Combined Effect of Histone Acetylation and Acetyl Mark Readers on Radiation Sensitivity

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ABSTRACT

We are interested in studying potential drugs that have the ability to make tumors more sensitive to radiation. There has been much interest in examining the effect of modulation to chromatin structure on the DNA damage response (DDR). We therefore examined histone deacetylase (HDAC) inhibitors, small molecules that modify the structural features of chromatin involved in the packaging of DNA. HDAC inhibitors are considered to be potential drug candidates to complement radiation therapy, yet the mechanisms underlying their effects have been elusive.

To study the DDR signaling effects of HDAC inhibitors, we developed a high-throughput automated microscopy assay to assess effects on elements of DNA damage-mediated signaling through quantification of H2AX phosphorylation, cell cycle profiling, and cell survival following irradiation. We then applied this assay to a library of diverse HDAC inhibitors as well as a collection of shRNAs targeting the individual HDACs. We next assessed DSB induction and repair kinetics on a selection of active HDAC inhibitors with CometChip, a novel high-throughput adaptation of the single-cell DSB comet assay. Finally, with the recent discovery of the role of the bromodomain chromatin reader protein Brd4 in the insulation of chromatin from the DDR, we explored combinatorial use of HDACi and JQ1 to achieve altered DDR through simultaneous alteration of chromatin structure and acetyl-lysine reading.

Our data suggest that class I HDAC inhibitors potently elevate H2AX phosphorylation, but without strong effects on DSB induction or repair kinetics. We find that many HDAC inhibitors also enhance G2/M cell cycle arrest and decrease proliferation, even in the absence of irradiation. These data suggest that HDAC inhibitors influence radiation effects primarily through altering DNA damage-induced signaling events. We have also found that overexpression of specific Brd4 isoforms can abrogate the elevated H2AX phosphorylation induced by HDAC inhibition and lead to preferential cell death. This may have important implications for the clinical use of these agents.
INTRODUCTION

Despite the targeted nature of current radiation therapy techniques, delivery of lethal tumor doses inevitably exposes healthy tissue to an attenuated but significant radiation dose. There has been much therapeutic interest in the development of small molecule radiosensitizers that preferentially target tumors. Such molecules induce an increased incidence of DNA double strand breaks (DSBs), disruption of the DNA repair pathways, or a modulation of signaling events in the DNA damage response (DDR) such that tumor cells are killed preferentially, thereby allowing a reduction in the dose required to successfully treat the tumor and increasing normal tissue sparing.

Epigenetic chromatin modifications are key determinants of transcriptional modulation. Such modifications have been implicated in cancer progression. More recently, epigenetic modifications have been demonstrated to affect the response to radiation-induced DNA damage\(^1\). Among these epigenetic modifications, those mediated by the interaction of histone acetyl transferase (HAT) and histone deacetylase activity (HDAC) have been well-studied\(^2\). Because aberrant HAT inactivation and HDAC activation has been associated with tumorigenesis, presumably due to the transcriptional repression of tumor suppressor genes, the development of small molecule HDAC inhibitors (HDACi) has become an area of great interest\(^3\). There is also evidence that several HDACs are overexpressed in many different types of cancers with an apparent inverse association with prognosis. Therefore, considerable attention has been given to the modulation of histone acetylation as a potential therapeutic target\(^4,^5\).

Another interesting though less well understood phenomenon surrounding HDACi comes from studies reporting an increased susceptibility of many cancerous cell lines to irradiation-induced cell death following pre-treatment with HDACi\(^2,^6\). Mechanisms surrounding this effect are unclear, but many of these studies reported enhanced and prolonged level of phosphorylation at serine 139 of histone variant H2AX. This phosphorylated H2AX, known as \(\gamma\)H2AX, has been used broadly as a highly sensitive
proxy for DNA damage signaling downstream of DSBs\textsuperscript{7-13}. Because the dephosphorylation of H2AX following irradiation correlates with the repair of DSB lesions\textsuperscript{14}, there has been much interest in whether the use of HDACi inhibits DNA DSB repair machinery and delays or prevents repair of DNA lesions\textsuperscript{15,16}. HDACi have therefore become an anti-cancer modality of great interest not only as a stand-alone therapies that target aberrant HDAC activity and associated transcriptional misregulation of tumor repressors, but also as potential radiosensitizers. The use of HDACi as radiosensitizing agents has garnered attention in the treatment of glioblastoma, where a recent phase 2 study of valproic acid used in conjunction with radiation therapy and temozolomide demonstrated a well-tolerated toxicity profile and encouraging clinical outcomes\textsuperscript{17}. Initial phase I studies regarding the safety of HDAC inhibitors such as panobinostat (LBH589) used in the context of radiotherapy or chemoradiotherapy in inoperable stage III non-small cell lung cancer and CUDC-101 in head and neck squamous cell cancer have also been reported\textsuperscript{18-20}.

Studies of the mechanisms of HDACi activity in cancer and the DDR have been further complicated by the discovery of multiple classes of HDACs, first with the identification of HDAC1, a histone deacetylase that shares homology with the yeast transcriptional regulator Rpd3\textsuperscript{21-23}. Based on sequence homology, 18 HDACs have subsequently been identified and subdivided into four groups: the zinc-dependent Class I (HDACs 1, 2, 3, and 8), Class IIA (HDACs 4, 5, 7, 9), Class IIB (HDACs 6, 10), and Class IV (HDAC11) and the zinc-independent Class III HDACs, or sirtuins\textsuperscript{24,25}. Class I HDACs have been reported to have ubiquitous expression and a predominantly nuclear localization, whereas Class II HDACs demonstrate more cell-type dependent expression and cytoplasmic pattern of localization\textsuperscript{23}.

