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Histone deacetylase inhibitors decrease NHEJ both by acetylation of repair factors and trapping of PARP1 at DNA double-strand breaks in chromatin

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Abstract

Histone deacetylase inhibitors (HDACi) induce acetylation of histone and non-histone proteins, and modulate the acetylation of proteins involved in DNA double-strand break (DSB) repair. Non-homologous end-joining (NHEJ) is one of the main pathways for repairing DSBs. Decreased NHEJ activity has been reported with HDACi treatment. However, mechanisms through which these effects are regulated in the context of chromatin are unclear. We show that pan-HDACi, trichostatin A (TSA), causes differential acetylation of DNA repair factors Ku70/Ku80 and poly ADP-ribose polymerase-1 (PARP1), and impairs NHEJ. Repair effects are reversed by treatments with p300/CBP inhibitor C646, with significantly decreased acetylation of PARP1. In keeping with these findings, TSA treatment significantly increases PARP1 binding to DSBs in chromatin. Notably, AML patients treated with HDACi entinostat (MS275) *in vivo* also show increased formation of poly ADP-ribose (PAR) that co-localizes with DSBs. Further, we demonstrate that PARP1 bound to chromatin increases with duration of TSA exposure, resembling PARP “trapping”. Knockdown of PARP1 inhibits trapping and mitigates HDACi effects on NHEJ. Finally, combination of HDACi with potent PARP inhibitor talazoparib (BMN673) shows a dose-dependent increase in PARP “trapping”, which correlates with increased apoptosis. These results

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Authorship

Contributions: C.R., P.N. designed, performed experiments and wrote the paper. F.R. designed experiments and wrote the paper, N.P., A.A. performed experiments, I.G. furnished patient materials and advice in patient-based studies, P.C. and M.D.J. synthesized and furnished p300 small molecule inhibitors.

Conflicts of interest

The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.leukres.2016.03.007>.

provide a mechanism through which HDACi inhibits deacetylation and increases binding of PARP1 to DSBs, leading to decreased NHEJ and cytotoxicity of leukemia cells.

Keywords

HDAC; DSB; repair; NHEJ; PARP1

1. Introduction

Histone deacetylases (HDACs) catalyze the removal of acetyl groups from the amino-terminal lysine residues of histone proteins, while histone acetyltransferases (HATs) promote their addition, leading to local remodeling of chromatin, a critical step for the access of regulatory proteins to DNA [1]. In addition to their activity on histone proteins, HDACs and HATs also target non-histone proteins. Transcription factors, transcription regulators and DNA repair proteins have been shown to be directly or indirectly regulated by HDACs and HAT enzymes [2]. In leukemias, specific oncogenic fusion proteins recruit HDACs to the promoters of their target genes. These events appear to contribute to leukemogenesis [3], suggesting that these diseases are particularly good candidates for treatment with HDAC inhibitors (HDACi) [4]. Indeed, clinical trials of HDACi in hematological malignancies, such as lymphoma [5] and myelodysplastic syndromes [6] are yielding promising results. Recently, U.S. Food and Drug Administration (FDA) approved HDACi panobinostat for treatment of multiple myeloma [7]. Also, Vorinostat and Romidepsin received FDA approval for treatment of cutaneous T-cell lymphoma [8–10].

Although HDACi treatment results in apoptosis of cancer cells *in vivo*, the mechanism underlying this effect is still unclear. It has been reported that HDACi induce apoptosis by upregulation of both death receptors and their ligands in leukemia cells [11,12], and by activation of the mitochondrial/intrinsic apoptotic pathway through selective activation of Bid and induction of reactive oxygen species (ROS) [13]. In addition, we and others have shown that HDACi cause DNA damage that is significantly increased in solid tumors [14] and leukemias [15] compared with normal cells. Increases in γ H2AX and ATM phosphorylation, that are early indicators of DNA damage [16,17], occur rapidly following HDACi administration [15]. Furthermore, treatment with HDACi such as trichostatin A (TSA) leads to a persistence of DNA double strand breaks (DSBs) [14,15], and sensitize solid tumor and leukemia cells to ionizing radiation [14,18–20].

There are two major mechanisms for the repair of DSBs [21], homologous recombination (HR) and the classical DNA-dependent protein kinase (DNA-PKcs)-dependent non-homologous end-joining (c-NHEJ or NHEJ). Recent studies have also identified an alternative and highly error-prone repair pathway, Alt-NHEJ [22–24] that appears upregulated in cancer and leukemia cells [25], and is implicated as a mechanism for genomic instability [22–24]. Poly ADP-ribose polymerase-1 (PARP1), that participates in several DNA repair pathways, including single strand break (SSB) repair, is also a key player for initiation of Alt-NHEJ [26].

