Selective Inhibition of HDAC1 and HDAC2 as a Potential Therapeutic Option for B-ALL

Matthew C. Stubbs1, Wonil Kim1, Megan Bariteau1, Tina Davis1, Sridhar Vempati1, Janna Minehart2, Matthew Witkin2, Jun Qi3, Andrei V. Krivtsov1,2, James E. Bradner3, Andrew L. Kung1,4, and Scott A. Armstrong1,2,4

Abstract

Purpose: Histone deacetylase inhibitors (HDACi) have recently emerged as efficacious therapies that target epigenetic mechanisms in hematologic malignancies. One such hematologic malignancy, B-cell acute lymphoblastic leukemia (B-ALL), may be highly dependent on epigenetic regulation for leukemia development and maintenance, and thus sensitive to small-molecule inhibitors that target epigenetic mechanisms.

Experimental Design: A panel of B-ALL cell lines was tested for sensitivity to HDACi with varying isoform sensitivity. Isoform-specific shRNAs were used as further validation of HDACs as relevant therapeutic targets in B-ALL. Mouse xenografts of B-cell malignancy–derived cell lines and a pediatric B-ALL were used to demonstrate pharmacologic efficacy.

Results: Nonselective HDAC inhibitors were cytotoxic to a panel of B-ALL cell lines as well as to xenografted human leukemia patient samples. Assessment of isoform-specific HDACi indicated that targeting HDAC1-3 with class I HDAC-specific inhibitors was sufficient to inhibit growth of B-ALL cell lines. Furthermore, shRNA-mediated knockdown of HDAC1 or HDAC2 resulted in growth inhibition in these cells. We then assessed a compound that specifically inhibits HDAC1 and HDAC2. This compound suppressed growth and induced apoptosis in B-ALL cell lines in vitro and in vivo, whereas it was far less effective against other B-cell–derived malignancies.

Conclusions: Here, we show that HDAC inhibitors are a potential therapeutic option for B-ALL, and that a more specific inhibitor of HDAC1 and HDAC2 could be therapeutically useful for patients with B-ALL. Clin Cancer Res; 21(10); 2348–58. ©2015 AACR.

Introduction

There is growing evidence that epigenetics, or heritable non-DNA sequence-based gene-expression alterations, and the chromatin modification proteins involved, are crucial players in cancer formation and survival (1). These chromatin-modifying enzymes are of particular interest in leukemias, in which they have been linked to gene-expression alterations leading to leukemogenesis (2). As many leukemias are dependent on oncogenic fusion proteins that consist of transcriptional regulators (3, 4), epigenetic therapies could prove useful as treatment options. Therefore, the idea of targeting these chromatin-modifying enzymes with small-molecule inhibitors as a putative antileukemia option is growing.

Histone deacetylases (HDAC) are one such family of chromatin-modifying enzymes whose aberrant activity has been linked to hematologic malignancy (4). HDACs regulate gene expression by removing acetyl groups from lysine residues of numerous proteins, including histones. In humans, there are 11 classical HDAC isoforms, grouped into four classes. The classical HDACs (excluding Sirtuins) are in class I (HDACs 1–3, 8), II (IIa—HDACs 4, 5, 7, 9; IIb—6, 10), and IV (HDAC11). HDACs 1–3 are enzymatically active members of transcriptional coexpression complexes, responsible for chromosomal compaction and gene repression through removing acetyl groups from lysine residues on histones. Interestingly, HDAC6 is mainly a cytoplasmic protein, with functions independent of histone deacytylation (5).

Histone deacetylase inhibitors (HDACi) define a promising class of cancer drugs whose mechanism of action is not completely understood, though they are widely touted as an epigenetic therapy (6). Of the many possible ways HDACi influence cell survival, there are data amassing that HDACs regulate genome stability and repair (7–9). HDACi may induce apoptosis by preventing chromatin compaction, facilitating an accumulation of DNA breaks that would be irreparable. Although several other mechanisms have been studied, a definitive route to apoptosis induction is still lacking.