Most HDAC inhibitors demonstrate remarkably unselective binding profiles, complicating the study of on-target effects in cancer cells\textsuperscript{23,26}. Some HDACs such as HDAC6 are also implicated in the deacetylation of non-histone proteins such as alpha-tubulin\textsuperscript{27}. It seems plausible that HDACi with
potential as cancer therapeutics through modulation of the chromatin state should preferentially target HDACs implicated in chromatin dynamics. Other cellular off-target effects could lead to unwanted toxicity. This phenomenon is demonstrated by the translation of pan-HDAC inhibitors such as vorinostat (SAHA) into phase I and II trials. These relatively broad-spectrum inhibitors induced deleterious effects such as fatigue, nausea, diarrhea, bone marrow suppression, coagulopathies, electrolyte abnormalities, and arrhythmias\textsuperscript{23, 28}. It is therefore of increasing importance to characterize effects of individual HDACs with respect to specific cellular functions such as the DDR. In this way, HDAC inhibitors for specific applications can move forward toward the goal of specific on-target effects, and less potential for off-target toxicity.

**Interaction of HDACi and bromodomain Brd4 isoform B activity in the modulation of the DDR**

The process of chromatin structure relaxation in response to DNA damage has been established as an important mediator in the cellular ability to repair DNA lesions, presumably due to the necessity for repair complexes to localize to areas of open chromatin surrounding damage-induced DSBs and initiate repair signaling\textsuperscript{29, 30}. HDACi induce structural relaxation of chromatin through the hyperacetylation of core histone lysine residues. Recent reports identified another mechanism of achieving increased chromatin relaxation\textsuperscript{31, 32}. Inhibition of the acetyl lysine-binding bromodomains of certain Brd4 proteins can also relax chromatin structure\textsuperscript{31}. JQ1, a potent BET bromodomain inhibitor, effectively inhibits the ability of Brd4 isoform-B to bind to acetylated histone lysine residues, most likely due to the inhibition of the acetyl lysine binding of bromodomain 1 on Brd4 isoform B\textsuperscript{31, 33}. As both HDACi and JQ1 act through mechanisms that potentially increase the accessibility of DNA lesion sites to repair machinery, we sought to explore the altered DDR that resulted from co-treatment with both agents as well as the relative ability of HDACi to alter the DDR in the presence of enhanced Brd4 isoform B activity.
RESULTS

High content image-based screen reveals Class I HDAC inhibitors as potent DDR modulators

Past efforts to characterize the role of chromatin modulation in the DDR have led to the development of a high-throughput, high-content quantitative microscopy assay assessing both early and late timepoints in the DDR, allowing for an interrogation of an RNA-mediated interference (RNAi) library focused on proteins known to interact with and modify chromatin\textsuperscript{31, 34, 35}. The assay has been validated with both small molecule inhibitors of DDR signaling as well as shRNA targeting known components of the DDR\textsuperscript{31}. To expand the investigation to include less well understood small molecule modulators of the DDR, the multiplex assay was modified to utilize a library of HDAC inhibitors arrayed in 384-well format (Fig. 1a). For each time point U2OS osteosarcoma cells were fixed and stained for γH2AX to monitor early signaling events in the DDR, along with Hoechst 33342 to monitor both proliferation of cells as well as cell-cycle progression following irradiation (Fig. 1b). Six representative images were acquired from each well at each timepoint and image segmentation of nuclei and γH2AX foci was performed using CellProfiler to provide a quantitative time-dependent, drug exposure-dependent assessment of the DDR\textsuperscript{34-37}.

This assay was used to interrogate the large panel of 20 HDAC inhibitors, arrayed from well to well in ascending concentrations. Treatment of cells with HDAC inhibitors for 16 hours prior to irradiation with 10 Gy caused concentration-dependent, compound-specific increases in the mean intensity of γH2AX foci at early and late timepoints, even causing an increase in the mean γH2AX foci intensity when no irradiation was applied (Fig. 2a). The potency of each HDAC inhibitor in enhancing the early DDR as evaluated through the proxy of γH2AX was evaluated by calculating the EC\textsubscript{50}, the concentration of compound that elevated the mean γH2AX foci intensity to 50% of the maximum value (Fig. 2b). Ranking the HDAC inhibitors by EC\textsubscript{50} revealed that HDAC inhibitors that have evidence of
potent targeting of multiple class I HDAC targets, as assessed by in-vitro assays utilizing specific purified HDACs, are the most potent activators of IR-induced H2AX phosphorylation, with sub-micromolar EC\textsubscript{50} values\textsuperscript{26,38-43}. HDAC inhibitors with less preferential profiles toward Class I targeting, such as tubacin (a Class IIb HDAC inhibitor) demonstrated appreciably larger EC\textsubscript{50} values, perhaps understandably given the higher range of reported in vitro IC\textsubscript{50} values related to targeting Class I HDACs. One Class I HDAC inhibitor, FK228, demonstrated an EC\textsubscript{50} with regard to mean γH2AX foci intensity elevation that was higher than the reported in vitro IC\textsubscript{50} range for all four Class I HDACs, and yet demonstrably less than the lowest in-vitro IC\textsubscript{50} for a non-Class I HDAC reported by Bradner et al. using the same HDACi library\textsuperscript{26}. For each of the 20 HDAC compounds, the EC\textsubscript{50} values were universally higher than the lowest literature reported IC\textsubscript{50} for a Class I target.

On the other hand, many other HDAC inhibitors showing great efficacy at elevating the mean γH2AX foci intensity had EC\textsubscript{50} values well below the highest reported in vitro IC\textsubscript{50} for a Class I HDAC. LAQ824, for instance, has reported in vitro IC\textsubscript{50} values of 0.55 nM, 1.4 nM, 4.2 nM, and 340 nM for HDAC1, 2, 3, and 8, respectively\textsuperscript{26}. While these data suggested that inhibition of three of the four Class I HDACs could potentially be sufficient for enhancing the DDR through H2AX signaling, we could also not rule out the possibility that certain HDACs within class I may have a more influential role with regard to elevation of the mean γH2AX foci intensity following irradiation. Thus we characterized the effect of knocking down single HDACs and observing the resulting early DDR signaling. As part of a wider effort characterizing lentiviral shRNA knockdown of proteins thought to play a role in chromatin structural modulation, a similar high-throughput high-content image-based screen was performed to examine potential early and late modulators of the DDR\textsuperscript{31}. As part of this effort multiple lentiviral shRNA hairpins were used to examine DDR alterations induced by all individual HDACs and sirtuins. Taking 25 mM caffeine (ATM/ATR inhibitor) and the use of an shRNA targeting ATM as positive controls for reduced H2AX phosphorylation following irradiation, as well as lentiviral Brd4 knockdown with shRNA as a
positive control for enhanced H2AX phosphorylation following irradiation, we found that knockdown of single HDACs and sirtuins did not have an appreciable effect on the induction of γH2AX foci at 1 hour following irradiation, though perhaps there were some hints of delayed resolution of γH2AX foci at 6 hours, particularly for HDAC3.