Several studies have investigated mechanisms by which HDACi modulate DSB repair [27–30]. Mouse embryonic fibroblasts mutated for NHEJ proteins Ku80 or DNA Ligase IV (LigIV), but not for the HR protein BRCA2, are hypersensitive to TSA [31]. In human prostate cancer cells, HDACi cause hyperacetylation of Ku proteins and decrease the binding of these proteins to DNA [32]. Vorinostat, which is also a pan-HDACi like TSA, downregulates DNA-PKcs in melanoma cells [14]. We previously observed increased co-localization of DNA-PKcs and γ H2AX in HDACi-treated cells indicating that NHEJ components localize in vicinity of DSBs after drug treatment [15]. However, acetylation of Ku70 by HDACi decreases overall NHEJ activity [27–29]. Thus, it is unclear whether repair factors assembled around DSBs in chromatin following HDAC inhibition are able to access DSBs and perform repair. In this study, we investigated whether HDACi impact recruitment of DSB repair proteins to chromatin, particularly those involved in NHEJ.

We show that pan-HDACi TSA treatment of acute leukemia cells results in differential acetylation of Ku70, Ku80, and PARP1, leading to decreased NHEJ activity. We also show that HAT p300/CBP inhibition using the pharmacological inhibitor C646 reverses these effects, specifically affecting PARP1 acetylation. Moreover, PARP1 is significantly enriched in chromatin following HDACi, compared with Ku proteins, in a mechanism similar to PARP1 trapping observed with PARP inhibitors (PARPi). Notably, PARPi in combination with HDACi lead to further increases in PARP trapping that correlates with increased apoptosis. This suggests that HDACi result in both a physical and functional alteration of PARP1 binding at DSBs, potentially preventing access of NHEJ factors, and therefore resulting in decreased NHEJ repair.

2. Methods

2.1. Cell culture, patients and treatment

K562 and HL60 cell lines from American Type Culture Collection (ATCC, Manassas, VA) were grown in RPMI 1640 medium with 25 mM HEPES (Cellgro, Mediatech Inc., Manassas, VA) supplemented with 2 mM glutamine (Cellgro), 1% penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS, ATCC) at 37°C and 5% CO₂. A derivative of K562 that stably expresses DRneo construct (K562DRneo) [25] or pimEJ5GFP was grown in the same medium as K562 but under constant selection with 350 μ g/ml hygromycin (Calbiochem, EMD Chemicals, Gibbstown, NJ).

Exponentially growing cells were resuspended in complete medium at a density of 0.5×10^6 cells/ml prior to the addition of drugs from concentration stock solutions at doses and times indicated on the figures. Stock solution of Trichostatin A (TSA, Sigma-Aldrich Corp, St. Louis MO), PARP inhibitor NU1025 (8-Hydroxy-2-methylquinazoline-4-one, Alexis Biochemicals, Plymouth Meeting, PA), HAT p300 small molecule inhibitors (C646, CBPi) cordially provided by Dr. Phil Cole, and [33] and BMN673 (BMN, Talazoparib, Medivation, San Francisco, CA) were prepared in EtOH (solvent) for TSA and DMSO (solvent) for the others drugs. Final concentrations of EtOH and DMSO did not exceed 0.1% and did not interfere with the assays.

Bone marrow mononuclear cells (BMMNC) from adults with acute myeloid leukemia (AML) and treated *in vivo* with entinostat 6 or 10 mg/m² at day 1 and 8 of 4 week cycle as described earlier [34] were kindly provided by Dr. Ivana Gojo. All patients signed informed consent in accordance with the policies of the Institutional Review Board at the University of Maryland School of Medicine, Baltimore.

2.2. Nuclear extracts, chromatin extracts, immunoprecipitation and immunoblotting

Cells were washed twice with PBS prior to preparation of nuclear extracts using the CellLytic™ NuCLEAR™ Extraction kit (Sigma, protocol without detergent). Protein concentration was measured using the Bradford reagent (Sigma) according to the manufacturer's instructions.

Immunoprecipitation with nuclear extracts was performed as described previously [25]. Membranes were incubated overnight at 4 °C with a rabbit polyclonal Acetylated-Histone 4 (AcH4, 1:1000, Upstate, Millipore, Billerica, MA), pan-H3 (1:30000, EMD Milipore, MA), acetyl-H3 (1:10000, EMD Milipore) mouse monoclonal Ku70 (1:2000, Santa-Cruz, Santa-Cruz, CA), Ku80 (1:2000, Calbiochem), PARP1 (1:1000, EBiosciences, San Diego, CA), H3 (used in Fig. 6) and β -Actin (1:5000, Abcam, Cambridge, MA) diluted in TBST-BSA5%.

Extraction of chromatin-bound proteins was performed using the protocol for the subcellular protein fractionation kit for cultured cells (Life Technologies, Carlsbad, CA), with minor modifications. Micrococcal nuclease digestion was carried out for 15 mins at 37 °C. For siRNA knockdown experiments, 4×10^6 cells were treated with either non-targeting control siRNA (GE Healthcare-Dharmacon®) or ON-TARGETplus PARP1 siRNA (GE Healthcare-Dharmacon®) for 24 h and cells were then split into 6-well plates and treated with DMSO (vehicle) or TSA for 72 h, as described above. Cell pellets were collected at respective time points and later processed for immunoblot analysis.