There are more than a dozen HDACi presently being studied as chemical probes and therapeutic agents, which may be subdivided into families based on chemical structure and biochemical spectrum of activity (10). The hydroxamic acid family is the most prevalent, with SAHA (Vorinostat; Zolinza; Merck) being the most clinically successful as of yet. SAHA is known to inhibit the class I

1Division of Hematology/Oncology, Department of Pediatric Oncology, Boston Children’s Hospital, Dana-Farber Cancer Institute, and Harvard Medical School, Boston, Massachusetts. 2Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, New York. 3Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. 4Harvard Stem Cell Institute, Boston, Massachusetts.

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Current address for M.C. Stubbs: Incyte Corporation, Wilmington, Delaware.

Corresponding Author: Matthew C. Stubbs, Incyte Corporation, 1801 Augustine Cut-off, Wilmington, DE 19803. Phone: 302-498-5716; Fax: 302-425-2759; E-mail: mstubbs@incyte.com

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Translational Relevance

Currently, two nonspecific HDAC inhibitors are approved for use in cutaneous T-cell lymphoma, with trials underway in several hematopoietic malignancies. However, the mechanism of action for these histone deacetylase inhibitors (HDACi) is not completely understood, and may not have a basis in epigenetics. We have found that BALLs are very sensitive to an HDACi that only targets the HDAC1 and HDAC2 isoforms, and that other B-cell–derived malignancies are far less responsive to this compound. This is important for several reasons. First, new HDACi that are more isotype specific should limit side effects seen with the current nonspecific HDACi. Second, inhibition of both HDACs 1 and 2 provides more insight into the mechanisms of leukemia maintenance in B-cell acute lymphoblastic leukemia (B-ALL). Third, an HDAC1 and 2 inhibitor could be a new therapeutic possibility for B-ALL that would likely act through epigenetic regulation.

HDACs as well as HDAC6 at low nmol/L concentrations (11) and is clinically approved for use in treating cutaneous T cell lymphomas (CTCL). The cyclic peptide family is most well known for the depsipeptide HDACi romidepsin (FK228, Istodax; Celgene), which is also clinically approved for CTCL. Romidepsin is a potent, class I–selective HDACi that exhibits on modest activity against HDAC6 at high concentrations may have a greater specificity for the class I enzymes, but also seems effective against HDAC6 (12, 13). The benzamide family of HDACi also exhibits class I selectivity, with inhibition of HDAC1, 2, and 3 apparent at pharmacologically achievable doses. Several benzamides are presently progressing through clinical trials (14). Only recently have selective inhibitors of HDAC6 been developed, such as tubacin, which demonstrate low potency for nuclear, class I deacetylases and exhibit toxicity when combined with proteasome inhibitors in preclinical models of multiple myeloma (15, 16). In addition, ongoing research is being performed to determine which transcriptional repressor complexes associate with various inhibitors to help establish a mechanistic understanding of biologic effects observed broadly in cancer, inflammatory, and neurodegenerative models (17).

We are interested in extending HDACi epigenetic therapy to B-acute lymphoblastic leukemia (B-ALL). Although there is evidence that nonselective HDACi may be effective against B-ALL (18–23), we also feel that a more isotype-specific HDACi may be useful against B-ALL for several reasons. First, we have shown that fusion oncoproteins such as MLL-AF4 alter the epigenetic landscape of pro- or pre-B cells to bring about transformation (24), and therefore inhibiting epigenetic regulators should be useful therapeutically. Second, as nonselective HDACi are thought to facilitate genomic damage (25, 26), a more specific inhibitor may function in a manner more consistent with epigenetic therapy. As the current clinically approved cancer targets of HDACi are T cell in origin, moving these drugs toward another lymphoid malignancy is logical.

Here, we have found that B-ALLs are uniformly sensitive to class I HDAC inhibitors both in vitro and in vivo. Treated cells rapidly accumulate DNA damage and undergo apoptosis. The doses necessary to prevent proliferation in vitro are in line with all other hematopoietic malignancies tested. HDAC6 inhibitors were effective against B-ALL cells, but only at high doses exceeding the dose range in which selective inhibition of HDAC6 is observed. The antiproliferative activity of class I–specific benzamide inhibitors prompted the study of a chemical probe that only targets HDAC1 and HDAC2. Interestingly, selective HDAC1 and HDAC2 inhibition demonstrated potent inhibition of B-ALL cell growth in vitro and in vivo, whereas other hematopoietic malignancies were much less sensitive.

