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HDAC inhibition induces microRNA-182 which targets Rad51 and impairs HR repair to sensitize cells to sapacitabine in acute myelogenous leukemia

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Abstract

Purpose—The double strand breaks elicited by sapacitabine, a clinically active nucleoside analog prodrug are repaired by Rad51 and the homologous recombination repair (HR) pathway which could potentially limit its toxicity. We investigated the mechanism by which HDAC inhibitors targeted Rad51 and HR to sensitize AML cells to sapacitabine.

Experimental Design—Chromatin immunoprecipitation identified the role of HDACs in silencing microRNA-182 in AML. Immunoblotting, gene expression, overexpression or inhibition of miR-182 and luciferase assays established that miR-182 directly targeted Rad51. HR reporter assays, apoptotic assays and colony forming assays established that the miR-182, as well as the HDAC inhibition-mediated decreases in Rad51 inhibited HR repair and sensitized cells to sapacitabine.

Results—The gene repressors, HDAC1 and HDAC2, became recruited to the promoter of miR-182 to silence its expression in AML. HDAC inhibition induced miR-182 in AML cell lines and primary AML blasts. miR-182 targeted Rad51 protein both in luciferase assays and in AML cells. Overexpression of miR-182, as well as HDAC inhibition-mediated induction of miR-182 were linked to time- and dose-dependent decreases in the levels of Rad51, an inhibition of HR,

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increased levels of residual damage and decreased survival after exposure to double strand damage inducing agents.

Conclusions—Our findings define the mechanism by which HDAC inhibition induces miR-182 to target Rad51 and highlights a novel pharmacological strategy that compromises the ability of AML cells to conduct HR, thereby sensitizing AML cells to DNA damaging agents that activate HR as a repair and potential resistance mechanism.

Keywords

HDAC inhibition; nucleoside analog; panobinostat; sapacitabine; homologous recombination repair; Rad51; AML

Introduction

Sapacitabine is an orally available nucleoside analog prodrug that is structurally related to cytarabine. It is active against AML both in elderly patients and in relapsed, refractory disease (1, 2). Sapacitabine is metabolized into its active form 2'-C-Cyano-2'-deoxy-1-β-D-arabino-pentofuranosylcytosine (CNDAC) by amidases. Following intracellular phosphorylation, the resulting triphosphate is incorporated into replicating DNA. In contrast to other nucleoside analogs, this does not terminate replication. Rather, after additional nascent DNA strand elongation, a β-elimination reaction occurs which causes the analog to rearrange to a 2', 3'-dideoxyconfiguration. This terminates the 3'-end resulting in a single stranded nick which becomes converted to a double strand break upon a subsequent round of DNA replication (3, 4). The double strand DNA breaks generated by CNDAC (5) or ionizing radiation (6), are partly repaired by the homologous recombination repair (HR) pathway. HR is governed by multiple proteins such as ATM, the Mre11-Rad50-Nbs1 complex (7), Brca1/2 and Rad51, a key protein that directs the homology search and DNA strand exchange that is critical for successful HR repair (8). High levels of Rad51 are linked to resistance to DNA damaging therapies, relapsing disease and poor survival (9, 10). Conversely, cells lacking Rad51 or its paralog Rad51D have an increased sensitivity to DNA damaging agents (11) including CNDAC (5). Unlike breast and ovarian tumors that often harbor intrinsic defects in HR, AML cells usually exhibit a functional HR pathway (12) which may allow them to limit the toxicity of DNA double strand break inducing agents.

The histone deacetylases (HDACs) are a class of chromatin modulating proteins that become recruited to target promoters to compact chromatin and silence gene expression. Conversely, inhibitors of HDACs (HDACi) function by promoting the acetylation of proteins, including histones at gene promoters to induce a transcriptionally active chromatin configuration and reverse HDAC-mediated gene expression (13, 14). HDACi's such as vorinostat (SAHA) and belinostat are approved for the treatment of cutaneous T cell leukemia (15) whereas panobinostat (PS) is approved for the treatment of refractory multiple myeloma (16, 17). In AML, HDACi's showed synergy with CNDAC in xenograft models (18) and were effective in Phase I/II trials when combined with demethylating (19) or other chemotherapeutic agents (20). Mechanistically, PS exposure led to decreases in the levels of ATM, Brca1 and Rad51 to sensitize cells to cytarabine, daunorubicin or ionizing radiation in solid tumor cell lines (21, 22). HDACi treatment also leads to the depression an important class of regulatory

genes, the microRNAs (miRNA, miR) (23–25). miRNA bind to complimentary sequences in target RNA to either destabilize it or prevent its transcription in a cell and context specific manner (26, 27). Specific miRNA such as miR-182 are overexpressed in breast cancer and intrinsically compromise HR in those cells (28). Other miRNA, such as miR-103, miR-107, miR-96 and miR-182 target Rad51 and other components of the HR pathway in solid tumors (28–31); however it is not known whether these miRNA target Rad51 in AML. Since Rad51 is well expressed in AML, an investigation of the expression levels of miRNAs that potentially target Rad51 and the mechanisms that regulate their expression becomes important.

In this study, we investigated whether HDAC inhibition up-regulated the expression of miRNA genes that targeted Rad51, and if the resultant impairment in HR sensitized cells to sapacitabine, a clinically relevant double strand break-inducing drug in AML.

Materials and Methods

Materials

Panobinostat (PS) was provided by Novartis. Vorinostat (SAHA) was purchased from Cayman Chemical) and Decitabine (DAC) was from Sigma. All reagents were dissolved in 100% dimethyl sulfoxide (DMSO; Burdick & Jackson) to a stock concentration of 10^{-2} M and stored at -80°C . CNDAC was supplied by Dr. A. Matsuda (Hokkaido Univ., Sapporo, Japan).