2.3. LacZ α reactivation NHEJ repair assay

The assay was conducted as described previously [35]. Briefly, EcoR1-linearized pUC18 plasmids (ThermoScientific, Glen Burnie, MD) were transfected into cells using Amaxa Nucleofector Kit V. Plasmid DNA was extracted (Qiagen Plasmid Mini Kit) and used to transform *Escherichia coli* strain DH5 α (Invitrogen). After plating on agar plates containing X-gal and IPTG, the total number of white (misrepaired) and blue (correctly repaired) colonies were counted. For each experiment, plasmids from approximately 15 white colonies were analyzed. The results are representative of three independent experiments $\pm$ SEM.

2.4. Chromosomal-based EJ5 NHEJ assay

The assay was conducted as described by the Gunn et al., [36]. pimeJ5GFP was a gift from Jeremy Stark (Addgene plasmid # 44026). K562-EJ5 cell line was treated with HDAC inhibitors or DMSO (vehicle) for 24 h before nucleofection with 2.5 μ g of pCBASceI construct, a gift from Maria Jasin (Addgene plasmid # 26477) using Amaxa Nucleofection Kit V (Lonza). To control for transfection efficiency, cells growing in the same conditions were transfected with pMax-GFP (Lonza, Walkersville, MD) instead of pCBASceI

transfections. 56 h after transfection of pCBASce1, 0.2 µg/ml aphidicolin was added to all samples to synchronize the cell population. After 72 h of pCBASce1 or pMax-GFP treatment, cells were collected, washed twice in 1× PBS and GFP+ cells were quantified using flow cytometric analysis at the UMGCC Flow Cytometry Core services. Approximately 100,000–200,000 events/sample were analyzed for this assay. To measure Alt-NHEJ, cells were collected post-flow cytometric sorting (as above) and as demonstrated in Supplementary Fig. 4, DNA was isolated and PCR for GFP+ cells was performed. Thereafter, half of the PCR product was re-digested with *I-SceI* enzyme (NEB). *I-SceI* negative (or resistant: 'S-') DNA was excised from the agarose gel followed by TOPO-cloning for sequence analysis (UMGCC Sequencing Core Facility). % of DSB repair junctions with microhomologies (>3 nt), described previously [35,37] were characterized as Alt-NHEJ products.

2.5. MTS cell viability assay & apoptosis

Cell viability was measured using CellTiter 96® AQueous One Solution Cell Proliferation Kit (Promega, Madison, WI). The assay was conducted as described in [37]. Apoptosis was measured by flow cytometric determination (at UMGCC Flow Core) of Annexin V-APC positive population (BD Biosciences, San Jose, CA).

2.6. Chromatin immunoprecipitation (ChIP)

DSBs were induced in K562DRneo by transient transfection of a plasmid encoding the *I-SceI* restriction enzyme (1 µg/10⁶ cells) [25] using Amaxa Nucleofector kit V (Lonza). Thereafter, proteins were cross-linked to DNA by addition of 1% formaldehyde in PBS for 10 min at room temperature. The reaction was quenched by adding 0.125 M glycine for 10 min at room temperature and cells were washed 3 times in 10 ml 1× PBS, containing protease inhibitors.

For each ChIP, 10⁷ cells were re-suspended in 1 ml SDS lysis buffer containing PI and sonicated using a Branson Sonifier® 450-A in order to obtain chromatin fragments of 200–1000 bp. One hundred µg of DNA was re-suspended in 3.6 ml of ChIP dilution buffer containing PI. Five µg of mouse monoclonal anti-PARP1 (F1-23, Santa Cruz), anti-phosphoserine 139 of H2A.x (γH2A.x, Upstate, Millipore), rabbit polyclonal anti-AcH4, anti-XRCC1 (Millipore), or isotype control were added to the ChIP solution to a final volume of 4 ml and incubated overnight at 4 °C. Immune complexes were collected with 100 µl of 3:1 Protein A/G magnetic Dynabeads. After incubation for 3 h at 4 °C, the beads were washed 4 times in low salt immune complex wash buffer (Upstate) containing PI, once in high salt immune complex wash buffer (Upstate) containing PI, and once in TE buffer containing PI before elution at 65 °C for 15 min in elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% SDS). Cross-links were reversed by incubation overnight at 65 °C and then the samples were digested with 0.2 mg/ml RNase A (Calbiochem) for 2 h at 37 °C followed by 0.2 mg/ml Proteinase K (New England Biolabs, Ipswich, MA) for 2 h at 55 °C. DNA was then recovered using QIAquick PCR purification kit (Qiagen) according to the manufacturer's instruction.

Real-time PCR was performed using 1 μ l of the input and 3 μ l of the actual immunoprecipitated samples using Quanti-Tect SYBR Green PCR kit (Qiagen) on a Mastercycler® ep *realplex* apparatus (Eppendorf, Westbury, NY), according to the manufacturer's instruction. 2 primer sets were designed and used to amplify a 145 bp product from 160 to 304 (200 bp; Forward primer: 5'-GCTTCCTCGTGCTTTACGGTATC-3' and Reverse primer: 5'-TATCAGATCCGAACAAACGACCC-3', Integrated DNA Technology, Coralville, IA) and a 199 bp product from 1205 to 1404 (1.3 kb; Forward primer: 5'-TCGCTGCGGCCGATCTTAGC-3' and Reverse primer: 5'-GCAGTCCTCGGCCCAAAGCA-3', Integrated DNA Technology) adjacent to the I-SceI cut site in DRneo construct. Results were analyzed using the C_t method [38]. All results were normalized to input and expressed as fold increase compared to IgG control. ChIP experiments were performed at least three times and results are representative of the mean of three independent experiment $\pm$ SEM.