Materials and Methods

Cell lines and vectors

B-ALL, B-lymphoma, and multiple myeloma cell lines were maintained in RPMI (Invitrogen) with 10% FCS (Invitrogen) and supplemented with 100 U/ml penicillin–streptomycin (Invitrogen) and 2 mmol/L L-glutamine (Invitrogen). Cells (293) were maintained in DMEM (Invitrogen) supplemented with 10% FCS (Invitrogen) and 100 U/ml penicillin–streptomycin (Invitrogen). RS4;11, REH, RPMI-8226 cells were from the ATCC. The 697 and OPM-2 cells were from DSMZ. SEMK2 cells were a generous gift from the laboratory of Dr. Stanley Korsmeyer (DFCI, Boston, MA). MM1.S cells were a generous gift from Dr. Constantine Mitsiades (Harvard Medical School, Boston, MA). B-lymphoma lines were generously given by Dr. Guo Wei (Broad Institute, Cambridge, MA). Each cell line has been tested and authenticated by short tandem repeat at the Molecular Diagnostics Laboratories at Dana Farber Cancer Institute (Boston, MA). All lentiviral vectors contained the pLKO.1 backbone and were obtained from the RNAi Consortium (Broad Institute, MIT). RNAi Consortium clones were HDAC1: TRCN0000004817, TRCN0000004818; HDAC2: TRCN0000004819, TRCN0000004821; HDAC3: TRCN0000004824, TRCN0000004828. Lentiviruses were produced as described previously (27). The viral titer was determined by infection of BAF3 cells. All viruses used had a viral titer of approximately 5 × 10^5 infectious units per ml.

Cell proliferation, survival, and annexin V analyses

To assay for sensitivity to the HDAC inhibitors LAQ824, MS-275, WT-161, and Merck60, the colorimetric Cell Proliferation Ki-67 assay kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazo- mid (MTT; Roche) was used. Cells were plated at a density of 10^5 cells per 100 ml media (RPMI, Invitrogen; supplemented with 10% FCS and 100 U/ml penicillin–streptomycin) into 96-well microtiter plates (Corning) The MIT assay was then performed as per the manufacturer’s protocol. For high-throughput HDACi screening, 1,000 cells were plated in 50 ml RPMI/10% FCS in 384-well plates (Nunc). All drugs in the high-throughput screen were made in house and added to cell plates using a Janus liquid handler (PerkinElmer). To analyze cell fitness, CellTiter Glo was used (Promega). EC50 values were determined using Prism GraphPad software. ZVAD-fmk was purchased from InvivoGen.

For cell viability assays after infection and puromycin selection (48 hours. at 2 μg/ml), cells were counted with a hemacytometer (VWR) using light microscopy and Trypan Blue (Invitrogen) exclusion. For apoptosis analyses, cells were harvested, washed with PBS (Invitrogen), and resuspended in annexin V buffer [140 mmol/L NaCl (Fisher), 10 mmol/L HEPES (Invitrogen), 2.5 mmol/L CaCl2 (Fisher)] with annexin V-Cy3 reagent (Biovision) and analyzed on a FACSCalibur (Becton Dickinson).
Western blots
Cells were harvested 72 hours after infection and equivalent cell numbers were lysed in RIPA buffer (150 mmol/L NaCl, 1% IPEGal, 0.5% deoxycholate (Sigma), 0.1% SDS (Invitrogen), 50 mmol/L Tris pH 8.0 (Sigma)] with Complete protease inhibitor cocktail (Roche). Histones were extracted by lysing cells with Triton X-100 in PBS (Invitrogen). Nuclei were spun at 6,500 rpm for 5 minutes, and pellets containing histones were incubated with 0.2N HCl overnight. Extracts were denatured and run on a 10% Acrylamide Bis-Tris gel (Invitrogen), 50 mmol/L Tris pH 8.0 (Sigma)] with Complete protease inhibitor cocktail (Roche). Histones were extracted by lysing cells with Triton X-100 in PBS (Invitrogen). Nuclei were spun at 6,500 rpm for 5 minutes, and pellets containing histones were incubated with 0.2N HCl overnight. Extracts were denatured and run on a 10% Acrylamide Bis-Tris gel (Invitrogen) and transferred to nitrocellulose (Whatman) for Western analyses.