As single knockdown studies on HDACs were insufficient to explain the efficacy of HDAC inhibitors targeting Class I at elevating H2AX phosphorylation, we performed combination knockdown studies to recapitulate the targeting profile of a typical Class I HDAC inhibitor, i.e. predominantly targeting HDAC1, 2, and 3 with greater potency than HDAC8. We could then assess the possibility of redundant or compensatory function among Class I HDACs, and rule out the possibility of elevation of H2AX phosphorylation through an off-target mechanism. Thus the combination of siRNA-mediated knockdown of HDAC1, HDAC2, and HDAC3 was assessed and compared with the results of single knockdown both by immunoblotting and by immunofluorescence (Fig. 2c and 2d). The elevation of DDR signaling as assessed by γH2AX signal on western blot and γH2AX mean foci intensity on immunofluorescence suggested that an inhibitory profile targeting HDAC1, HDAC2, and HDAC3 is sufficient for altering the early signaling events in the DDR.

Using quantification of Hoescht 33342 staining from the image-based screen, we assessed the effect of the panel of HDAC inhibitors on proliferation and cell cycle progression (Figure 3). Interestingly, virtually all of the HDAC inhibitors tested in the library, with the possible exception of the Class IIb HDAC inhibitor tubacin, demonstrated a concentration-dependent reduction in the number of U2OS cells detected at baseline and follow-up, even without irradiation (Fig. 3a). The effect of the panel of HDAC inhibitors on cell counts thus suggests that these agents do not act in a purely radiosensitizing manner in U2OS, i.e. evidence of HDACi-induced toxicity is not dependent on radiation. Wortmannin, a potent PI3K inhibitor included in the small molecule drug screen that is known to have radiosensitizing activity.
demonstrated a more typical behavior of a purely radiation sensitizing agent in that there was little evidence of concentration-dependent reduction in cell count when cells were exposed to wortmannin in the absence of irradiation, yet the mean cell count fell relative to the DMSO control when cells were treated with 10 Gy. Pooling the quantified Hoescht 33342 intensity from several wells spanning the active concentration ranges of selected HDAC inhibitors in the image-based screen allowed for the generation of DNA histograms at all timepoints to provide an assessment of cell cycle progression (Fig. 3b). 16 hour pre-irradiation exposure to HDAC inhibitors typically characterized as acting on Class I HDACs was associated with increased evidence of G2/M arrest at 6 hours, also with potentially more cells arrested at the G2/M checkpoint at baseline in the non-irradiated setting. Tubacin, on the other hand, did not demonstrate a cell cycle progression markedly different from the DMSO control. These data suggesting HDACi-induced cell cycle arrest are consistent with the proliferative pattern shown in Fig 3a, in that exposure of U2OS cells to HDAC inhibitors with a potent effect on Class I HDACs is associated with baseline toxicity and reduced proliferative potential in the presence or absence of irradiation.

As the majority of HDAC inhibitors interrogated in the image-based screen demonstrated an elevated and prolonged H2AX phosphorylation following 10 Gy, we sought to determine whether or not the increased induction of γH2AX signal and delayed resolution were reflective of the increased induction and/or reduced repair of DNA double-strand breaks (DSBs). To examine this, the neutral CometChip assay was used to measure the relative amount of DSB induction and the kinetics of repair when U2OS were exposed to selected HDAC inhibitors representing the major classes covered in the image-based screen (Fig. 4). CometChip has an advantage over the traditional comet assay in providing a method to isolate comets in the proper focal plane, facilitating accurate and reliable image segmentation and quantification in an automated fashion. Even in the case where aspiration of excess cells from the macrowells is incomplete, the presence of an arrayed grid of comet heads provided
by the CometChip microwells facilitates automated identification of out-of-focus cells and artifacts outside the optimal focal plane for comet analysis, thereby allowing subsequent removal of spurious signals from automated comet segmentation and tail length quantification (Fig. 4a, left). Exposure to ionizing radiation induces an acute increase in the number of DSBs and an associated increased tail length following DNA electrophoresis under neutral conditions (Fig. 4a, middle). While an increase in DSB induction would be evident with an increased tail length immediately after irradiation and neutral lysis, agents associated with delayed DSB repair through the inhibition of the process of non-homologous end-joining, such as the DNA-PK inhibitor Nu7441, produce a comet tail length profile characterized by continued elevation of the tail length at later timepoints, followed by delayed resolution\textsuperscript{45,47,48} (Fig. 4a, right). When testing for the effect of HDAC inhibitors on the induction and repair rate of DNA DSBs, U2OS cells were pre-treated for 16 hours, irradiated, and allowed to incubate post-IR in the presence of the same conditioned media. Treatment of U2OS cells with a selection of HDAC inhibitors did not markedly alter the irradiation-dependent induction level of DSBs, as the tail length immediately after 50 Gy was virtually indistinguishable between all conditions tested (Fig. 4b). Treatment with HDAC inhibitors prior to irradiation also did not markedly alter the kinetics of comet tail length resolution compared to the DMSO control, certainly not to the level of the delayed repair induced by Nu7441 treatment. At least at the resolution provided by the neutral CometChip assay, the increased DDR signaling as reflected by increased H2AX phosphorylation and delayed resolution is not reflected to the same degree at the most upstream measurement of DNA damage, i.e. the DSB induction and repair kinetics.