2.7. Immunofluorescence

BMMNC (0.5×10^6) were cytospun onto glass slides for 5 min at 200 rpm in Hanks medium on a Shandon Cytospin 4. Cells were then fixed for 10 min in 1% formaldehyde, permeabilized for 10 min in 70% EtOH, washed 3 times, in TBS-T0.2% and then blocked overnight in TBS-T0.2% containing 10% serum. After incubation with mouse monoclonal anti- γ H2A.x (1:100) and rabbit polyclonal anti-PAR (1:200, Alexis Biochemicals) or isotypes controls for an hour at 37 °C in TBS-T0.2%, cells were washed and then incubated with Cy3-anti-mouse (1:300) and Cy2-anti-rabbit (1:100, Jackson ImmunoResearch) for an hour at 37 °C prior to counterstaining with 4',6 diamidino-2-phenylindole, dihydrochloride (DAPI, 0.3 μ g/ml, Promega, Madison, WI) in TBS-T0.2%. Slides were mounted in DPX mountant (VWR international, West Chester, PA) and examined using a Nikon fluorescent microscope Eclipse 80i (100 $\times$ /1.4 oil, Melville, NY). Images of at least 50 cells/slide were captured using a CCD (charge-coupled device) camera and the imaging software NIS Elements (BR 3.00, Nikon). Immunostaining was performed in triplicate for each sample and results are representative of the mean of three independent experiment $\pm$ SEM.

3. Results

3.1. HDACi decrease NHEJ repair efficiency

Several lines of evidence suggest that inhibition of HDACs decrease the expression of DSB repair proteins involved in NHEJ (*i.e.* Ku70, Ku80, DNA-PKcs) [14,20,39]. These effects contribute to decreased NHEJ but the exact mechanism is not clear [40]. We first tested whether pan-HDACi TSA decreases total cellular NHEJ activity in well-established leukemia cell lines. Using the previously published LacZ α reactivation NHEJ repair assay [25], we found that indeed treatment of K562 (Fig. 1A) and HL60 (Fig. 1B) leukemia cells with HDACi TSA (300 nM, 1 and 6 h) resulted in a significant decrease in NHEJ repair efficiency compared to controls ($p < 0.05$). Similar results were also obtained with another class I HDAC inhibitor- Entinostat, which has been used in a number of clinical trials [4,34] (300 nM, 6 h, $p < 0.05$), (Fig. 1A and B). To verify that these results are not biased for describing only the repair of complementary DSB ends, and to more accurately represent

repair *in vivo*, we measured total NHEJ using the chromosomally integrated GFP reporter pimEJ5GFP in K562 cells (K562-EJ5) [36,41]. NHEJ of *I-SceI* induced DSBs re-constitute GFP expression, and the% GFP-positive cells were measured by flow cytometry. We found that 600 nM TSA significantly decreased NHEJ activity in comparison to controls ($p < 0.05$) (Fig. 1C). Moreover, decreases in total NHEJ capacity in K562 is associated with an increase in cytotoxic DSBs, as measured by immunoblotting for γ H2AX, (Fig. S1A and S1B) and a decrease in cell viability (Fig. 1D and E).

3.2. Ku70/80 and other repair factors are differentially acetylated in acute leukemia cells

Earlier reports suggested that HDACi differentially acetylate DNA-PKcs dependent Ku proteins in prostate cancer cells [32]. We determined whether Ku70 and Ku80 are differentially acetylated in leukemia cells following HDACi treatment. Immunoprecipitation of acetylated lysine (AcK) residues was performed on nuclear extracts from K562 and HL60 cell lines before and after treatment with TSA (300 nM, 1 and 6 h) followed by immunoblotting using antibodies directed against Ku70, Ku80, and AcH4. In accord with the observations of Chen et al., [32] Ku70 and Ku80 demonstrated differential acetylation in TSA treated extracts from K562 (Fig. 2A and Fig. S2) and HL60 cells (Fig. 2B), with increased acetylation observed within 1 h and 6 h treatments, compared with controls. As expected, acetylation of H4 is also increased with TSA treatment at both time points (Fig. 2C and D). Given that Ku70/80 and PARP1 proteins compete for binding to DSBs for repair [42,43], using the same methodology, we next determined whether TSA modulates PARP1 acetylation. Indeed, PARP1 is differentially acetylated in TSA treated K562 (Fig. 2E, S2) and HL60 (Fig. 2F) leukemia cells, compared with untreated controls. In contrast, other DNA repair proteins, DNA LigIII α and WRN, which participate together with PARP1 in Alt-NHEJ pathway, are not affected by HDACi treatment (Table 1, Fig. S3).

We next questioned whether PARP1 acetylation by HDACi leads to changes in microhomology-mediated end joining (MMEJ) repair, which is often used to estimate total Alt-NHEJ activity, and is characterized by short stretches of microhomology-sequences. The MMEJ assay was performed using two different approaches as described in Fig. S4 and S5 [41]. Interestingly, TSA treatment does not affect overall MMEJ activity in cells, as the sequence analysis of DSB repair junctions did not reveal any differences between vehicle (DMSO) and HDACi treated cells in two independent experimental analysis, EJ5-*I-SceI* and Puc18-*EcoRI* plasmid systems (Fig. S4 and S5 respectively).