Western blots were performed using mouse α–pan-actin (Chemicon), p21 (Santa Cruz Biotechnology) γH2A.X and Histone H3 (Abcam), HDAC1, HDAC2, HDAC3 (Cell Signaling Technology), AChH3K9 and AChH3K56 (Upstate Millipore), Ac-tubulin (Sigma), HRP conjugated anti-mouse and anti-rabbit antibodies (Amerham), and developed with the Western Lightning Enhanced Chemiluminescence Kit (PerkinElmer).

In vivo drug assays
Bioluminescence assays were carried out as described previously (28). For testing LBH589, SCID Beige mice were injected with 2 × 107 MLL-AF4–bearing patient sample cells (B-ALL) and were treated at 10 mg/kg i.p. daily or vehicle control for 14 days. For testing Merck60, the first cohort of NOG mice was injected with 7,000 MLL-AF4–bearing patient sample cells (B-ALL), and treated with 45 mg/kg i.p. or vehicle control for 14 days over an 18 day span. Subsequent cohorts of mice were injected with 2 to 3.5 × 107 cells from B-ALL patients without MLL rearrangements and random cytogenetics. These mice were treated with 30 mg/kg i.p. Merck60 or vehicle control for 5 days, followed by 2 days off drug, followed by 5 more days on drug. For both LBH589 and Merck60, treatment began when peripheral blood hCD45 levels exceeded 1%. Peripheral blood was treated with red blood cell lysis buffer (BD Biosciences), and white blood cells were incubated with anti-human CD45-FITC antibody (BioLegend), and then analyzed on a FACScalibur (BD Biosciences).

Results
B-ALL cells are sensitive to pan-HDAC inhibition
In an effort to determine the utility of HDAC inhibitors as a therapeutic option for B-ALL, we incubated the RS4;11, REH, SEMK2, and 697 cell lines in serial dilutions of the potent, nonselective HDAC inhibitor LAQ824 (Novartis). After 48 hours, we observed growth suppression by MTT assay in each cell line in nonselective HDAC inhibitor LAQ824 (Novartis). After 48 hours, SEMK2, and 697 cell lines in serial dilutions of the potent, therapeutic option for B-ALL, we incubated the RS4;11, REH, B-ALL cells are sensitive to pan-HDAC inhibition, but not to HDAC6 inhibition
To further characterize the response of the B-ALL cell lines to HDAC inhibition, we performed a screen to determine whether or not an HDAC inhibitor that possesses class specificity would effectively inhibit growth in our B-ALL lines. We assessed the antiproliferative activity of a panel of HDAC inhibitors that possess varying degrees of isoform specificity (Supplementary Table S2) against B-ALL cell lines. We found that all pan-HDAC inhibitors were very effective against the B-ALL lines. Interestingly, benzamide derivatives, including MS275, MGCD0103, and CI-994 possessed activity, indicating that inhibiting the class 1 isoforms is sufficient for growth repression in B-ALL cells. The HDAC6 inhibitor tubacin, however, lacked antiproliferative activity within its selective dose range, only inhibiting cell growth at relatively high concentrations, where evidence of class I deacetylation inhibition was evident by immunoblot analysis for acetylated histones.

To further verify the data that specifically targeting HDAC6 may not be sufficient to prevent B-ALL cell growth, we also treated our cell lines with WT161 (30), a structurally distinct HDAC6 inhibitor. Using serial dilutions, we found that WT161 is only effective against our cell lines at doses above the range that would specifically target HDAC6 (Fig. 2A). Immunoblot analyses show that, although tubulin is acetylated at low WT161 concentrations, Histone H3 acetylation is markedly increased at 1 μmol/L, a concentration still below the amount necessary to inhibit growth at 48 hours (Fig. 2B). These data indicate that HDAC6 inhibition alone is likely not sufficient for growth inhibition in B-ALL cell lines.