Cell lines

OCI-AML3 and MV4–11 AML cells were obtained from ATCC maintained in RPMI supplemented with 5% FCS and 1% glutamine in a 37°C incubator containing 5% CO_2 . OCI-AML3 cells were authenticated at the Characterized Cell Line Core facility at M.D. Anderson Cancer Center. MV4–11 and HeLa-DR-13 cell lines were authenticated by sequencing at the Ohio State University. The HeLa-DR-13 (HeLa-DR) and HEK cells were a gift from Dr Parvin (Ohio State Univ.) and maintained as described (32). Primary AML cells and nucleic acids from normal bone marrow were collected under an IRB approved protocol and obtained from the Leukemia tissue bank at OSU. Primary AML blasts were maintained in RPMI with 20% FCS and $1\times$ Stem Span CC100 (Stem Cell Technologies).

Cytotoxicity assays

OCI-AML3 and MV4–11 cells were exposed to $2\text{ }\mu\text{M}$ CNDAC and increasing concentrations of PS or SAHA for 48 h following which annexin assays were conducted by incubating cells with annexin V–fluorescein isothiocyanate and propidium iodide (BD Biosciences) for 15 min and accumulating the fluorescence of at least 10,000 cells on a BD Biosciences FACSCalibur flow cytometer to determine the percentage of apoptotic and viable cells. Drug interactions were analyzed using the CalcuSyn (BioSoft). A combination index (CI) value of 1 indicated an additive drug interaction, whereas a CI value >1 suggested antagonism and a value <1 denoted synergism (33).

miRNA expression array

miRNA microarray analysis was carried out by LC sciences (<http://www.lcsciences.com/>) as described in Suppl. methods.

Immunoblotting

Immunoblotting was performed with antibodies against Rad51 (Millipore, Billerica, MA), H3K9Ac (Millipore), PARP (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), γ -H2AX and H2AX (Cell Signaling Technology) or GAPDH (Cell Signaling Technology).

Chromatin immunoprecipitation (ChIP) and protein immunoprecipitation

Equal amounts of cross-linked chromatin from OCI-AML3 or primary AML cells were used to carry out ChIP directed against HDAC1, HDAC2 (Millipore), H3K4me3 and H3K9AC. Rabbit IgG was used as a non-specific control (Jackson Laboratories)(34). DNA eluted from immunoprecipitates were purified and analyzed by PCR probes specific for the miR-182 promoter. For immunoprecipitation, HDAC1, HDAC2, and IgG immune complexes were prepared by incubating extracts from OCI-AML3 cells with antiserum for 1 h, followed by 45 min precipitation with protein A agarose beads, washed and assayed for the presence of HDAC1 or HDAC2.

Real-Time PCR

Total RNA was extracted from cells using the *mir*-Vana RNA extraction kit (Applied Biosystems). The expression of miR-182 was determined using 5 ng of total RNA, the step loop reverse primer and the *mir*-Vana Reverse transcription kit followed by qPCR using primer-probes from Applied Biosystems. Expression levels were quantitated by the comparative C_T method using snRNA RNU48 levels for normalization. Similarly, the levels of Rad51 were measured using the one step RT-PCR procedure probes from Applied Biosystems.

miRNA, antimiR and siRNA transfection

Pre-miR-182, anti-miR-182 and Pre-miR-negative control #1 were obtained from Life Technologies. Oligonucleotides were transfected into OCI-AML3 or HeLa-DR cells at 150 nM using the Nucleofection system, (Solution T, X-001) (35) (Amaxa) following manufacturer's instructions for 72–96 h, after which cells were harvested for RNA and protein analysis.

Colony forming assay

OCI-AML3 were exposed to drugs for 24 h, washed with PBS (37°C), and plated in methylcellulose medium in triplicate. Clonogenicity was determined after seven days. OCI-AML3 cells were exposed to scrambled (scr), miR-182 or antimiR-182 for 48h, then exposed to CNDAC for an additional 24 h before being assayed for the ability to form colonies after seven days.

Homologous recombination directed Repair Assay (HDR)

HeLa-DR cells were transfected with 300 or 500 nM miR-182 or scr for 48h before being transfected with the pCBASceI plasmid. For the experiments with miR-182 and scr, we performed a second round of transfection along with the pCBASceI plasmid. After 96 h, cells expressing green fluorescence were measured using a Gallios FACSCalibur instrument as described (32). For experiments testing HDAC inhibitors, cells were exposed to 10 or 15 nM PS for 24 h before transfection with pCBASceI and assayed for HDR as described (32).

Luciferase assay

The pmiRTarget vector with *RAD51* WT insert (full-length 3'UTR) or mutated miR-182 3'UTR (containing a deletion of the miR-182 target site) were co-transfected into HeLa or HEK cells with miR-182 or scr in HeLa cells. Cells were lysed 48 h after transfection and luciferase and Red fluorescence protein intensity (used as a transfection control) were measured 36 to 48 h and data expressed as the mean $\pm$ SEM from triplicate determinations from two to four independent transfections.

Statistical Analysis

Quantitative data are shown as the mean $\pm$ SEM for at least 3 independent experiments. Comparative statistics used the paired Student t test or one-way ANOVA with Tukey posthoc tests to evaluate differences between experimental groups. $P < 0.05$ and lower was considered statistically significant.

