C inhibition is associated with further decrease in TIGAR expression potentially through impact on p53 expression (Fig. 2E and F). This attenuation of ROS scavenging process provides a potential mechanistic explanation for redox deregulation and associated synergistic induction of ROS by the combination. These results highlight that decitabine in combination with MUC1-C inhibition intensifies ROS generation and decreases ROS-scavenging capacity leading to disturbance in intracellular redox homeostasis.

Combined therapy with decitabine and MUC1-C inhibitor inhibits DNMTs altering Nos4 and Duox2 expression and activating Smad signaling
Having observed a diminution in ROS scavenging abilities, we investigated the effects of the combination on ROS generating mechanisms. Decitabine has been demonstrated to disrupt redox...
homeostasis through its modulation of ROS generative enzymes such as Nox. Accordingly, we assessed the impact of the combined therapy on hypomethylation and the resultant transcriptional changes that could increase ROS levels in this setting. In this study, we demonstrate that exposure of CTCL cells to GO-203 down-regulates DNMT 1 and 3b expression. Consistent with this finding, combined therapy with GO-203 and decitabine markedly reduced the expression of these proteins compared to either agent alone (Fig. 3A and B). We hypothesized that the resulting hypomethylation would increase expression of proteins such as trimethyl histone 3 (H3K27me) and NADPH oxidases, a family of oxidases that induces superoxide production with two of its isoforms having putative CpG islands in its promoter (13). Consistent with our hypothesis, we demonstrated that combined therapy with decitabine and GO-203 increased expression of H3K27me and isoforms Nox4 and Duox2 providing a mechanistic link between combined hypomethylating effect of these agents and subsequent ROS-mediated killing of CTCL cells. To validate the effects of the increased oxidative stress exerted by the combination on established downstream signaling changes that promote apoptosis, we investigated the effect on Smad signaling pathway. We also observed an increase in expression of Nrf2, which prevents excessive cellular damage produced by oxidative stress under homeostatic conditions, supporting previous observations. ROS is known to regulate the expression of the Smad family of transcription factors (18). Accordingly, increased phosphorylation of Smad2 and Smad3 proteins leading to their activation was observed with the combination. Further, expression of the c-Myc oncoprotein, a known downstream target gene of the Smad and MUC1-C signaling pathways was downregulated in the combination treated cells (19). These results highlight the potential mechanisms by which ROS is induced by the combination and the role of redox disruption in altering expression of key transcriptional proteins regulating proliferation and cell cycle.

**Combination of decitabine and MUC1-C inhibitor induces synergistic decrease in DNA methylation**

Based on downregulation of DNA methyltransferases associated with combination treatment, we investigated the effects of the combination on DNA methylation patterns. Indeed, in the combination treated H9 and HuT-78 cells, there was a significant decrease in the percentage of DNA methylation at each of the

![Figure 1](image-url)
four CpG positions in the long interspersed nucleotide element (LINE-1) repeats, a surrogate marker of global DNA methylation (Fig. 4A and D). This supports a role for the combination in inducing greater global DNA methylation compared to the single agents. Having observed a global decrease in methylation and increase in protein expression of Nox4 and Duox2, we investigated the effects of the combination on methylation of the Nox4 and Duox2 promoter. In line with our hypothesis, we noted a statistically significant decrease in the percentage of DNA methylation at each of the eight CpG positions in the Nox4 promoter (positions −386 to −296; Fig. 4B and E) and seven CpG sites in the Duox2 promoter (positions −902 to −860) (Fig. 4C and F). Locations of the CpG sites that were analyzed in the promoter of these genes with respect to the remaining to the structure of the genes are represented in Supplementary Figs. S3 and S4. The present results extend the importance of the combination in

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Figure 2.

Decitabine and GO-203 combination decreases NADP, NADPH, GSH, and TIGAR levels leading to decrease in ROS scavenging abilities in CTCL cells. A and B, H9 cells were left untreated, treated with 3 μmol GO-203 each day for 3 days, single dose of 40 nmol Dac or the combination. Cells were analyzed for NADP, NADPH, and GSH levels at 96 hours. The results are expressed as fold change in total NADP and NADPH and GSH levels relative to the control (mean ± SD of three determinations). C and D, HuT-78 cells were also analyzed for NADP, NADPH, and GSH levels at 96 hours under the same conditions mentioned above (mean ± SD of three determinations). E and F, H9 and HuT-78 cells were left untreated, treated with 3 μmol GO-203 each day for 3 days, single dose of 40 nmol Decitabine or the combination. Cells were harvested at 96 hours. Lysates were immunoblotted with the indicated antibodies.
enhancing global and gene-specific DNA demethylation including epigenetic changes in redox homeostatic genes.