To determine the cellular effects of inhibiting class 1 HDACs (here only referring to HDAC1, 2, and 3) on B-ALL cells, we performed MTT assays and immunoblot analyses after treatment with MS275 (Fig. 2C and Supplementary Fig. S2). As with LAQ824, the cells treated with MS275 showed increased levels of acetylated H3K9, p21, and γH2A.X. However, levels of acetylated tubulin did not change, even with doses of drug 10-fold over
the EC50 for these cell lines, indicating that HDAC6 is not nonspecifically targeted and does not factor into the sensitivity of these cells to MS275 consistent with its reported biochemical specificity (10). We extended these studies more broadly to other B-lineage malignancy cell lines (Fig. 2D). Although the EC50 values are not greatly disparate between leukemia, lymphoma, and myeloma (Supplementary Table S1), it is evident that the B-ALL cells are more sensitive at higher dosages than the other cell types. Possibly, the lack of HDAC6 inhibition decreases the sensitivities of the lymphoma and myeloma cell lines to MS275. These data demonstrate that selective inhibition of class I–selective HDACi effectively inhibits proliferation in B-ALL cells.

Suppression of specific class I HDACs induce apoptosis in B-ALL cells

To better understand the importance of individual class I HDAC isoforms to B-ALL survival, we transduced the B-ALL cell lines with lentiviruses encoding shRNAs specific for HDAC1, HDAC2, or HDAC3. Immunoblot analyses indicated that suppression of HDAC1 or HDAC2 levels result in increased p21, but little or no increase in either DNA damage (as assessed by γH2A.X) or acetyl H3K9 accumulation, indicating that these two isoforms may have some functional redundancy or compensatory abilities (Fig. 3A). This is similar to what has previously been shown in other cell lines (31–33), where upregulation of HDAC1 occurs when HDAC2 levels drop, and vice versa. Upon reduction of HDAC3 protein levels, p21 was induced, similar to what we saw with suppression of either HDAC1 or HDAC2 suppression. Acetylated-H3K9 and γH2A.X were also observed with HDAC3 suppression (Fig. 3A) with HDAC1 or 2 suppression yielding a far weaker possible induction of γH2A.X in SEMK2 cells. Interestingly, knockdown of HDAC3 does not lead to higher levels of HDAC1 or HDAC2 (Supplementary Fig. S3A). We next monitored cell growth and apoptosis in
transduced B-ALL cell lines. Figure 3B shows the change in proliferation for RS4;11 cells transduced with shRNAs targeting HDAC1, 2, or 3 as compared with control (GFP shRNA). Supplementary Fig. S3B shows similar data for the REH, SEMK2, and 697 cells, with statistics shown in Supplementary Table S3. Suppression of HDAC1 or HDAC2 leads to a decrease in growth of B-ALL cells, whereas HDAC3 suppression resulted in complete proliferative arrest. Each of the B-ALL cell lines began to undergo increased apoptosis at some point later than 2 days of HDAC1 or HDAC2 suppression, and the amount of apoptosis increased further after 6 days (Fig. 3C). HDAC3 suppression resulted in rapid induction of apoptosis, with essentially no viable cells remaining after 6 days. These data indicate that HDAC1 or HDAC2 suppression leads to an antiproliferative phenotype, with HDAC1 knockdown likely causing a G1 arrest and HDAC2 knockdown yielding multiple possible growth delays (Supplementary Fig. S3C), as well as an increase in apoptosis. HDAC3 suppression mimics treatment with a nonselective HDAC inhibitor, causing nearly complete loss of viable cells within a few days.

B-ALL lines are sensitive to a selective HDAC1/HDAC2 inhibitor

Having demonstrated that nonselective HDAC inhibition might be a useful therapeutic option for B-ALL, we next sought to determine whether more specific inhibitors might demonstrate comparable antileukemic activity. The robust antiproliferative activity of HDAC1 or HDAC2 suppression establishes the plausibility that agents selective for these two isoforms might retain antileukemic activity while obviating toxicity associated with HDAC3 inhibition. Currently, there are no available HDAC1-specific or HDAC2-specific inhibitors, so we treated our cell lines with a compound capable of inhibiting both HDAC1 and HDAC2 with more than 200-fold more specificity than HDAC3 (HDAC1 IC50 7 nmol/L; HDAC2 IC50 49 nmol/L; HDAC3 IC50 10 μmol/L).