Results

HDAC inhibitors sensitize AML cells to the cytotoxic action of CNDAC

The pan HDAC inhibitor, vorinostat (SAHA) has been shown to synergize with CNDAC, the active metabolite of sapacitabine, in killing MV4–11 AML cells both in vitro and in a xenograft mouse model (18). To determine whether panobinostat (PS), a related HDACi, would synergize with CNDAC in AML cells we exposed OCI-AML3 and MV4–11 cells to either 2 μ M CNDAC, 0.1, 0.2 or 0.5 nM PS, or to 0.1, 0.2 or 0.5 μ M of SAHA and observed minimal cytotoxicity (Fig. 1A and 1B and Suppl. Fig. 1A and Suppl. Fig. 1B). However, when these cells were exposed to the combination of 2 μ M CNDAC with increasing concentrations of PS up to 0.5 nM (Fig. 1A and Suppl. Fig. 1A) or SAHA up to 0.5 μ M (Fig. 1B and Suppl. Fig. 1B) there was a greater than additive loss in cell survival (p value between $p < 0.01$ and $p < 0.05$). Drug interactions were analyzed by the Chou-Talalay method; the combination index (CI) values in OCI-AML3 cells for the interaction of 2 μ M CNDAC with 0.1, 0.2 or 0.5 nM PS and 0.1, 0.2 or 0.5 μ M SAHA were below 0.68 ± 0.24 (Suppl. Table 1). Similarly, the CI value in MV4–11 cells for the interaction of 2 μ M CNDAC with 0.1, 0.2 or 0.5 nM PS and 0.1, 0.2 or 0.5 μ M SAHA were below 0.72 ± 0.02 indicating synergy (Suppl. Table 1). We then chose the highest concentrations of PS (0.5 nM) and SAHA (0.5 μ M) that were synergistic with CNDAC in the apoptotic assays and determined whether they could synergize with CNDAC in colony forming assays. Concentrations of 0.5 μ M CNDAC were chosen for the cloning assay because exposure to 1 μ M or higher CNDAC inhibited colony growth in the OCI-AML3 cell line (5) attesting to the higher sensitivity of

the clonogenic assays. OCI-AML3 cells were exposed to 0.5 μ M CNDAC, 0.5 nM PS, 0.5 μ M SAHA or to combinations of CNDAC and PS or CNDAC and SAHA for 24 h. Our results indicate that the combination of 0.5 nM PS as well as 0.5 μ M SAHA was synergistic with 0.5 μ M CNDAC in inhibiting colony growth ($p < 0.0001$ and $p < 0.001$ respectively) (Fig. 1C and 1D). Taken together, our data indicate that HDAC inhibitors synergize with CNDAC to limit the survival of AML cell lines.

HDAC inhibition results in a decrease in the levels of Rad51 protein

In colon cancer cells, HDAC inhibition was linked to decreases in Rad51 and an inability to complete repair by HR (21). To determine the action of HDAC inhibition on Rad51 in AML, we exposed OCI-AML3 cells to increasing concentrations of PS, SAHA or combinations of PS or SAHA with 2 μ M CNDAC for 48 h. HDAC inhibition resulted in the hyperacetylation of histones H3 and H4 at lysine residues (K9/14). This was accompanied by dose dependent decreases in the levels of the Rad51 protein in cells exposed to either PS, SAHA and to combinations of PS or SAHA with 2 μ M CNDAC (Fig. 1E), whereas cells exposed to CNDAC alone did not significantly decrease Rad51 protein. Similar results were obtained when MV4-11 cells were exposed to increasing concentrations of PS either alone or in combination with 2 μ M CNDAC (Suppl. Fig. 1C). We then conducted time course experiments where OCI-AML3 and MV4-11 cells were exposed to 2 μ M CNDAC or 2 μ M CNDAC in combination with 0.5 nM PS for up to 48 h or to 0.5 nM PS alone for 48 h. Our results demonstrate that cells exposed to CNDAC minimally decreased levels of Rad51 in both cell lines. However, those exposed to either a combination of CNDAC with PS or PS alone significantly decreased Rad51 levels (Suppl. Fig. 2A and Suppl. Fig. 2B) indicating that it was HDAC inhibition that was responsible for the observed losses in Rad51 protein.

Multiple mechanisms such as downregulation of mRNA transcript (36), caspase-3 mediated cleavage (37) and microRNA mediated targeting regulate the abundance of any given protein (36). To identify the mechanisms by which HDAC inhibition decreased Rad51 protein, we exposed OCI-AML3 to 0.2 or 0.5 nM PS for varying times. Exposure of 0.2 nM PS or 0.5 nM PS for up to 48 h led to minimal decreases in the levels of Rad51 mRNA (Suppl. Fig. 3A). The levels of Rad51 protein on the other hand decreased by 20% by 48 h in response to 0.2 nM PS and by over 60% within 24 h in response to 0.5 nM PS (Fig. 1F, Suppl. Fig. 3B). Loss of Rad51 was accompanied by the appearance of cleaved caspase-3 and some PARP cleavage in cells exposed to 0.5 nM but not 0.2 nM PS (Fig. 1F). In contrast to Rad51, the levels of the closely related HR repair proteins Rad50, ATM and Nbs1 remained constant for up to 48 h (Suppl. Fig. 3B and Suppl. Fig. 4) suggesting that within the HR pathway, Rad51 was selectively targeted by HDAC inhibition. Because Rad51 is a known substrate of caspase-3 (37) and cells exposed to 0.5 nM PS showed processing of caspase-3, we exposed OCI-AML3 cells to 0.5 nM PS with or without the presence of the pan caspase inhibitor, z-vad-fmk. The results indicated that levels of Rad51 declined to the same extent regardless of inhibition in caspase-3 processing, indicating that caspase-3 mediated cleavage was not a major mechanism for the observed declines in Rad51 in response to HDAC inhibition (Suppl. Fig. 3C and Suppl. Fig. 3D).