3.3. Inhibition of ku70 acetylation by histone acetyltransferase (HAT) p300 inhibitor C646 increases NHEJ repair efficiency

Given that impairment in NHEJ activity is seen with increasing doses of TSA, we next set out to determine whether this effect is dependent upon the acetylation status of NHEJ factors and/or PARP1. HAT p300/CBP proteins have been shown to acetylate PARP1 and Ku70 [29,44] and, thus, inhibitors of HAT p300/CBP proteins should in theory decrease acetylation of these proteins. K562 cells were treated with increasing doses of the p300/CBP inhibitor (CBPi), C646, followed by measurement of Ku70 and PARP1 acetylation. Whereas Ku70 acetylation decreases slightly with the CBP inhibitor 20 μ M C646 treatment, acetylation of PARP1, in contrast, decreases significantly (>2-fold), compared to respective

controls (Fig. 3A and Fig. S6). Next, we examined NHEJ efficiency in K562-EJ5 treated with CBPi compared to control. K562-EJ5 treatment with CBPi significantly increases the NHEJ activity, particularly with 20 μ M C646 ($p < 0.05$), as demonstrated by an increase in overall *GFP+* cell population of K562-EJ5 (Fig. 3B). These results suggested that the acetylation status of PARP1, perhaps more than Ku70/Ku80, may affect the overall NHEJ activity in leukemia cells.

3.4. Binding of repair factors to DSB is increased with HDACi

To investigate whether TSA treatment alters the binding of Ku70/Ku80 or PARP1 to DSBs, we used chromatin immunoprecipitation (ChIP) in K562 cells stably transfected with DRneo construct expressing a single inducible *I-SceI* DSB (K562DRneo cells) [25] (Fig. 4A). K562DRneo cells were transfected with *I-SceI* endonuclease construct, followed by treatments with TSA (300 nM, 6 h) or vehicle control (EtOH, 6 h), and ChIP of genomic sequences surrounding the DSB, was performed, as described in the Methods section and Fig. 4A. First, to confirm that HDACi treatment results in increased protein acetylation, AcH4 was used as a surrogate marker in ChIP experiments at 160–304 bp region surrounding the putative DSB in HDACi treated *versus* control K562DRneo cells. In HDACi treated cells, significant acetylation of H4 was observed within this region, compared to controls (Fig. 4B). Also, to verify that expression of the *I-SceI* endonuclease construct induces a DSB within the putative DSB region of DRneo, ChIP was performed for γ H2AX, in K562DRneo cells transfected with *I-SceI* and treated with TSA (300 nM, 6 h), compared with controls. As shown in, Fig. 4B, γ H2AX is significantly increased at the site of *I-SceI* induced DSB in TSA-treated K562DRneo cells, compared to controls. Intriguingly, we find that while both Ku70 and PARP1 are bound to the region containing the induced *I-SceI* DSB, only PARP1 appears to be significantly increased (~5-fold, $p < 0.05$), relative to the control (DMSO) treatment, while Ku70 is slightly and not significantly enriched (Fig. 4C). To determine whether the presence of PARP1 is specific to the induced DSB, we also examined a genomic region 1.3 kb from the DSB. We find that the level of PARP1 at 1.3 kb from the DSB is similar to that of the control (Fig. 4D), suggesting that indeed PARP1 binding at the DSB site is specific and induced by HDACi treatment. We therefore hypothesized that increased binding of PARP1 at DSBs may block the ability of DSB repair factors such as Ku70/Ku80 to bind DSB ends to perform NHEJ.

3.5. Increased PAR and γ H2AX foci co-localization in AML patient samples after *in vivo* HDACi treatment

We next examined bone marrow mononuclear cells (BMMNC) obtained from AML patients that were treated on a single agent Phase I study of HDACi entinostat [34], for levels of DSBs and co-localization of poly ADP-ribose (PAR) clusters at DSBs, compared to pre-treatment controls. BMMNC from 4 AML patients treated with 6 mg/m² entinostat and 2 AML patients treated with 10 mg/m² on days 1 and 8 of a 4-week cycle were examined [34]. As suggested by our previous data [15], γ H2AX foci levels are increased significantly following entinostat treatment, compared to pre-treatment controls, in 3 out of 4 AML patients treated with 6 mg/m² entinostat ($n = 4$; Fig. 5A) and 1 out of 2 AML patients treated with 10 mg/m² entinostat ($n = 2$; data not shown). To examine whether PARP1 localized with DSBs more frequently following HDACi treatment, cells were immunostained for poly-

ADP ribosylation (PARylation), a marker that is widely used as a measure of not only total cellular PARP1 activity but also in regulation of DSB repair [45]. Entinostat treatment increases the percentage of cells with co-localization of PAR staining and γ H2AX foci, compared to pre-treatment controls (Fig. 5B). The same effect could be seen in an AML patient that responded to treatment with 10 mg/m² entinostat (data not shown). This data confirms our *in vitro* data indicating a response to increased DNA damage associated with HDAC inhibitors.