We synthesized and characterized a chemical probe first reported in a medicinal chemistry study by Merck Research Laboratories as Compound 60, which for these studies we will describe as Merck60 (Fig. 4A; ref. 34), also reported as "Compound 60" (35). We performed an MTT assay on our B-ALL cells and found that treatment for only 48 hours had a marginal effect on our B-ALL line panel. However, consistent with our knockdown data,

Figure 2. B-ALL cell lines respond to drugs targeting class I HDACs. A, MTT assays were carried out after treating cell lines for 48 hours with the HDAC6-specific inhibitor WT161. B, Western blots show acetylated tubulin as a marker of HDAC6 inhibition and acetyl-H3K9 at higher WT161 concentrations. C, MTT assays were carried out after treating cell lines for 48 hours with a class I HDAC inhibitor MS-275 (Syndax). D, MTT assays with MS-275 were performed and are displayed as a heatmap as in Fig. 1C.
where growth impairment and apoptosis were more prevalent with HDAC1 or HDAC2 knockdown after 4 to 6 days, treatment with Merck60 for 96 hours led to growth inhibition that was evident at doses in the low to mid nmol/L range (Fig. 4B). Immunoblot analysis showed that acetylated H3K56 (AcH3K56), a mark known to be regulated by HDACs 1 and 2 (8), was accumulating within the cells (Fig. 4C), and p21 was also induced as seen with nonselective HDACi. AcH3K56 occurred within 18 hours. Interestingly, γH2A.X was also induced by Merck60. However, this mark did not appear until after 48 hours (Supplementary Fig. S4A), corresponding with when growth suppression and apoptosis began (Supplementary Fig. S4B and S4C). Although it is of note that we can detect the AcH3K56 mark with 100 nmol/L Merck60 at 18 hours, and yet apoptosis is not apparent until after 48 hours with 500 nmol/L Merck60, this disconnection is consistent with what has been seen with inhibition of other epigenetic regulators as well (e.g., DOT1L inhibition; ref. 36). Because of the later induction of γH2A.X when compared with nonspecific HDACi, we sought to verify whether the γH2A.X mark was due to Merck60-driven DNA damage or due to apoptosis-related DNA degradation. RS4;11 cells were grown in Merck60 with or without the caspase/apoptosis inhibitor ZVAD-fmk. Cells in ZVAD were more viable and showed very little induction of γH2A.X (Supplementary Fig. S5A). In contrast, cells treated with LAQ824 showed an increase in γH2A.X with or without ZVAD, even when cells are nearly completely viable (Supplementary Fig. S5B). Therefore, Merck60 appears to be inducing apoptosis in a different manner than the nonspecific HDACi.

To determine whether HDAC1/HDAC2 inhibition would also inhibit growth of other B-cell–derived malignancies, we performed MTT assays on our B-lymphoma and multiple myeloma cell line panel as in Figs. 1C and 2E. We found that, although our B-ALL lines are sensitive to Merck60 at well under 1 μmol/L (Supplementary Table S1), our lymphoma and myeloma lines are far less sensitive to this drug (Fig. 5A).
where all lines tested were similarly sensitive. Western analyses indicate that lines that are not sensitive to Merck60 (MM1S-LN, OPM2) still induce AcH3K56 and p21 when treated with the drug (Fig. 5B). However, γH2AX levels do not increase upon Merck60 treatment, indicating a differential response between sensitive and insensitive cells (Fig. 5B). When the insensitive lines were treated with the DNA-damaging agent doxorubicin or LAQ824, they are able accumulate γH2AX (Supplementary Fig. S6). These lines are able to show elevated γH2AX levels, but not when treated with Merck60.

Next, we wanted to investigate whether this sensitivity bias occurs in vivo. Immunodeficient (NOG) mice were injected with either SEMK2 or MM1S-LN cells that stably express firefly luciferase (28). Mice with established cancer burden were treated with Merck60 (45 mg/kg daily) or vehicle control. As shown in Fig. 5C, a decrease in bioluminescence was observed in SEMK2-bearing mice injected with Merck60. Mice bearing MM1S-LN tumors demonstrated no response to daily therapy with Merck60 compared with vehicle controls. Thus, Merck60 was not effective against the multiple myeloma cells, whereas the B-ALL cells did respond to HDAC1/HDAC2 inhibition in vivo.