HDAC inhibition induces a microRNA signature that target proteins of the HR pathway

Antagonizing the action of the HDACs leads to a reversal of silencing of a large number of genes, including those of miRNA (23, 24). To determine whether HDAC inhibition could induce the expression of epigenetically silenced miRNA that were predicted to target Rad51 we exposed OCI-AML3 cells to 0.5 nM PS for 6 h. The expression of a total of 163 miRNA was altered in response to HDAC inhibition; the miRNA significantly up- or down-regulated ($p < 0.01$) are shown (Fig. 2A). Many of the miRNA induced in response to PS putatively targeted multiple genes of the HR repair pathway, such as Rad51, its paralogs (Rad51D, Rad51B, XRCC2/3), Brca1 and Nbs1 (Suppl. Table 2); miRNA predicted to target Rad51 in silico by a minimum of two algorithms (Target Scan and miRanda) are highlighted in bold (Suppl. Table 2). To validate the results of the microarray, we chose three miRNA, miR-182, miR-211 and miR-320 that were induced after HDAC inhibition as well as predicted to target Rad51 for further evaluation. Following exposure of OCI-AML3 cells for varying times with 0.5 nM PS, the expression of these miRNA was induced between 4–6 fold in response to HDAC inhibition (Fig. 2B–D).

DNA hypermethylation acts in conjunction with the histone deacetylases to epigenetically silence gene expression (38). To determine the contribution of DNA hypermethylation to the silencing of miR-182 in AML, we exposed MV4–11 AML cells to 0.5 μ M decitabine (DAC, a DNA methylation inhibitor), 10 nM PS, or a combination of PS and DAC for up to 24 h. We exposed MV4–11 cells to 10 nM PS based on the concentrations utilized to demonstrate synergy with other DNA damaging agents in this cell line (39). The results show that exposure to DAC alone for up to 48 h induced miR-182 by less than two-fold (Fig. 2E and Suppl. Fig. 5). However, exposure to PS alone or in combination with DAC induced miR-182 levels by seven-fold within 24 h, indicating that HDACs and not DNA promoter hypermethylation played a dominant role in the silencing of miR-182 in AML (Fig. 2E). Correspondingly, the PS-induced increases in miR-182 in cells exposed to PS alone or in combination with DAC led to reciprocal decreases in the levels of Rad51 (Fig. 2F).

miR-182 targets Rad 51 in AML

The ability of a miRNA to target a given mRNA is highly context and tissue specific (26, 27) making it important to validate the interaction between miRNA and its targets in the relevant tissue of interest. Therefore, we focused on 2 miRNAs, miR320 and miR-182 that had highly conserved binding sites clustered within the central portion of the 3'UTR of Rad51. The Rad51 gene 3'-untranslated region (UTR) has two binding sites for miR320a/c and a single binding site for miR-182 (Fig. 3A). Mimetics for miR-182 and miR-320 were transfected into OCI-AML3 cells along with the non-targeting controls (scr) for 48 h. Of these, miR-320 did not reduce Rad51 whereas miR-182 consistently reduced the levels of Rad51 by 40–50% after 48 h compared to cells expressing scr oligonucleotides (Fig. 3B–3E) ($p < 0.01$, paired t-test, $n = 6$). Time course experiments then confirmed that cells expressing miR-182 targeted Rad51 protein even up to 72h (Fig. 3C). Next, to definitively determine the role of miR-182 in targeting Rad51, we over-expressed antagomirs to miR-182 for 48 h. Our data indicate that in the presence of antimiR-182, the levels of Rad51 were preserved whereas expression of miR-182 caused the expected loss in Rad51 levels (Fig. 3D and 3E). Finally, to test whether miR-182 directly targeted Rad51, HeLa-DR as well as HEK cells

were transfected with the pmirTarget luciferase reporter containing either the full length 3'-UTR or one with the Rad51 binding site deleted with miR-182 (Fig. 3F, underlined) or non-targeting controls at 300 nM. Ectopic expression of miR-182 caused a 30% decrease in luciferase activity ($p < 0.05$, paired t-test, $n = 4$) in HeLa cells and a 70% decrease in luciferase activity ($p < 0.05$, paired t-test, $n = 2$) in HEK cells in comparison to the scr, whereas deletion of the 3'-UTR binding site for miR-182 restored the luciferase activity in both cell types (Fig. 3G and H). This indicates that miR-182 directly targets Rad51.

HDAC1 and HDAC2 bind the miR-182 promoter in OCI-AML cells and primary AML blasts

We compared the expression of miR-182 in normal bone marrow and AML (Suppl. Table 3) and found that the expression of miR-182 was 10–1000 fold lower in AML blasts (Fig. 4A). Since we had determined that HDAC inhibition led to an upregulation of miR-182 in AML, we evaluated whether HDAC1 and HDAC2 were recruited to the miR-182 promoter to silence its expression. miR-182 is an intergenic microRNA transcribed as part of a cluster (40) contains a major (S1) recruitment site for HDAC1 and HDAC2 (Fig. 4B), the major Class I HDACs. HDAC1 and HDAC2 are found together in repressive transcriptional complexes which suggest a high degree of functional redundancy between the two proteins (41). Reciprocal immunoprecipitations from nuclear extracts using antibodies against HDAC1, HDAC2 or IgG were resolved on gels and immunoblotted for the presence of HDAC1 or HDAC2. The results demonstrated that HDAC1 and HDAC2 form a complex in nuclei (Fig. 4C). Chromatin immunoprecipitations (ChIP) assays demonstrated that HDAC1 and HDAC2 were recruited specifically over IgG at the miR-182 promoter in OCI-AML3 cells (Fig. 4D). Conversely, HDAC inhibition led to the accumulation of transcriptionally permissive chromatin modifications such as the trimethylation of histone H3 (H3K4me3) over non-specific IgG at the miR-182 promoter (Fig. 4E). This was accompanied by a 6–8 fold increase in the expression of miR-182 by 18 h (Fig. 2D).