3.6. HDACi treatment leads to PARP1 trapping in chromatin, and HDACi combined with a PARPi leads to significant increases in apoptosis

Increased DNA binding of PARP1 with HDACi treatment (Fig. 4C) suggested that PARP1 is either transiently bound to DNA or is involved in a mechanism similar to PARP trapping that is observed with potent PARP inhibitors, in which cytotoxic PARP-DNA complexes are formed [47]. To determine whether HDACi increase PARP trapping, K562 cells were treated with TSA (75, 150, 300 and 600 nM) for 6 h and 72 h followed by Western blotting for PARP1 in chromatin fractions. While a slight increase in PARP1 bound chromatin is observed 6 h post TSA treatment (Fig. 6Ai and Aii), a 3–5 fold increase ($p < 0.05$) in PARP1 trapping is seen after 72 h (TSA, 150–600 nM). Notably, TSA-induced PARP1 levels in chromatin drop significantly with siRNA knockdown of PARP1 (Fig. 6Bi and Bii).

If HDACi can lead to trapping of PARP1 in chromatin, we predicted that combining HDACi with potent PARP inhibitor BMN673 (BMN, talazoparib) would enhance these effects. Indeed, our results indicate that HDAC inhibition (300 nM and 600 nM), in BMN673-pretreated (20 nM) cells significantly increases PARP1 in chromatin within 6 h of treatment (6-fold, $p < 0.05$) (Fig. 6Ci and Cii). These results together with earlier data, suggest that the increased presence of PARP1 in chromatin may prevent access of NHEJ proteins to DSB sites, leading to a decrease in overall NHEJ activity in cells. We verified this hypothesis by conducting intrachromosomal EJ5-*ISceI* NHEJ assays in HDACi treated cells, following knockdown of PARP1 (PARP1 KD). HDAC inhibition in PARP1-depleted cells did not decrease NHEJ, as in PARP1-proficient cells (Fig. S7). Importantly, combination of PARPi (BMN) with HDACi (TSA or MS275) significantly increased apoptosis induction at 6-day treatments, in comparison to HDACi alone (Fig. 6Di and Dii). These observations suggest that persistence of PARP1 in chromatin following HDAC inhibition may be critical in blocking access to the NHEJ machinery and plays an important role in HDAC inhibitor toxicity.

4. Discussion

Acetylation and deacetylation is a dynamic process that can have profound effects on the outcome of DSB repair. Several lines of evidence demonstrate that HDACi lead to decreased NHEJ activity [40,31], but the mechanisms by which these inhibitors promote their effects on DSB repair remain unclear. We show here that pan-HDACi (TSA) inhibits the deacetylation of not only Ku70 or Ku80, but also PARP1, which functions in multiple DNA repair pathways including SSB and DSB repair. While HDACi treatment decreased total c-NHEJ activity, it did not affect repair *via* MMEJ. Inhibition of HATs by P300/CBP inhibitor

C646, in contrast, increases c-NHEJ activity, while significantly decreasing acetylation of PARP1 [44]. Moreover, PARP1 appears significantly enriched in chromatin following HDACi, compared with Ku proteins. The increased presence of PARP1 in chromatin with HDACi resembles PARP trapping seen with PARPi. Notably, PARPi in combination with HDACi treatment increases PARP1 trapping and apoptosis induction. This suggests a novel concept that HDACi result in both a physical and functional alteration of PARP1 binding at DSBs, impeding access of repair factors and leading to decreased NHEJ repair and inducing toxicity.

Acetylation of PARP1 by p300/CBP is known to promote its functions as a transcriptional co-activator [44]. Moreover, three lysine residues K498, K521 and K524 situated in the auto-modification domain of PARP1 are targets for PAR(ylation) [48] but can also be targets for acetylation [44]. PARP1 is normally recruited to DNA damage sites and activated by PAR(ylation), thereby recruiting critical DNA repair proteins. Following the recruitment of repair proteins, poly (ADP-ribose) glycohydrolase (PARG) removes PAR polymers, making PAR(ylation) a very dynamic and transient post-translational modification. Following this rapid turnover, PARP leaves DNA damage sites, allowing repair to occur. We found that HDACi increases the binding of PARP1 to *I-SceI*-induced sites of DSBs, relative to Ku70 and Ku80, in leukemia cell lines. Thus, while it is unclear whether the two post-translational functionalities *i.e.* acetylation and PAR(ylation) cooperate with each other in regulating the outcome of DSB repair, persistent binding of PARP1 at DSBs may block access of factors to repair these DNA damage sites.

One potential outcome of a transient increase in chromatin-association of PARP1 is increased DNA repair activity, including microhomology-mediated end-joining (MMEJ). Thus, we postulated that HDACi treatment could increase total repair activity associated with MMEJ, which requires PARP1 [35]. MMEJ represents a subset of total Alt-NHEJ activity in cells, which itself only represents ~10% of DSB repair activity in mammalian cells [49]. Contrary to expectation, our data utilizing two different plasmid-based approaches indicates that MMEJ is not affected by HDAC inhibition. It is likely that while PARP1-DNA interaction is stabilized with HDACi, the signaling mechanism that activates Alt-NHEJ is not induced, thus undermining the role of Alt-NHEJ in HDACi response. These findings are in agreement with observations of Manova et al., demonstrating that chromatin acetylation status does not influence Alt-NHEJ repair activity [50].