An HDAC1/HDAC2 inhibitor reduces leukemic burden in mouse models of human primary B-ALL

We also pursued using HDAC1/HDAC2–specific inhibition to inhibit growth of B-ALL patient samples. NOG mice were injected with cells from a pediatric patient with an MLL-rearranged leukemia. Engraftment was monitored by human CD45 levels in the peripheral blood as in Fig. 1. When levels reached 1%, mice were treated either with Merck60 or vehicle control. After 14 days of treatment, there was a significant drop in the percentage of and number of human leukemia cells found in the bone marrow (Fig. 6A), indicating that HDAC1/HDAC2–specific inhibition can inhibit growth of patient-derived primary leukemia cells. Marrow was harvested 2 hours after the final dose of Merck60 was administered for analyses. Immunoblots revealed that marrow from mice treated with Merck60 exhibited a pharmacodynamic elevation of AcH3K56 (Fig. 6B), when compared with marrow from mice receiving vehicle control. Merck60 was, therefore, inhibiting HDAC1 and HDAC2 in vivo, leading to a diminished leukemic burden within those mice. To verify further the ability of Merck60 to inhibit growth of primary B-ALL cells without MLL rearrangements in vivo, NSG mice were injected with cells from

Figure 4.
B-ALL cells are sensitive to an inhibitor specific for HDAC1 and HDAC2. A, structure of Merck60. B, MTT assays were carried out after treating cell lines for 48 (left) and 96 hours (right) with the HDAC1/HDAC2–selective HDAC inhibitor, Merck60 (Merck). C, Western blots show increased levels of acetyl-H3K56 (a mark specifically controlled by HDAC1/HDAC2), γH2AX (DNA damage), and p21 in response to 72 hours of Merck60 treatment.
three separate adult B-ALL patient samples, and treated with Merck60 or vehicle control. Figure 6C shows spleen weights as surrogates for leukemic response to Merck60. In two of the three samples, mice treated with Merck60 showed a statistically significant ($P < 0.05$) decrease in spleen weight when compared with vehicle controls, indicating that Merck60 is also able to inhibit the growth of primary B-ALL cells without MLL rearrangements in vivo.

**Discussion**

Our goal has been to assess the potential efficacy of therapeutic approaches that target epigenetic mechanisms against B-ALL cells. We chose to assess HDACi for several reasons. First, B-ALL is frequently driven by transcriptionally active fusion oncoproteins, thus altering the transcriptional landscape might be a useful approach in this disease. Second, knowing that nonspecific HDACi leads to accumulation of dsDNA lesions, and that B-ALL cells are extremely sensitive to DNA damage, we felt this might also increase the potential efficacy of these inhibitors against B-ALL cells. Finally, Vorinostat (Merck) and Istodax (Romidepsin—Celgene) are already approved for a lymphoid malignancy, CTCL.

Here, we show that B-ALL cells are sensitive to class I HDAC inhibition, including inhibition of only of HDACs 1 and 2. This is of particular interest in light of the fact that genetic inactivation of HDAC1, 2, or 3 does not hamper growth of H-Ras/T antigen–transformed fibroblasts (37), yet we see growth inhibition and apoptosis in B-ALL cells upon suppression of HDAC1 or 2, and large-scale apoptosis from HDAC3 knockdown. This shows that HDACs may play a much more critical role in B-ALL cells survival than in other cell types. Our experiments provide further support for this argument, as B-ALL cells are sensitive to HDAC1 and HDAC2 inhibition whereas other cell types are either far less sensitive or insensitive. Also, as compared with other B-lineage malignancies, B-ALL cells are more sensitive to class 1–specific inhibitors. It is important to note that HDAC inhibitors that avoid inhibition of HDAC3 could be clinically beneficial. And as demonstrated in our in vivo studies, a lack of HDAC3 inhibition does not appear to cause a significant loss of efficacy in B-ALL patient sample xenograft models (Fig. 1D vs. Fig. 6A and C). Outside of the ability of HDAC3 to regulate genomic stability (7), mouse knockout studies have also shown that HDAC3 is necessary for maintenance of several metabolic functions in both cardiac and hepatic tissues (38, 39). One particular problem with current clinically available HDAC inhibitors is that they also bind and inhibit the hERG ion...
channel, which has been linked with QT prolongation (40). This off-target binding can lead to cardiac arrhythmia and is potentially fatal. Newer inhibitors are now being screened not only for their abilities to block HDAC function, but also for their abilities to block hERG activity as well (40, 41). An HDAC inhibitor that structurally cannot inhibit HDAC3 and cannot bind hERG, then, would be of considerable therapeutic interest.