**Decitabine and GO-203 combination increase expression of Nox4 and Duox2 mRNA**

To investigate whether the combination regulates transcription of Nox4 and Duox2 following promoter demethylation, qRT-PCR was performed. A statistically significant increase in Nox4 and Duox2 mRNA levels was observed in the combination treated H9 and HuT-78 CTCL cells compared with control and single agents (Supplementary Fig. S5).

**Decitabine and GO-203 demonstrate synergistic killing of MUC1-C positive versus MUC1-C negative CTCL cell lines in vitro**

In keeping with increase in oxidative stress, a significant increase in cells manifesting late apoptosis/necrosis was observed following exposure to decitabine and GO-203 as compared to either agent alone as determined by flow cytometric analysis of PI/annexin V–FITC staining (Supplementary Fig. S6A and S6B). Representative flow cytometry plots are demonstrated in Supplementary Fig. S6C and S6D. The induction cell death was reversed by exposure of the cells to NAC highlighting the contribution of redox disruption in inducing cytotoxicity.

To formally assess synergy of cytotoxic killing by decitabine and GO-203, H9 and HuT-78 cells were treated with a single dose of decitabine in nanomolar concentrations corresponding to IC$_{50}$–IC$_{10}$ (10–40 nmol) and MUC1-C inhibitor, corresponding to IC$_{10}$–IC$_{25}$ (1.5–3 µmol). The combination demonstrated marked synergy with respect to induction of cytotoxic death over a range of concentrations in both cell lines (Supplementary Fig. S7A and S7B). The validated *Excess over Bliss Independence Model* was used to assess synergistic interaction. Values >10 represent synergy. Excess over Bliss (EOB) scores and

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**Figure 3.**

Decitabine and GO-203 combination decreases expression of DNMT1, 3b, increases Nox4, Duox2, activates Nrf2 and Smad signaling pathway leading to downregulation of c-Myc. **A** and **B**, H9 and HuT-78 cells were left untreated, treated with 3 µmol GO-203 each day for 3 days, single dose of 40 nmol Decitabine or the combination. Cells were harvested at 96 hours. Lysates were immunoblottedted with the indicated antibodies.
Results were consistent with escalation of doses across the cell lines. Similar results were observed in combinational index values presented in the adjoining tables (Supplementary Fig. S7C and S7D) indicating increasing synergy with escalation of doses across the cell lines. Similar results were confirmed in another MUC1-C expressing cell line, Myla (Supplementary Fig. S2C and S2D). SeAx and HH are two CTCL cell lines with low to negative expression of MUC1-C as previously demonstrated by us (7). The combination failed to induce cytotoxic killing in both these cell lines, providing support for cytotoxic specificity of the combination in MUC1-C positive CTCL cells (Supplementary Fig. S1C and S1D).

Combination of decitabine and MUC1-C inhibitor is cytotoxic in primary CTCL cells

The malignant clonal population was isolated from three primary CTCL samples by FACS of PBMCs for the double positive cells (TCR Vβ and MUC1-N) as previously described. FACS plots of all patients are presented in Fig. 5D. Sorted cells were treated with single minimally cytotoxic dose of decitabine (40 nmol) and daily GO-203 (3 μmol) for 3 days. Exposure to decitabine and GO-203 resulted in a substantial decrease in viability at 96 hours as compared to untreated and cells treated with either agent alone (Fig. 5A–C). EOB scores presented below confirmed synergy. An increase in ROS levels with the combination that was partially reversed by NAC was also confirmed in primary CTCL cells and is presented in Supplementary Fig. S8.