We next sought to characterize the effect of HDACi-mediated signaling changes in the broader context of chromatin structural alterations by investigating the interaction between HDACi and the chromatin insulator Brd4 isoform B. Co-administration of BET bromodomain inhibitor JQ1 and pan-class HDAC inhibitor LBH589 to U2OS cells 16 hours before irradiation with 10 Gy resulted in increased H2AX
phosphorylation 1 hour following irradiation relative to when treated with JQ1 or LBH589 alone, the effect seemingly more profound when examining an immunoblot readout of gross γH2AX rather than when examining the immunofluorescence readout of mean γH2AX foci intensity (Fig. 5a). Indeed, the additive increase in γH2AX signal on western blot was quite appreciable even in the absence of irradiation (Fig. 5a, left and middle). Examining the effect of JQ1 of LBH589 co-administration on cell viability and proliferation using CellTiter-Glo® as a readout revealed that while both compounds seemed to reduce the proliferative potential of U2OS, co-administration of the two drugs seemed to halt proliferation all-together (Fig. 5b). An examination of cell cycle progression using flow cytometry after the administration of JQ1, LBH589, or the combination of both agents 16 hours before irradiation revealed that both LBH589 and LBH589/JQ1 treated cells experienced an increased G2/M arrest at baseline before irradiation (Fig. 5c). Increased G2/M arrest was also seen 5 hours following the receipt of 10 Gy of irradiation, likely due to a head start in cell cycle arrest from the baseline effect of the drugs. The striking resemblance of the cell cycle progression of the LBH589 and LBH589/JQ1 treated samples suggests that the predominant mediator of G2/M cell cycle arrest was LBH589. These data suggest that the simultaneous inhibition of Brd4 isoform B acetyl lysine binding and hyperacetylation of chromatin is associated with an enhanced level of early DNA damage signaling, and yet the proliferative state of U2OS is impaired by the presence of these drugs.

As increased Brd4 isoform B activity induces a condensed chromatin state in U2OS, we sought to determine how HDACi-induced DDR alterations would be affected by the overexpression of specific Brd4 isoforms. The chromatin-relaxing alteration induced by HDACi treatment would potentially have a competing effect on the DDR with the chromatin-condensing alteration of artificial Brd4 isoform B overexpression. Overexpression of Brd4 isoform B or C resulted in decreased γH2AX signaling following 10 Gy ionizing radiation as assessed by γH2AX foci intensity, consistent with published results (Fig. 6a-b), yet interestingly, overexpression of Brd4 isoform B or isoform C abrogated the typical increase in
DDR as assessed by γH2AX foci intensity induced by a pre-irradiation treatment with LBH589. Using propidium iodide uptake in trypsinized cells as a proxy for membrane perforation and cell death, we compared the rates of irradiation induced death at 24 hours between U2OS cells overexpressing GFP-tagged Brd4 isoform B or C with a GFP control, in the presence and absence of 16 hour LBH589 pre-treatment (Fig. 6c). Brd4 isoform B or C overexpression seemed to exert a toxic effect on cells by itself, as the Brd4 overexpressing cells were more preferentially dead at 24 hours than the GFP control, even in the absence of irradiation. LBH589 seemed to produce an additional stress on the GFP-transfected cells, as both the Brd4 overexpressing cells and GFP control transfectants preferentially took up more PI staining at 24 hours in the presence of LBH589. In the presence of irradiation, however, the Brd4 overexpressing cells demonstrated increased vulnerability to irradiation-induced cell death compared with the GFP control, though perhaps the relative vulnerability compared to the GFP control was greater when the cells were exposed to DMSO only rather than 40 cumulative hours of LBH589 in addition to irradiation. These data suggest that the relative overabundance of the DNA damage insulator Brd4 isoform B has the potential to abrogate the normal HDACi-induced DDR enhancements, and that this abrogation of signaling correlates with increased death of cells acutely following DNA damage.
DISCUSSION

Our data suggest that Class I HDAC inhibitors are implicated in the early DDR as assessed by increased phosphorylation of H2AX following exposure to ionizing radiation in U2OS cells. Furthermore, our data suggest that the concentration-dependent amplification of γH2AX outstrips the effect seen when examining the kinetics of DSB repair when U2OS are exposed to HDACi. Many studies attribute the familiar elevated and prolonged γH2AX signal following irradiation induced by HDACi to delayed DSB repair, but the use of γH2AX as a proxy for the number of DSBs is imperfect due to the dependence of the downstream phosphorylation of H2AX on several kinases such as ATM and DNA-PK. The phosphorylation and dephosphorylation of H2AX are also markedly delayed relative to the formation and resolution of DSBs, so an accurate assessment of DSB induction and repair kinetics likely requires an examination of DSBs utilizing assays that measure a level closer to DSB lesions rather than a downstream signaling event. Studies that have attempted to do so provide some conflicting evidence. Miller et al. have reported that HDAC1 and HDAC2 knockdown with siRNA resulted in a decreased rate of comet tail resolution in U2OS, while another study reported that 24 hour exposure to an active concentration of SAHA did not markedly alter the cellular kinetics of DSB repair as assessed by pulsed-field gel electrophoresis. Other studies have attributed at least part of the delayed resolution of γH2AX signal to a reduction in the expression of Ku70, Ku80, DNA-PK, 53BP1, and RAD51 by HDAC inhibitors such as NaB, Trichostatin A, and SAHA. Such studies typically use pre-irradiation drug exposures of at least 24 hours. For instance, 53BP1 downregulation was observed 72 hours after SAHA treatment in prostate cells. It is possible that longer drug exposures result in alterations in DSB repair machinery expression to the point that alterations in the DSB repair kinetics are measurable, but our data suggests that with shorter drug exposure the γH2AX elevation and prolongation is most likely due to alterations of the downstream signaling.
Our data also suggest that HDACi may not act in a purely radiosensitizing manner in certain cell lines. This is perhaps not surprising, given the effects of HDACi as standalone agents in many cancer cell types with inherent overexpression of HDACs leading to transcriptional misregulation. For HDAC inhibitors to have particular efficacy as radiosensitizing agents rather than as cytotoxic therapeutics to be used as an adjunct to radiation therapy, the concentration dependent effect on normal, healthy tissue has to be limited. One advantage of utilizing a radiosensitizer is that even if the agent is delivered systemically and all tissues are exposed, the use of radiotherapy contoured for a particular clinical tumor volume adds a second level of specificity such that only tissue in the vicinity will experience the radiosensitizing effect of the drug. Hopefully improvements in the understanding of which HDACs in particular modulate the DDR, complemented with the development of potent small molecule inhibitors specific to those HDACs will lead to reductions in dose-dependent toxicity that has been seen in many of the phase I and II trials utilizing HDAC inhibitors with broad inhibitory profiles.