Several mechanisms may explain the persistence of PARP1 at DSBs following HDAC inhibition (Fig. 7). It is well established that treatments with HDACi increases reactive oxygen species (ROS) [2,51,52]. HDACi also downregulate base excision repair (BER), which represents a major form of SSB repair activity in cells [52]. Dianov et al. reported that for DNA ligation to occur, PARP1 needs to leave the SSB, and inhibition of PARP1 activity blocks PARP1 dissociation [53], thus preventing repair [54,55]. Our data show that increased acetylation of PARP1 increases its presence at DSBs after 6 h of HDACi treatment. This underscores the idea that acetylation of PARP1 induces a prolonged presence of PARP1 at the DSB site compatible with a delayed or inhibitory effect of PARP1 acetylation on DNA repair activity.

Notably, our data suggests that prolonged exposure (72 h) to HDACi or 6 h HDAC inhibition in cells pre-treated with a potent PARPi increases PARP trapping in chromatin [47]. These results suggest that either DNA damage and/or acetylation effects of HDACi on PARP1 stabilize its DNA binding. One explanation is that acetylation of PARP1 changes its charge and conformation that leads to increased binding to chromatin as an allosteric effect. Data supporting this model have previously been reported to explain the mechanism of PARP inhibitor action [56]. Another explanation is that HDAC inhibition changes the dynamics of energy metabolism. Recently, Hopkins et al. demonstrated that DNA damage from alkylating agent MMS in NAD⁺ depleted cells increases PARP trapping [56]. These findings highlight the possibility that HDAC inhibition potentially traps PARP1 by decreasing its dissociation from DNA. Interestingly, PARP inhibitor's ability to trap PARP1 in chromatin is postulated to correlate with the measure of their cytotoxicity [47]. Therefore, it is not implausible that a combination of HDACi and PARPi has a synergistic effect on PARP1 trapping in chromatin as shown by our results in leukemia cells. Interestingly, while HDACi decreases NHEJ, in PARP1-depleted cells NHEJ is not decreased. Thus, we believe that the persistence of PARP1 on DSBs and the trapping effect have a critical role in diminishing NHEJ following HDAC inhibition. Therefore, the model of HDACi increasing PARP1 binding at DSBs, impeding access of repair factors, and leading to decreased NHEJ repair, is an attractive one.

Our studies in cells from AML patients treated with entinostat as part of a clinical trial [34], showed that HDACi treatment *in vivo* also increases the levels of DSBs in leukemia cells, confirming our previous data [15]. Furthermore, entinostat treated cells demonstrate increased association of PAR cluster with γ H2AX, indicating a significant DNA damage response, which could be potentially used as a biomarker for cytotoxic effects of these drugs. Importantly, our data suggests that combining HDACi and PARPi in leukemias *in vivo* should be an attractive therapeutic strategy. Future studies investigating the temporal recruitment of DSB repair proteins to DSB sites in chromatin will yield insights into mechanisms through which NHEJ is reduced that will aid development of better therapeutic strategies for the treatment of leukemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

HDACis decreases NHEJ repair efficiency. (A and B) LacZ α reactivation NHEJ repair assays in K562 (A) and HL60 (B) cells treated with TSA (300 nM) for 1 h (Grey bars), and 6 h (Black bars) or entinostat (300 nM, Light grey bars) for 6 h compared to controls (White bars). DNA repair efficiency is presented as fold decrease compared to control $\pm$ SEM, * $p < 0.05$ based on *t*-test. (C) Intra-chromosomal NHEJ assays in K562 cells with stably integrated pimeJ5GFP. *I-SceI* was induced by transfection and 6 h later, treated with indicated doses of TSA (72 h). NHEJ was measured 3 days post-treatments, using flow cytometry for GFP. % GFP cells indicate NHEJ efficiency. (D & E) MTS cell viability assay- K562 cells seeded at a density of 1000 cells per well were treated with HDACi. (D) TSA and (E) Entinostat (or MS275) at indicated doses. Cells were allowed to proliferate in the drug-containing or drug-free media (control) and MTS dye was added at day 7, followed by colorimetric analysis. Results are representative of three independent experiments $\pm$ SEM.

Fig. 2.

HDACis induces differential acetylation of NHEJ repair proteins. Immunoprecipitation with AcK antibody or IgG were performed on K562 (A, C, E) or HL60 (B, D and F) cells treated with TSA (300 nM, 1 and 6 h) or with control. Immunoblotting was then performed with antibodies directed against Ku80 and Ku70 (A, B) and PARP1 (E,F) proteins. Level of expression of IgG and β-actin were used as loading controls for immunoprecipitated and input samples respectively. Results are representative of three independent experiments.