Decitabine and MUC1-C inhibition demonstrate synergy in a xenograft murine model of CTCL

To assess in vivo antitumor activity of the combination, H9 cells were inoculated subcutaneously in the flank of NSG mice. After the xenograft tumors reached a volume of approximately 100 mm³ as determined by 3D ultrasonography, cohorts of animals were assigned to control (treated with PBS); treatment with decitabine alone; GO-203 or combined therapy. Tumor volume was quantified by serial assessment by ultrasonography. Rapid tumor growth was observed in the control cohort that was blunted modestly in animals treated with either decitabine or GO-203 alone. In contrast, combination therapy resulted in a statistically significant inhibition of tumor growth after completion of 3 weeks of therapy (Figs. 6A and B). One week after completion of therapy, mice were sacrificed for histological examination of the xenograft tumors and stained for TUNEL and trimethyl histone 3 analysis. Consistent with the in vitro studies, tumors isolated from mice treated with decitabine and GO-203 demonstrated higher levels of apoptotic cells and trimethyl histone as compared to animals treated with either agent alone (Figs. 6C–F). P values <0.05 represent statistical significance.

Discussion

We have demonstrated that the MUC1-C oncoprotein is selectively expressed by CTCL cells as compared to normal T cells and...
plays a vital role in protecting tumor cells from ROS-mediated cell death. Consistent with these findings, inhibition of MUC1-C signaling using a cell penetrating peptide that blocks dimerization, nuclear translocation, and downstream signaling by the MUC1-C subunit results in increased ROS levels and apoptotic cell death in in vitro and in vivo models. Although it demonstrated therapeutic efficacy, treatment with the MUC1-C inhibitor alone did not fully eradicate disease in a xenogenic mouse model, suggesting that its effect in promoting ROS-mediated cell death is incomplete. As such, we have investigated strategies to further enhance MUC1-C inhibition-mediated cytotoxicity in an effort to develop effective therapies without detrimental effects for this challenging T-cell lymphoma. The hypomethylating agent, decitabine, modulates redox balance in leukemia models (13). Based on our recent observation that MUC1 contributes to regulation of oxidative stress in CTCL cells, we sought to systematically explore the effect of the MUC1-C inhibitor with decitabine on redox signaling. To minimize toxicity of combination treatments, we specifically chose minimal inhibitory concentrations of both compounds. Herein, we demonstrate that exposure to decitabine and GO-203 results in increased ROS levels and increased expression of pro-apoptotic proteins such as phospho-p38 and phospho-JNK as compared to either agent alone. Given the pleiotropic effects of MUC1-C targeting and HMAs, we hypothesized that the combination may have an effect on ROS-scavenging ability. In this context, the combination led to a decrease in levels of the scavenger molecules, NADP, NADPH, and GSH. The downregulation of TIGAR resulted in its inability to inhibit glycolysis and protect from increase in intracellular ROS levels. To investigate the mechanistic basis for enhanced generation of ROS with the combination, we first examined the impact of MUC1-C inhibition on DNMT1 and 3β, essential for maintaining DNA methylation. We observed that targeting MUC1-C affects methylation patterns via its regulation of DNMT expression. We hypothesized that combining MUC1 inhibition with decitabine would further increase the degree of hypomethylation, resulting in enhanced transcription of genes that could modulate redox signaling. Indeed, we found that targeting MUC1-C in combination with

Figure 5. Decitabine and GO-203 combination is synergistically cytotoxic in CTCL primary cells. A–C, MUC1-N and TCR Vβ rearranged double positive malignant population was FACS sorted from three patients with L-CTCL. Cells were left untreated, treated with 3 μmol GO-203 each day for 3 days, single dose of Dac 40 nmol or the combination (GO plus Dac). Cell Titer Glo was added and viability was measured at 96 hours. The results are expressed as relative percentage viability (mean ± SD of 3 determinations) compared to the control. Excess over Bliss independence Model was used to calculate synergy co-efficients reported in the respective tables. Values greater than 10 represent synergy. D, FACS plots of the three patients with L-CTCL are depicted demonstrating sorting of the double positive, TCR Vβ, and MUC1-N population from PBMCs for in vitro cytotoxicity experiments.
Decitabine resulted in the near abrogation of DNMT 1 and 3b levels supporting our hypothesis. The combination induced marked decrease in global DNA methylation as reflected in the reduction of DNA methylation percentage of all CpG sites in the LINE-1 element. Further, the combination treatment was associated with a greater diminution in DNA methylation of Nox4 and Duox2 promoter that play a critical role in cellular redox balance. These results provide evidence for synergistic effect of the combination in inducing DNA demethylation and varied molecular effects leading to the intensified antitumor activity of the combination. Decrease in the expression of other proteins such as DNMT1 and 3b was also seen in the xenograft tumors but was not statistically significant probably due to heightened apoptosis mediating clearance of tumor cells.