Our data concerning the interaction between HDACi and the activity of the acetyl lysine binding Brd4 isoform B demonstrate the importance of the broader role of chromatin structure in the DDR. Since Brd4 isoform B and HDAC inhibition have been demonstrated to facilitate chromatin relaxation\textsuperscript{31}, one plausible explanation for the increase in DDR as represented by the enhanced and prolonged phosphorylation of H2AX is the increased accessibility of DSB sites to the early mediators of DNA damage signaling. In the case of Brd4 isoform B inhibition, increased and prolonged γH2AX has been shown not to be reflective of the DSB repair kinetics following IR, as pulse-field gel electrophoresis has demonstrated negligible difference following shRNA knockdown of Brd4\textsuperscript{31}; rather, the relative conformational state of chromatin and the ability to recruit early signaling mediators seems the more likely explanation for the increase in DDR signaling as assessed by H2AX phosphorylation.
On the other hand, overexpression of specific Brd4 isoforms prevented the action of HDACi in increasing the early DDR. The observation that these cells experienced rapid and preferential death perhaps goes against traditional characterization of γH2AX signal as reflective of the DSB profile of the cell, with a high signal correlating with a cell rich with DNA lesions. In the cells overexpressing Brd4, one plausible explanation for the preferential cell death despite the reduction in γH2AX is that the DNA lesions induced by irradiation are not effectively repaired in the absence of necessary DNA damage signaling, leading the cells to die from mitotic catastrophe or a similar mechanism. Interestingly, the dominant effect when HDACi and Brd4 isoform overexpression were combined seemed to be the overactivity of the chromatin insulator, as even HDACi pre-treatment could not elevate the γH2AX signal relative to the DMSO control. Our data suggest that predicting the level of early DDR signaling following DNA damage could very well depend on the cell specific expression and activity of chromatin insulator Brd4 isoform B or C. This could also be one potential explanation of the differential response of particular cancer cell lines to DNA damage in the presence of HDACi pre-treatment, i.e. cells that have a robust elevation of DDR signaling following administration of HDACi may have correspondingly dampened activity of chromatin insulator Brd4 isoform B. Many investigators have expressed interest in the search for cellular biomarkers that may provide an indication for which tumor types may have a particular indication for HDACi therapy in conjunction with radiation\textsuperscript{53, 54}. While our data are limited in focusing solely on a single perturbation to a model cell line with regard to its Brd4 expression, the interaction of histone acetylation and a reader of acetyl marks shown in this model system provides evidence that both epigenetic writers and readers affect chromatin structure and the ability of the DSB repair machinery to propagate signaling information regarding DSBs to downstream effectors. This is likely to play a role in the differential response of various cell lines to epigenetic strategies aimed at increasing the susceptibility of cancer cells to irradiation.
CONCLUSIONS

Using a high-throughput image-based screen we obtained evidence that the combined activity of individual Class I HDACs are implicated in the early DNA damage response as assessed by time-dependent phosphorylation of H2AX. Our data suggest that rather than functioning as purely radiosensitizing agents in U2OS, Class I HDACi induce G2/M arrest and reduced proliferation even in the absence of irradiation. Our data using CometChip suggests that the elevated and prolonged γH2AX signal induced by HDAC inhibitors is not reflected to the same degree at the level of DNA double strand breaks, suggesting that the alterations induced by HDAC inhibitors have a primary effect on the regulation of downstream DNA damage signaling rather than influencing the rate of double strand break induction or repair. Our examination of the role of HDAC inhibitors in combination with alterations to the activity of specific Brd4 isoforms revealed that while simultaneous inhibition of HDACs and Brd4 isoforms increased early phosphorylation of H2AX, overexpression of specific Brd4 isoforms abrogated the typical increase in DDR signaling typically induced by HDACi. These data suggest that the relative relationship between the activity of epigenetic writers and readers is likely to play a role in the chromatin structure and early DDR signaling that results following irradiation.
SUMMARY

There has been much therapeutic interest in the development of small molecule radiosensitizers that preferentially target tumors. Epigenetic modifications have been demonstrated to affect the response to radiation-induced DNA damage, particularly those mediated by the interaction of histone acetyl transferase (HAT) and histone deacetylase (HDAC) activity. Utilizing a high-throughput image-based screen assessing early and late mediators of the DNA damage response (DDR), we obtained evidence that HDAC inhibitors (HDACi) that target Class I HDACs demonstrate a potent ability to enhance and prolong the level of phosphorylation at serine 139 of histone variant H2AX, a marker of early DDR signaling. This phenomenon was not recapitulated when using a lentiviral shRNA library to assess DDR signaling following knockdown of each HDAC and sirtuin individually. Rather, combination siRNA experiments demonstrated that enhanced early DDR signaling was only found when a combination of three HDACs in Class I (HDAC1, 2, and 3) were knocked down together, suggesting that Class I HDAC inhibitors achieve their effects through class-wide inhibition of Class I HDACs. We also found that HDAC inhibitors do not function purely as radiosensitizing agents in U2OS, as they induce G2/M arrest and dose-dependent reductions in proliferation even in the absence of irradiation. Our examination of the DNA double-strand break (DSB) kinetics following administration of a selection of HDAC inhibitors revealed that HDAC inhibitors do not markedly the rate of DSB induction or repair, implying that the alterations in the DDR induced by HDACi are primarily on downstream signaling phenomena. Our examination of the role of HDACi in combination with alterations to the activity of Brd4 isoform B revealed that while simultaneous inhibition of HDACs and Brd4 isoform B increased early phosphorylation of H2AX, overexpression of specific Brd4 isoforms abrogated the typical increase in DDR signaling typically induced by HDACi. These data suggest that the relative relationship between the activity of acetyl lysine writers and readers is likely to play a role in early DDR signaling that results following irradiation.
METHODS

Cell culture. U2OS osteosarcoma cells (ATCC HTB-96) were cultured in DMEM supplemented with 10% v/v FBS (complete media) at 37°C in a humidified incubator supplied with 5% CO2 in a BL2 facility. For the high-throughput, high content image-based screen all cells were obtained at passage 10-15 grown and were grown in media supplemented with Pen/Strep. Cells were tested for mycoplasma by PCR before seeding and infection. All infections were performed in a BL2+ facility.