Similarly, HDAC1 and HDAC2 were specifically recruited over IgG to the promoter for miR-182 in primary AML blasts (Fig. 4F). The recruitment of HDAC1 and HDAC2 at the miR-182 promoter did not change significantly before or after exposure to PS in AML cell lines or primary samples (Suppl. Fig. 6). The IC_{50} of PS against AML blasts is less than 20 nM (39). Based on this finding and other published results (42) we exposed AML blasts to 10 nM PS which led increases in the levels of the activating chromatin mark, AcH3K9 in the primary AML samples (Fig. 4G), a 10–50 fold increase in the levels of miR-182 in primary AML samples (Fig. 4H) as well as reciprocal and sustained losses in the levels of Rad51 in all primary AML cells (Fig. 4I).

Targeting of Rad51 by miR-182 or HDAC inhibition impairs HR and delays DNA repair in AML

Because the loss of Rad51 was associated with an attenuation of DNA repair in solid tumor cells (21), we evaluated the consequences of miR-182 overexpression or of HDAC inhibition on the HR repair process using the HDR assay. We utilized HeLa-DR cells that had the p-DR-GFP (with 2 inactive copies of GFP) integrated into its genome (43). Transfection with the Scl1 endonuclease caused a single double stranded break (DSB), which if repaired using homologous sequences from the donor GFP gene resulted in a functional GFP (32). HeLa-

DR cells were transfected with scrambled (scr) or miR-182 oligonucleotides at 300 nM or 500 nM for 48 h before being transfected with the Sce1 endonuclease. Overexpression of miR-182 but not scr resulted in a >50% decrease in Rad51 in HeLa-DR cells (Fig. 5A). Correspondingly, transfection of the scr oligos together with Sce1 endonuclease and resulted in 20% GFP positive cells indicative of successful HR repair (Fig. 5B, 5C and 5E). In contrast, transfection of miR-182 in conjunction with the Sce1 endonuclease led to a >50% inhibition of HR as measured by a reduction in the percentage of GFP positive cells (Fig. 5B, 5D and 5F, $p < 0.001$). Inhibition of HR would be expected to lead to higher levels of unrepaired damaged DNA, which can be assayed by measuring the levels of γ -H2AX that persist after DNA damage (44). Therefore, OCI-AML3 cells were exposed to either 0.5 μ M CNDAC, or to miR-182 or scr oligonucleotides for 48 h before being challenged with 0.5 μ M CNDAC for 24 h. Cells exposed to CNDAC alone or those expressing scr did not decrease Rad51, but responded with some increases in γ -H2AX after exposure to CNDAC for 24 h (Fig. 5G). In contrast, cells transfected with miR-182 showed the expected decrease in Rad51 and exhibited higher levels of γ -H2AX when exposed to CNDAC for 24 h (Fig. 5G). We then tested the consequence of the miR-182 mediated decrease in Rad51 and inhibition of DNA repair on the survival of cells exposed to CNDAC. OCI-AML3 cells were left untreated, exposed to 0.5 μ M CNDAC for 24 h, or transfected with scr, miR-182 or anti-miR-182 oligonucleotides after which a portion was exposed to 0.5 μ M CNDAC for 24 h before being assayed for colony growth. Untreated cells or those expressing scrambled showed similar numbers of colonies (280 ± 41 and 282 ± 14) (Fig 5H). Expression of miR-182 decreased colony formation by 23% to 218 ± 7 in comparison to cells expressing scr ($P < 0.05$, two-tailed p test) whereas expression of anti-miR-182 increased the number of colonies to 243 ± 6 in comparison to those expressing miR-182 ($P < 0.05$, two-tailed p test). Untreated cells or those expressing scr showed a 40% decrease in colony formation to 60 ± 9 and 62 ± 7 after exposure to CNDAC ($P < 0.001$, two-tailed p test). However, exposure of cells expressing miR-182 CNDAC caused a greater than 65% inhibition in colony formation to 38 ± 9 in comparison to cells expressing scr that were exposed to CNDAC ($P < 0.001$, two-tailed p test). Finally, exposure of cells expressing anti-miR-182 to CNDAC supported 185 ± 6 colonies (decrease of 24% vs 65% in cells exposed to miR-182+CNDAC) ($P < 0.01$, two-tailed p test) (Fig. 5H). Finally, to determine whether the miR-182-mediated loss in Rad51 augmented the toxicity of PS in combination with CNDAC, we exposed OCI-AML3 cells to scrambled oligonucleotides, miR-182 or anti-miR-182 for 48 hr before challenging cells to a combination of 0.5 nM PS with 0.5 μ M CNDAC. Transfection with scrambled oligonucleotides elicited $17 \pm 2\%$ annexin positive cells. In comparison, cells transfected with miR-182 showed an increase in annexin positive cells to $28 \pm 7\%$ whereas those transfected with anti-miR-182 showed decreases in cell death to $13 \pm 11\%$. Addition of CNDAC to cells transfected with scrambled, miR-182 and anti-miR-182 increased annexin positivity to $52 \pm 2\%$, $66 \pm 12\%$ and $32 \pm 22\%$ respectively ($p = \text{ns}$, Suppl. Fig. 7).