The Nox family of which there are currently seven identified isoforms (Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2) produce ROS (13, 20). They use NADPH as an electron donor and are widely distributed in cancer cells. Nox4 and Duox2 have putative CpG islands around its promoter region invoking the possibility of hypomethylation by DNMT inhibitors such as decitabine. We found that the combination of decitabine and MUC1-C inhibitor is associated with upregulation of Nox4 and Duox2.

**Figure 6.**
Decitabine and GO-203 combination treatment results in greater decline of the xenograft CTCL tumors. 

**A** and **B**, Two and a half million H9 cells were injected subcutaneously in the flank of NSG mice (n = 20). In vivo ultrasound (US) images were acquired starting day 4 after inoculation of cells. After the mean diameter of the tumor reached 5 to 7 mm they were randomized to control, treatment with GO-203 alone, treatment with decitabine (Dac) alone or combination cohorts (n = 5 in each cohort). Control mice were treated with normal saline daily injected intradermally. In the GO-203 alone arm, drug was administered daily at the dose of 14 mg/kg i.d. for 21 days. In the decitabine alone arm, drug was administered twice per week at the dose of 0.4 mg/kg intraperitoneally (i.p.) for 3 weeks. In the combination arm, Dac and GO-203 were administered at same doses, schedule, and route as single agents for 3 weeks. Both cohorts were imaged before starting treatment and subsequently weekly. The US system acquired sequential images for each tumor, resulting in a three-dimensional (3D) representation of the tumors. The 3D images were used to calculate the estimated tumor volume, and generate a tumor response curve (A). Representative pictures with the area of the tumor outlined by blue line are shown (B). **C** and **D**, Xenograft tumors were harvested from the different cohorts 1 week after completion of treatment. The tumors were stained and analyzed by TUNEL for apoptosis and quantified for percentage of apoptotic cells in combination arm in comparison with other cohorts (C). Representative photomicrographs (20×) of TUNEL positive cells, averaging 10 per 100× hpf in the control group, 10 per 100× hpf in the Decitabine group, 12.3 per 100× hpf in the GO-203 group, and 18.8 in the combination group are shown (D). Xenograft tumors were also stained for trimethyl histone 3 (H3K27 me; E). Representative photomicrographs of H3K27 me positive cells, averaging 1.54 per 100× hpf in the control group, 2.04 per 100× hpf in the decitabine group, 2 per 100× hpf in the GO-203 group, and 2.5 in the combination group are shown (F). P values <0.05 represent statistical significance.
that many of the profound molecular changes incited by the therapeutic index without adverse events. It is also interesting to see the combination exerts pleiotropic antitumor effects by disrupting redox balance and modifying the expression of key regulatory proteins involved in apoptosis, cell adhesion, and cell cycle. Of note, our studies do not exclude the possibility that mechanisms outside of redox disruption can provide alternative molecular basis for the synergy observed. Transcription of tumor suppressive microRNAs with hypermethylated promoters unique to CTCL could be one such possibility and will be the focus of subsequent studies (11, 12).

It is worth noting the low concentrations of decitabine and MUC1-C inhibitor that were specifically selected for the experiments. The translational relevance of this observation raises the potential con

In conclusion, the present results support the potential of targeting MUC1-C and DNMT inhibitors in CTCL. The combination (1) impairs ROS repair mechanism through decrease of NADPH, GSH, and TIGAR (2) is associated with down regulation of DNMTs, decrease in global and gene-specific promoter DNA methylation and upregulation of Nox4 and Duox2 protein expression (3) collectively these lead to increase in overall cellular ROS levels and, (4) ROS-mediated late apoptosis/necrosis through modulation of different genes including oncoproteins and tumor suppressors. MUC1-C inhibitor has entered phase I/II evaluation in patients with AML (https://clinicaltrials.gov/ct2/show/NCT02204085) and based on the above results warrants an early phase study in CTCL patients. Efficacy of this regimen in the clinic will provide the impetus needed to combine agents with robust scientific rationale and preclinical data in this poorly understood neoplasm. Our observations have led us to use this combination as a backbone in developing novel strategies targeting redox regulation in CTCL.

Disclosure of Potential Conflicts of Interest

Donald Kufe has ownership interest (including patents) for Genus Oncology; and also is a consultant/advisory board member in Genus Oncology. No potential conflicts of interest were disclosed by the other authors.

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References

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