Small molecule drugs. 384-well plates with serial dilutions of the 20 HDAC inhibitors presented in Fig. 2A-C and Wortmannin were provided as a gift from Dr. James Bradner, Dana Farber Cancer Institute, Boston, MA, USA. Dr. Bradner performed the assembly and synthesis of the library as described26. Subsequent LBH589 was obtained from BioVison (catalog number 1612-5, lot number 50112). BET bromodomain inhibitor JQ1 was synthesized as described33 and was provided as a gift from J. Bradner.

Antibodies and stains. Mouse monoclonal antibodies against H2AX were from Upstate/Millipore (catalogue number 05636) and Actin (Sigma, catalogue number A5441). Rabbit polyclonal antibodies against H2AX were from Cell Signaling (catalogue number 9718), HDAC1 (Abcam, catalogue number ab7028), HDAC2 (catalogue number ab7029), and HDAC3 (Abcam, catalogue number ab 16047). DNA stains were Hoechst 33342 (Life Technologies, catalogue number H1399) and propidium iodide (Life Technologies, catalogue number P1304MP). Fluorescent antibodies were from Life Technologies: goat anti-rabbit and goat anti-mouse Alexa 488, 555 and 647 (catalogue numbers A11001, A21422, A21235, A21238, A21428 and A21244).

shRNA library. shRNA was applied to cells using a high-titre arrayed lenti-viral library maintained in the pLKO_TRC001 vector34.
Image-based screens. U2OS cells were seeded with a MicroFill (Biotek) in 384-well black, clear bottom plates (Greiner) at a density of 500 cells/well in 50 μL of media, and allowed to attach overnight at 37°C in a 5% CO2 incubator. The day after plating, HDAC inhibitors at different concentrations in 100 nL DMSO were pin transferred to cells with a CyBio robot, and cells were propagated for 16 hours. Five plates were created in replicate for the timepoints outlined below and in Fig. 1A. All plates were treated with 10 Gy of 667 keV X-rays from a $^{137}$Cs source in a Gammarcell irradiator (Atomic Energy of Canada, Ltd). Two control plates (0 hour and 24 hour) were not irradiated. The plates were returned to the incubator and fixed with 4.4% w/v paraformaldehyde in phosphate-buffered saline (PBS) at 1, 6, and 24 hours post irradiation. Plates were stored in PBS at 4°C prior to staining. Fixed plates were washed 3 times with PBS and blocked with 24 μL of GSDB (0.15% goat serum, 8.33% goat serum, 120mM sodium phosphate, 225 mM NaCl) for 30 minutes. All five plates were incubated with 1:300 dilutions in GSDB of primary mouse monoclonal anti-H2AX (Ser 139). All plates were incubated overnight at 4°C, washed, and stained with a secondary antibody mix containing 10 μg/mL Hoescht 33342, 1:300 goat anti-mouse polyclonal-Alexa Fluor 488 in GSDB. After a second overnight incubation at 4°C, the plates were washed 3 times in PBS and stored in 50 μL/well 50 μM Trilox (Sigma) in PBS at 4°C. Plates were allowed to equilibrate to room temperature for 30 minutes and imaged on a Cellomics ArrayScan VTI automated microscope with a 20x objective. The acquisition parameters were the same for each well of the chemical library. Six fields per well were imaged, with two channels/field (DAPI and fluorescein) for a total of 12 images per well. Images were segmented and analyzed with CellProfiler cell image analysis software\textsuperscript{35}. The imaging pipeline used to segment the images is available on request. Cell morphology and intensity data were acquired on a per image and per cell basis, and exported into a mySQL database. The data were visualized with SpotFire (TIBCO). shRNA image-based screens were performed as described\textsuperscript{31}. 
**Constructs, siRNA, and transfection.** Full-length constructs of Brd4 isoform B (accession number BC035266) and C (accession numberNM_014299.2) were cloned into pEGFP-C1 (Clontech) by PCR. Cells were transfected with X-tremeGENE 9 (Roche) according to the manufacturer’s instructions. Silencer Select siRNA against HDAC1 (s73), HDAC2 (s6493), HDAC3 (s16876), and control No. 1 (4390843) were purchased from Life Technologies. For siRNA experiments, cells were transfected with Lipofectamine RNAiMax (Life Technologies) according to the manufacturer’s instructions.

**SDS-PAGE and Western blotting.** Cells were treated with 10 Gy ionizing radiation with a $^{137}\text{Cs}$ source in a Gammacell irradiator (Atomic Energy of Canada). Whole cell lysates were prepared by trypsinizing cells and preparing lysates in laemmli buffer (4% SDS, 120mM Tris, pH 6.8) with protease and phosphatase inhibitors (Complete mini EDTA-free and PhosSTOP, Roche Applied Science). SDS–PAGE and western blot was performed using the methods of Laemmli and Towbin and a LI-COR® Odyssey® scanner.

**Immunofluorescence microscopy.** U2OS cells were plated on number 1 glass coverslips (VWR) and cultured in DMEM + 10% v/v FBS (complete media) at 37°C in a 5% CO$_2$ atmosphere, then exposed to 10Gy ionizing radiation from a $^{137}\text{Cs}$ source in a Gammacell irradiator (Atomic Energy of Canada), fixed in ice cold methanol, and processed for immunofluorescence using the antibodies indicated above. Images were captured on an EVOS® FL Cell Imaging System. CellProfiler was used for image feature quantification.$^{35}$