Fig. 3. HAT p300/CBP inhibitors increase NHEJ repair efficiency. (A) Immunoprecipitation with AcK antibody was performed on K562 cells treated with HAT p300/CBP inhibitor (CBPi, 20 μ M), TSA (300 nM) or in the presence of control for 6 h. Immunoblotting was then performed against PARP1 and Ku70 proteins. Level of IgG and β -actin expression were used as loading controls for the immunoprecipitated and input samples respectively. (B) NHEJ assay in K562-EJ5 cells treated for 72 h with C646 (CBPi) at indicated doses, compared to controls. % GFP cells indicate NHEJ efficiency. Results are representative of three independent experiments $\pm$ SEM, * p <0.05 based on t -test.

Fig. 4.

TSA induces increased binding of PARP1 to DSBs. (A) Schematic of ChIP after DSB induction in K562DRneo cells engineered with an I-Sce1 inducible DSB. Primer sets located 200 or 1300 bp from the inducible DSB were used for the real-time PCR analysis of ChIP experiment. (B, and C) Real-time PCR analysis of immunoprecipitated DNA located 200 bp from the DSB, from ChIP experiments performed with antibodies directed against γ H2A.x, AcH4 (B), PARP1 and Ku 70 (C) on K562DRneo cells treated with TSA (300 nM, 6 h, Black bars), compared to control (White bars). (D) Real-time PCR analysis of

immunoprecipitated DNA located 1300 bp from the DSB, from ChIP experiments performed with antibody directed against PARP1 on K562DRneo cells treated with TSA (300 nM, 6 h, Black bars), compared to DMSO control (White bars). Bound/Input ratio is presented as fold increase compared to control and results are representative of the mean of three independent experiments $\pm$ SEM, * $p < 0.05$ based on *t*-test.

Fig. 5.

Entinostat increases co-localization between PAR and γ H2A.x in AML patient. BMMNC from 3 AML patients treated with entinostat (6 mg/m²) at day 1 and 8 and collected at day 0 and 8 as shown in were cytopun onto glass slides and fixed cells were co-immunostained with antibodies directed against PAR and γ H2A.x proteins. At least 50 cells/slide were analyzed in triplicate, and the number of cells expressing ≥ 50 γ H2A.x foci/cells (A) and the number of cells expressing γ H2A.x foci co-immunostained for PAR (B) were counted in samples treated for 7 days with entinostat (black bars, D8) *versus* pre-treatment control (white bars, D0). Results are representative of the mean of three independent experiments and presented as fold increase compared to pre-treatment $\pm$ SEM, * $p < 0.05$ based on *t*-test.

Fig. 6.

HDACis increases PARP trapping in chromatin. K562 cells treated with TSA or DMSO (vehicle) at indicated doses for either 6 h or 72 h were pelleted and subcellular fractionation of proteins was conducted as described in Methods. (Ai) 5 µg of chromatin-bound proteins was analyzed by western blotting. H3 is used as a loading control protein for chromatin. (Aii) Densitometry quantitation of PARP1 enrichment from three independent experiments. (Bi) 5 µg of chromatin-bound proteins – PARP1 knockdown using targeted siRNA (siPARP) or non-targeting control siRNA (siCTRL), followed by TSA treatments for 72 h, as

described in Methods (Bii) Densitometry analysis of three-independent knockdown experiments (Ci) PARP inhibition by 20 nM BMN673 (BMN) for 16 h, followed by 6 h TSA treatments. (Cii) Densitometry quantitation of three independent experiments. (D) Flow-cytometry analysis of % Apoptosis. Exponentially growing cells were treated with either the indicated doses of HDACi (Di) TSA or (Dii) Entinostat, pre-treated (for 12 h) with or without 20 nM BMN for 6-days followed by staining with Annexin 5- APC dye. The data represents only the Annexin 5 positive cells indicating early apoptosis induction (*indicates $p < 0.05$, based on *t*-test).

Fig. 7.

Simplified model of HDACi effect on PARP1 and NHEJ in cancer and leukemia cells.

HDACi influence multiple biological and physiological responses in cancer/leukemia cells including the primary effect of acetylation of histone and non-histone proteins, such as DNA repair proteins Ku70, Ku80 and PARP1. Secondly, HDACi also induce ROS generation and direct/indirect increase in DNA strand breaks (SSBs and DSBs). Both of these responses cause an increase in the PAR(ylation) and activation of PARP1 binding to histones.

However, the downstream consequence of PARP acetylation/and or DNA damage is increased and persistent binding of PARP1 to broken DNA ends in chromatin signified by PARP trapping. Mechanistically, PARP trapping in cells from HDAC inhibition could be due to acetylation-mediated conformational changes in PARP1 and allosteric binding or NAD⁺ pool depletion due to hyperactivation of PAR(ylation). The net effect is increased DSBs and cytotoxicity of leukemia cells.

Table 1

DNA-PKcs dependent NHEJ (c-NHEJ) and Alt-NHEJ proteins differentially acetylated in K562 cells treated with TSA (300 nM, 6 h) versus control.

DNA REPAIR PATHWAY	PROTEINS	DIFFERENTIAL ACETYLATION
DNA-PK dependent	Ku70	Yes (Fig. 2 and S2)
NHEJ	Ku80	Yes (Fig. 2 and S2)
Alt-NHEJ	PARP-1	Yes (Fig. 2 and S2)
	DNA LigIII α	No (Fig. S3)
	WRN	No (Fig. S3)