We then tested the consequence of HDAC inhibition on the ability of HeLa-DR cells to carry out HR. Cells transfected with the Sce1 endonuclease alone showed 24% GFP positive cells indicating ongoing HR, whereas those pre-exposed to 10 and 15 nM PS responded with a 75 to 80% inhibition in HR (Fig. 6A–E). We exposed HeLa-DR-13 cells to concentrations of 10 and 15 nM PS because those concentrations decreased Rad51 protein by > 50% (Fig. 6F).

We then tested whether HDAC mediated inhibition of HR would affect the ability of cells to resolve DNA damage. When OCI-AML3 cells were exposed to IR, an agent that causes instantaneous DSB, there was a rapid increase in the levels of γ -H2AX which decreased to baseline within 4–6 hr indicating the successful repair of DNA damage. However, if these cells were pre-treated with 0.5 nM PS before being exposed to IR, the γ -H2AX signal persisted indicating a delay in DNA repair (Fig. 6G). In parallel, when OCI-AML cells were exposed to CNDAC which causes DSB after 2 replication cycles there was an increase in the γ -H2AX starting at 24–48 hr. However, pretreatment with PS resulted in γ -H2AX accumulating earlier (16 h) and to a greater level suggesting that the HDACi-mediated loss in Rad51 led to a delay in DNA repair and higher levels of residual DNA damage (Fig. 6H).

Discussion

The HDAC inhibitors PS and SAHA have been shown to synergize with cytarabine, daunorubicin (22) and CNDAC using cell viability assays (18). We utilized cell viability as well as colony forming assays to demonstrate that PS and SAHA synergized with CNDAC in killing AML cells. Pharmacokinetic evaluations show that the maximum serum plasma concentrations of vorinostat are $\sim 1.4 \mu\text{M}$, 1–3 h post dosing in AML (45, 46) and $\sim 19 \text{ nM}$ for PS (46) indicating that the concentrations of vorinostat and PS achieved in patients are more than sufficient to inhibit both the proliferative capacity as well as kill AML cells. Correlative studies during therapy would help validate the action of this combination on the proliferative index and survival of circulating AML blasts.

To elucidate the mechanism underlying the synergy between HDACi and CNDAC, we focused on Rad51, a key component of the homologous repair pathway in AML cell lines and primary AML lymphocytes. The one published study did not elucidate the mechanism underlying the observed synergy between SAHA and CNDAC (18), whereas two additional publications showed that PS at concentrations of 10 nM decreased Rad51, Brca1 and ATM transcripts (22) and protein to inhibit HR (21). However, at the lower concentrations of PS (0.5 nM) used in our study, Rad51 transcript levels did not decrease. Rather, we identified an alternative mechanism whereby Rad51 protein levels decreased due to targeting by miR-182. MicroRNAs have been shown to regulate components of the HR pathway. In breast cancer cells, miR-182 was found to target BRCA1, P53BP1 as well as CHEK (28, 30). Additionally, miR-182, miR-96, miR-103 and miR-106 exhibited high levels of expression in breast cancer cells to target Rad51 (29, 31) and intrinsically suppress HR (28, 31). AML differs from these solid tumors in that the miRNAs targeting Rad51 are under-expressed in AML leading to robust levels of expression of Rad51 as well as a functional HR pathway. Additionally, miRNA target proteins in a context dependent manner and miRNA usually express a reverse correlation with their cellular targets (29, 44). Therefore, although miR-103 and miR-106 targeted Rad51 in solid tumors (29), it was unlikely they would do the same in AML because they and their target, Rad51 were expressed at high levels in OCI-AML3 cells (Suppl. Table 2). This conclusion is supported by our findings with miR-320 which is expressed at high levels in OCI-AML3 cells (Suppl. Table 2) and failed to target Rad51 even though Rad51 was a predicted target.

Thus, we focused on miR-182 because it was expressed at low levels in AML whereas its predicted target Rad51 had robust expression. Mechanistically, targeting the HDACs with PS or SAHA rapidly reversed the silencing of miR-182 whereas targeting DNA hypermethylation did not indicating that while DNA promoter methylation has a key role in suppressing many microRNA in AML (38), miR-182 is not regulated by this mechanism. miR-182 directly targeted Rad51 at the protein level and in luciferase assays. Targeting Rad51, either via HDACi exposure or ectopic expression of miR-182 inhibited HR and strongly sensitized AML cells to the cytotoxic action of CNDAC in colony forming assays highlighting the importance of Rad51 as a determinant of the sensitivity of AML cells to double strand damaging agents. Manipulating the levels of miR-182 with mimics and anti-miRs moderately increased or attenuated the cytotoxicity caused by the combination of PS and CNDAC. This is not surprising given that the combination of PS and CNDAC induces a very robust cell death response. However, this observation raises the possibility that miR-182 may have additional targets the loss of which may co-operate with PS and CNDAC to impact AML survival.