**Microwell fabrication.** PDMS molds were created as described$^{45}$ and provided as a gift from Dr. Bevin Engelward, Massachusetts Institute of Technology, Cambridge, MA, USA. Following fabrication of silicon wafers (WaferNet) with SU-8 photoresist microwells (SU-8 2025, Microchem), Polydimethylsiloxane (PDMS) was cast on a negative silicon mold and baked for 1 hour at 50°C. Following removal of the silicon mold, the PDMS stamp retained patterned microposts with approximately 50 μm depth and 40 μm width. The PDMS stamp was used in the assembly of subsequent CometChips.
**CometChip assay.** The neutral lysis CometChip assay was performed using a modified version of the technique presented by Weingeist et al. Molten 1% normal melting point agarose (UltraPure™, Life Technologies, catalogue no. 16500100) was applied to a sheet of GelBond film (Lonza, catalog number 53761), and the PDMS mold was allowed to float until the agarose set. Excess agarose was removed and the 300 μm thick gel with arrayed microwells that remained following PDMS stamp removal was then clamped between a glass plate and a bottomless 96-well titer plate (Greiner BioOne) using 4 binder clips. U2OS cells exposed to 16 hours of small molecule drugs in DMSO were trypsinized and added to the macrowells (at least 10,000 cells per macrowell). Cells were allowed to settle by gravity in DMEM + 10% v/v FBS (complete media) at 37°C in a 5% CO₂ atmosphere for 15 minutes. Excess cells were aspirated, following which the 96-well plate was removed in order to cover the arrayed cells in a layer of 1% low melting point agarose. Following the setting of the agarose, the gel with cell-loaded macrowells was realigned between the glass plate and the bottomless 96-well titer plate and subsequently reclamped to form a liquid-tight seal. The original conditioned drug media was reapplied to each well prior to irradiation. Five identical CometChip plates were prepared corresponding to the five timepoints shown in Figure 4b. One CometChip plate was not irradiated and was lysed in neutral lysis buffer as described⁴⁵ (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 1% N-Lauroylsarcosine, pH 9.5 with 0.5% Triton X-100 and 10% DMSO added 20 min before use). The remaining four CometChip plates were treated with 50 Gy ionizing radiation with a $^{137}$Cs source in a Gammacell irradiator (Atomic Energy of Canada) and similarly lysed at the indicated timepoints following aspiration of conditioned media and separation of the 96-well bottomless and glass plates from the gel. Each CometChip gel was placed in a tray of 150 mL of neutral lysis buffer overnight a 43°C. Gels were then washed two times for an hour each with electrophoresis buffer (90 mM Tris, 90 mM Boric Acid, 2 mM Na₂EDTA, pH 8.5). Electrophoresis was conducted at 4°C for 1 h at 0.6 V/cm and 6 mA.
Comet imaging and comet analysis. After electrophoresis gels were neutralized and washed three times in 0.4 M Tris, pH 7.5 for 15 minutes each. Gels were stained with SYBR Gold (Life Technologies). Images from the gel were acquired automatically by a Cellomics ArrayScan Animated Microscope with a 5X objective and analyzed automatically using a modified version of the custom software written in MATLAB (The Mathworks) presented by Weingeist et al. with additional algorithms to optimize the automated comet grid detection and filtering of artifacts.

Flow cytometry. U2OS cells were plated and treated for 16 hours with small molecule drug as indicated, exposed to 50 Gy of ionising radiation from a a $^{137}$Cs source in a Gammanacell irradiator (Atomic Energy of Canada) (Atomic Energy of Canada, Ltd.), and harvested at varying times as indicated and fixed with ice cold 100% ethanol. Cells were resuspended in PBS with 1% BSA and 0.1 mg/mL propidium iodide and processed for flow cytometry. Data were analyzed using FlowJo (www.flowjo.com) software.

GFP expression and PI exclusion assay. U2OS cells were plated and cultured in DMEM + 10% v/v FBS (complete media) at 37°C in a 5% CO₂ atmosphere and transfected with pEGFP-C1 based constructs as described above. Following a 48 hour incubation, the indicated drug was added to the media for 16 hours prior to treatment with 10 Gy of ionizing radiation from a $^{137}$Cs source in a Gammanacell irradiator (Atomic Energy of Canada). Cells were harvested at varying times as indicated and resuspended in PBS. An equal volume of 0.5 mg/mL propidium iodide solution was added to samples 5 minutes before imaging using a Nexcelom Cellometer Auto 2000 Cell Viability Counter. Images obtained were processed for GFP and PI dual fluorescence using CellProfiler and data were analysed with Spotfire.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.
ACKNOWLEDGEMENTS

This work was supported in part by the Koch Institute Support (core) Grant P30-CA14051 from the National Cancer Institute. Additional support was obtained from a Howard Hughes Medical Institute Medical Research Fellowship and the Alexandra J. Miliotis Fellowship in Pediatric Oncology (B.A.G.); a SPARC grant (M.B.Y.); and a Holman Pathway Research Resident Seed Grant, American Society for Radiation Oncology Junior Faculty Career Research Training Award, Klarman Scholar Award, Koch Institute Clinical Investigator Award, and Burroughs Wellcome Career Award for Medical Scientists (S.R.F.). We thank A.E. Carpenter, H. Le, T.R. Jones, and M. Vokes for assistance with screening and image analysis. We thank C. Whittaker, S. Hoersch, and M. Moran for computing and data analysis assistance. We thank the Koch Institute Swanson Biotechnology Center for technical support, specifically the Barbara K. Ostrom (1978) Bioinformatics and Computing Facility.
References


Fig. 1. A high throughput time-dependent multiplex image-based screen identifies small molecule inhibitors that modulate the DDR. A, Assay scheme. U2OS cells in 384 well plates were treated with small molecule compound libraries exposed to 10 Gy IR from a $^{137}$Cs source, and co-stained for simultaneous readout of DNA content and γH2AX signal at 0, 1, 6, and 24 hrs. B, Automated image analysis. Cells were imaged by automated microscopy, segmented by custom image analysis, and quantified for DNA content (Hoescht stain) and early DDR signaling (γH2AX antibodies). Six images from each well in two fluorescence channels corresponding to DNA and γH2AX were acquired with an automated microscope and quantitative data from the images was extracted with CellProfiler image analysis software. Multiple features of γH2AX foci and DNA content were measured including integrated and mean intensity, shape, and area. Representative portions of the 20X fields stained and segmented for γH2AX are shown in the third and fourth rows for the 1 hour timepoint for each condition.
Fig. 2. HDAC inhibitors targeting multiple Class I HDAC targets are potent modulators of the DDR. 