The mechanism behind HDAC1/HDAC2 inhibition and the reasons why B-ALLs are more susceptible to HDAC1 and 2 inhibitors than other malignancies are as of yet unknown. We have seen that the kinetics of Merck60 action are much slower than that of the nonspecific HDACi, possibly due to the differing mechanisms of action between the two drugs. Here, the cell of origin of these cancer types might play an important role. HDAC1 and HDAC2 have been shown to control early B-cell development (33). A conditional double knockout in B-lineage cells yields a differentiation block in the pre-B stage with G1 arrest. B-ALLs are derived from pre-B cells so there may be a necessity for HDAC1 and 2 for cell proliferation. The same double knockout in more mature B-cell–derived malignancies such as B-lymphoma and multiple myeloma have less requirement for functional HDAC1 and 2, possess mutations that are hyperproliferative and antiapoptotic, and are therefore less sensitive to the inhibitor.

Our data indicate that inhibition of HDACs would potentially be a useful therapeutic addition to therapies for patients with B-ALL, and that an inhibitor that specifically targets HDAC1 and HDAC2 might be particularly beneficial. As such, potent and selective inhibitors of HDAC1 and HDAC2 are presently being developed in our laboratories for therapeutic application. It remains to be determined whether HDAC inhibitors, broad spectrum or specific, will synergize well with existing chemotherapeutic drugs for B-ALL. On the basis of the accumulation of DNA damage, as well as much prior work showing synergy between HDACi and DNA damage–inducing agents (11), it seems highly likely that HDAC1 and 2 inhibition would fit well into existing regimens. HDAC1 and 2 inhibition leads to accumulation of AcH3K56, a mark shown to be specifically regulated by HDAC1 and 2 (8). The AcH3K56 mark has been shown to have multiple different roles in response to DNA double-stranded breaks where the mark is either increased around sites of DNA damage (42), or decreased in response to DNA damage (43). HDAC inhibitors can also cause a decrease in levels of the repair proteins RAD50 and MRE11 (26) as well as homologous recombination genes (44). HDAC inhibitors have recently been linked to the DNA damage response as well as autophagy in budding yeast (9), and in-house
preliminary studies suggest that a functional DNA damage response pathway may at least partially be necessary for the growth inhibition and apoptosis caused by HDAC1- and 2-specific inhibition. In addition, HDAC1 and 2 have been shown to be necessary for dsDNA break repair by regulating the timing and placement of nonhomologous end joining proteins. Therefore, under the influence of an HDAC inhibitor, this mark will improperly accumulate throughout the genome, making it plausible that in B-ALL, an HDAC inhibitor may both help bring about DNA damage, and then somehow impair the cell’s ability to repair it. The possibility also exists for a more specific epigenetic role for HDAC1/HDAC2 inhibitors giving rise to an as of yet undiscovered mechanism for leading to cell death. The use of more specific HDAC inhibitors will hopefully lead to new strategies for patients with B-ALL.

Disclosure of Potential Conflicts of Interest

M.C. Stubbs is an employee of Incyte Corporation. S.A. Armstrong is a consultant/advisory board member for Epizyme Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M.C. Stubbs, W. Kim, A.L. Kung, S.A. Armstrong

Development of methodology: M.C. Stubbs, W. Kim, S. Vempati, A.V. Krivtsov, J.E. Bradner

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.C. Stubbs, W. Kim, M. Bariteau, T. Davis, J. Minehart, M. Witkin, J.E. Bradner

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.C. Stubbs, W. Kim, M. Witkin, J.E. Bradner, A.L. Kung, S.A. Armstrong

Writing, review, and/or revision of the manuscript: M.C. Stubbs, M. Bariteau, A.L. Kung, S.A. Armstrong

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Bariteau, J. Minehart, J. Qi, A.V. Krivtsov

Study supervision: M.C. Stubbs, M. Bariteau, S.A. Armstrong

Other (contributed to the animal studies; daily monitoring of animal health status, injections, harvesting of tissues/collating relevant endpoint data, etc.): M. Bariteau

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# Selective Inhibition of HDAC1 and HDAC2 as a Potential Therapeutic Option for B-ALL

Matthew C. Stubbs, Wonil Kim, Megan Bariteau, et al.


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