In conclusion, our findings define the strategy of blocking HDACs to induce miR-182 and limit Rad51 expression. Further, if miR-182 targets multiple components of the HR pathway in AML cells as shown for solid tumors (28, 30); epigenetic re-activation of miR-182 could be used to compromise HR at multiple levels. Because, HDAC inhibition induced miR-182 which targeted Rad51 in AML was sufficient to decrease HR by over 80% and sensitized cells to DNA strand breaking agents, it represents a novel pharmacologic strategy. HR is implicated in the repair of damage caused by diverse agents including topoisomerase I inhibitors (47), IR (6) DNA interstrand cross-linkers (48) as well as sapacitabine (5). This suggests that investigations in other malignancies could establish whether targeting Rad51 expression with HDAC inhibitors may also serve to augment the selective toxicity of double strand break-inducing DNA damaging agents across cancers. Thus, this mechanism-based strategy may have multiple applications for clinical evaluations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

Sapacitabine is a clinically active nucleoside analog prodrug that elicits double strand DNA breaks. Rad51 is a key protein in the homologous recombination repair (HR) pathway that repairs the DNA damage caused by sapacitabine to potentially limit its toxicity. In this study we identify that microRNA-182 targets Rad51 and that miR-182 is silenced by the histone deacetylases in AML. HDAC inhibition induces miR-182 to cause reciprocal decreases in Rad51 and inhibits HR to sensitize AML cells to sapacitabine. Our findings highlight a novel pharmacological strategy that targets Rad51 to compromise the ability of AML cells to conduct HR, and can be used to sensitize cells to DNA damaging agents that activate HR repair as a potential resistance mechanism.

Fig. 1. HDAC inhibitors synergize with CNDAC in OCI-AML3 cells and cause decreases in Rad51

(A) OCI-AML3 cells were treated with 0.1, 0.2 or 0.5 nM panobinostat (PS) alone, 2 μM CNDAC alone or a combination of PS and CNDAC for 48 h after which cell death was determined by determining the percentage of Annexin V/PI positive cells. Data represent mean ± SEM of six independent experiments (*= p<0.05, **=P<0.01, one way ANOVA). (B) OCI-AML3 cells were treated with 0.1, 0.2 or 0.5 μM vorinostat (SAHA) alone, 2 μM CNDAC alone or a combination of SAHA and CNDAC for 48 h after which cell death was determined by determining the percentage of Annexin V/PI positive cells. Data represent

mean $\pm$ SEM of three independent experiments (*= $p < 0.05$, **= $P < 0.01$, one way ANOVA). (C) OCI-AML3 cells were exposed to 0.5 nM PS, 0.5 μ M CNDAC or both drugs for 24 h in after which they were plated onto methylcellulose and monitored for colony growth. Graph represents mean $\pm$ SEM of three independent experiments in triplicate; the difference between CNDAC, PS and the combination was significant (****= $p < 0.0001$; one way ANOVA). (D) OCI-AML3 cells were exposed to 0.5 μ M SAHA, 0.5 μ M CNDAC or both drugs for 24 h in after which they were plated onto methylcellulose and monitored for colony growth. Graph represents mean $\pm$ SEM of three independent experiments in triplicate; the difference between CNDAC, SAHA and the combination was significant (**= $p < 0.001$; one way ANOVA). (E) Action of increasing concentrations of PS or SAHA in the presence or absence of 2 μ M CNDAC on Rad51. GAPDH was used a loading control and H3K9/14Ac was used to measure the hyperacetylation of histones following HDAC inhibition. Rad51 and GAPDH were quantitated to derive the ratios of Rad51/GAPDH. Figure and ratios of Rad51/GAPDH are representative of three independent experiments (F) Action of 0.2 nM and 0.5 nM PS for varying times on the levels of Rad51, cleaved caspase-3 (C-Casp-3), full length and cleaved PARP and GAPDH in OCI-AML3 cells. Figure is representative of three independent experiments.

Fig. 2. HDAC inhibition results in an induction of miRNA expression

(A) OCI-AML3 cells were exposed to 0.5 nM PS for 6 h following which the miRNA from 2 independent experiments were isolated and hybridized in duplicate to an oligonucleotide array to determine the effect of PS exposure on miRNA expression levels. The Fig. shows the miRNA that were either up-regulated or down-regulated with a p value of <0.01. (B–D) Validation of microarray results. OCI-AML3 cells were exposed to 0.5 nM PS for 8, 12 or 18h after which the induction of miR-211, miR-320 and miR-182 was determined. Assays were done thrice in duplicate. (E) MV4–11 cells were exposed to 0.5 μ M decitabine (DAC), 10 nM PS or a combination of DAC and PS for increasing times after which the levels of miR-182 were quantitated. Graph represents mean $\pm$ SEM of three experiments. (F) MV4–11 cells were exposed to 0.5 μ M DAC, 10 nM PS or a combination of DAC and PS for increasing times after which the levels of Rad51 were determined. GAPDH was used as a loading control and H3K9/14Ac was used to measure the hyperacetylation of histones following HDAC inhibition. Figure is representative of three experiments.

Fig. 3. miR-182 targets Rad51

(A) Represents the predicted location of the binding sites for miR182 and miR-320a/c within the Rad51 3'UTR. (B) Action of ectopic expression of the miRNA mimics, miR-182 and miR-320 for 48 h in OCI-AML3 cells on levels of Rad51. scrambled (scr) sequence was used for comparison and GAPDH was measured as a loading control. Figure is representative of six experiments. (C) Action of miR-182 on Rad51 in OCI-AML3 cells at 48 h and 72 h. Rad 51 levels after transfection with scr sequence and in untreated cells (C) were used for comparison. Fig. is representative of three experiments. (D and E) Action of Anti-miR-182 in blocking the miR-182 mediated decrease of Rad51 protein in OCI-AML3 cells; the graph represents the quantitated values for Rad51 protein from six experiments (**= $p < 0.01$, paired t-test). (F) The putative binding site of miR-182 on the Rad51 3'UTR. (G) The pmiRTarget vector with *RAD51* WT insert (full-length 3'UTR) or mutated miR-182 3'UTR (containing a deletion of the miR-182 target site) were co-transfected with miR-182 or Scr in HeLa cells. Luciferase activity was recorded after 36 to 48 h. The luciferase counts between Scr and miR-182 are statistically significant and represents the mean $\pm$ SEM from triplicate determinations from four independent transfections (*= $p < 0.05$, paired t-test). (H) The pmiRTarget vector with *RAD51* WT insert (full-length 3'UTR) or mutated miR-182 3'UTR (containing a deletion of the miR-182 target site as underlined) were co-transfected

with miR-182 or Scr in HEK cells. Luciferase activity was recorded after 24 h. Data represents the mean $\pm$ SEM from triplicate determinations from two independent transfections (*= $p < 0.05$, paired t-test).