A, Multiple HDAC inhibitors enhance both formation and persistence of γH2AX foci in a concentration-dependent manner. Mean γH2AX foci intensities at 0, 1, and 6 hours for each HDAC inhibitor are shown according to the assay scheme presented in Figure 1, with increasing concentration indicated by larger colored markers.

B, Top panel: HDAC inhibitors targeting multiple Class I targets potently increase H2AX phosphorylation after DNA damage. Mean γH2AX foci intensity as a function of log10 concentration is shown for selected HDAC inhibitors. Bottom panel: Bar chart shows EC50 (concentration of HDAC inhibitor resulting in 50% of the maximum mean γH2AX foci intensity) calculated from curve fits of the data. Asterisks denote compounds with EC50 exceeding 10 μM.

C-D, siRNA knockdown of single HDAC targets does not significantly alter DDR, however combination knockdown of HDAC1, HDAC2, and HDAC3 recapitulates enhanced phosphorylation of H2AX at 1 hour. Immunoblots (C) and immunofluorescence mean foci quantification (D) of γH2AX following 48 hours of siRNA treatment targeting the indicated combination of Class I HDACs and 10 Gy ionizing radiation. Representative γH2AX foci fields are shown for each condition at 1 hour. Error bars indicate S.E.M.
Fig. 3. HDAC inhibitors reduce proliferation in an irradiation-independent fashion. A, Multiple HDAC inhibitors induce reduced proliferation in a concentration-dependent manner. Cumulative numbers of nuclei segmented and counted in the six frames for each well corresponding to a specific concentration of HDAC inhibitor are assay shown according the scheme presented in Fig. 1, with increasing concentration indicated by larger colored markers. Only counts corresponding to non-irradiated cells are shown at 0 hours, but at 24 hours counts corresponding to irradiated and non-irradiated cells are shown in red and black, respectively. B, HDAC inhibitors targeting multiple Class I HDACs induce increased G2/M arrest after irradiation after 16 hour pre-irradiation drug exposure. DNA content was assessed through CellProfiler segmentation of nuclei and measurement of integrated Hoechst staining intensity. Shown are DNA histogram plots comprising 8 wells corresponding to the indicated concentration ranges of HDAC inhibitor from unirradiated cells at 0 hours, and at 1, 6, and 24 hours following 10 Gy IR.
Fig. 4. CometChip demonstrates 16 hour pre-irradiation exposure of U2OS cells to HDAC selected inhibitors does not markedly alter the short-term kinetics of DNA DSB induction of repair in response to 50 Gy. A. Image Analysis and Comet Scoring. Arrayed microwell comets were generated from DMSO and HDAC inhibitor treated U2OS cells lysed and electrophoresed under neutral conditions. Automated imaging was performed using a modified version of the Matlab software presented in Weingeist et al. designed to rapidly detect the comet grid and focal plane and eliminate all spurious artifacts. Comet head and tail lengths were then measured in Matlab or CellProfiler. The effect of various pre-irradiation drug exposures could be compared against the delayed comet tail resolution induced by Nu7441, a potent DNA-PK inhibitor. B, 16 hour pre-irradiation exposure to selected HDAC inhibitors does not markedly alter DSB frequency or repair kinetics. U2OS cells were pre-treated at the indicated concentration of selected active HDAC inhibitors representing Pan-Class, Class I, and Class IIb specificity. Drug concentrations were chosen to target the class specific HDACs only based on relative in vitro IC50 studies (Figure 2b). CometChips were irradiated in the presence of drug with 50 Gy from a $^{137}$Cs source, and chips were lysed at the indicated timepoints. Shown is the aggregate tail length constructed from 4 separate experiments. Error bars indicate standard error of the mean tail length.
Fig. 5. Combined Inhibition of Histone Deacetylase with HDACi and Acetyl Lysine Reading with JQ1 in U2OS cells induces enhanced and prolonged H2AX phosphorylation and reduced proliferation and increased G2/M arrest with and without IR. A, γH2AX signal at 1 hour following 10 Gy IR following 16 hour pre-treatment with combination of LBH589 and JQ1. Representative immunoblot for γH2AX signal is shown on the left, with a quantification of the relative γH2AX/β-actin integrated intensity shown in the middle plot (n=3). On the right is shown a quantification of mean γH2AX foci intensity at 1, 3, and 6 hours following 10 Gy IR for corresponding drug pre-treatments. Representative portions of 40X fields are shown for each condition at 1 hour. Error bars indicate S.E.M. B, Proliferation with and without IR following JQ1 and LBH589. CellTiter-Glo® assay was performed at multiple timepoints following exposure to 16 hours of drug and 10 Gy IR. Irradiated and unirradiated cells were left in conditioned media until lysis at indicated timepoints. Luminescence for each sample was read from quadruplicate wells. Error bars indicate S.E.M. C, Multi-timepoint cell cycle analysis following 16-hour LBH589 and JQ1 combination treatment and 10 Gy IR. Ethanol fixation followed by propidium iodide staining and flow cytometry was used to examine drug and IR-induced cell cycle effects.
Fig. 6. **Overexpression of Brd4 isoform B or C abrogates LBH589-induced enhancement of DNA damage signaling through γH2AX and leads to preferential cell death following 10 Gy IR.**

A. Representative 40X images demonstrating presence of GFP signal following 48 hour incubation following transfection of either empty GFP vector or GFP-tagged Brd4 isoform B or C, with 16 hours of drug exposure prior to receiving 10 Gy. Coverslips were fixed 1 hour after irradiation. Corresponding images demonstrating γH2AX staining are shown.

B. CellProfiler based quantification of 10 representative 40X frames for each condition shown above.

C. PI exclusion assay performed on live cells gated for positive GFP and positive PI uptake. Shown is the fraction PI positive of GFP positive cells that have successfully been transfected with either an empty GFP vector or a GFP-tagged Brd4 isoform B or C expressing vector. Samples were treated for 16 hours with drug prior to receiving 10 Gy. Cells were left in drug conditioned media until analysis at the indicated timepoints.