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Fig. 4. Role of HDACs in regulating miR-182 expression in AML cell lines and primary blasts
 (A) Comparison of miR-182 expression in normal bone marrow (n=4) and AML blasts (n=7). (B) Schematic depicting the putative binding sites for HDAC1 and HDAC2 on the miR-182 promoter. (C) Co-immunoprecipitation of HDAC1 and HDAC2 from the nuclei of OCI-AML3 cells. Figure is representative of three experiments. (D) Recruitment of HDAC1 and HDAC2 at the miR-182 promoter in OCI-AML3 cells by ChIP. Data represents mean $\pm$ SEM of four independent experiments (***=p<0.001, paired t-test). (E) Accumulation of the transcriptionally permissive mark H3K4me3 at the miR-182 promoter in OCI-AML3 cells after exposure to 0.5 nM PS for 6 h. Data represents mean $\pm$ SEM of four independent experiments (****=p<0.0001, paired t-test). (F) Recruitment of HDAC1 and HDAC2 at the miR-182 promoter in primary AML blasts by ChIP. Data represents mean $\pm$ SEM from three independent samples in triplicate (***= p<0.001, paired t-test). (G) Accumulation of H3K4me3 at the miR-182 promoter in primary AML blasts after exposure to 10nM PS for 6 h. Data represents mean $\pm$ SEM from three independent samples in triplicate (****=p<0.0001, paired t-test). (H) Induction in miR-182 expression in primary AML blasts exposed to PS for varying times. Assays were done in duplicate for each sample. (I) Decrease in the levels of Rad51 in AML blasts treated as in GAPDH was used as a

loading control and H3K9Ac was used to measure the hyperacetylation of histones as a positive measure of HDAC inhibition. Figure representative of duplicate immunoblots.

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Fig. 5. miR-182 targets Rad51 to impair homologous recombination

(A) HeLa-DR cells were treated with miR-182 or scrambled (scr) sequence to determine effects on Rad51. GAPDH was used as a loading control. (B) Quantitation of the % inhibition of HR in cells overexpressing miR-182 (300 or 500 nM) in comparison with scrambled in four independent experiments conducted in triplicate (***=p<0.001, paired t-test). (C-F) HR assay showing impairment of the HR process owing to miR-182 overexpression. HeLa-DR-13 cells were left untransfected, or transfected with the non-targeting miRNA controls (scr) and miR-182 at 300 nM and 500 nM for 48 h before being transfected with the Sce-I endonuclease to induce a defined double strand break. The percentage of GFP positive cells was measured by flow cytometry after 48 h after transfecting with Sce1 endonuclease. Figure representative of four independent experiments (G) Cells were transfected with Scr or miR-182 for 48 h before being challenged with 2 μM CNDAC for 24 h after which the levels of Rad51 were measured to confirm the Rad51 targeting action of miR-182. Following this, the levels of γ-H2AX was assessed as a measure of DNA damage. GAPDH was used as a loading control. Figure representative of three experiments. (H) OCI-AML3 cells were left untreated, or transfected with scr oligonucleotides, miR-182 and anti-miR-182 for 48 h before a portion from each condition was exposed to 0.5 μM CNDAC for 24 h. Cells were then washed and plated onto

methycellulose and monitored for colony growth. Graph represents mean ± SEM of three independent experiments in triplicate (*=P<0.05, **=P<0.01, ***=p<0.001; two-tailed p-tests).

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Fig. 6. Exposure to PS impairs homologous recombination and leads to a sustained increase in the intensity of γ -H2AX after IR or CNDAC-induced double strand breaks
(A–D) HR assay shows impairment of the HR process when cells are exposed to PS. HeLa-DR cells were left untransfected, transfected with the Sce-1 endonuclease alone to induce a defined double strand break or exposed to 10 nM and 15 nM PS before transfecting with the Sce-1 endonuclease after which the percentage of GFP positive cells was measured by flow cytometry at 48 h. Figure representative of three independent experiments. (E) The comparisons between HeLa-DR transfected with Sce-1 and HeLa-DR transfected with sce-1 in the presence of 10 nM or 15 nM PS are statistically significant ($***=p<0.001$, paired t-test) and represent the average values of triplicate determinations of three independent experiments. (F) Exposure to 10 nM PS leads to a decrease in the levels of Rad51 in the HeLa-DR cells. (G) OCI-AML3 cells were exposed to 3 Gy ionizing radiation (IR) alone, or to 0.5 nM PS for 18 hr before being challenged with 3 Gy IR after which the levels of γ -H2AX were determined over time. Total H2AX levels were assayed as a control. Figure representative of three independent experiments. (H) OCI-AML3 cells were exposed to 2 μ M CNDAC alone, to 0.5 nM PS alone or to a combination of both drugs and the levels of

γ -H2AX were determined over time. Total H2AX levels were assayed as a control. Figure representative of three independent experiments.

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