Research Article

Exposure to a Histone Deacetylase Inhibitor Has Detrimental Effects on Human Lymphocyte Viability and Function

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Abstract

Histone deacetylase inhibitors (HDACi) have been reported to increase tumor antigen expression, and have been successfully tested as adjuvants for melanoma immunotherapy in mouse models. In this work, we tested the effects of a pan-HDACi on human lymphocytes and melanoma cell lines. Effects of the pan-HDACi panobinostat (LBH589) on cell viability, cell cycle, apoptosis, and DNA damage were determined in peripheral blood mononuclear cells (PBMC) from 2 healthy donors, 13 patients with metastatic melanoma, 2 bone marrow samples from patients with different malignancies, and 12 human melanoma cell lines. Intracellular signaling in lymphocytes, with or without cytokine stimulation, was analyzed by phospho-flow cytometry in one of each type. The IC50 in PBMCs was <20 nmol/L compared with >600 nmol/L in melanoma cell lines; >40% apoptotic cell death in PBMCs versus <10% in melanoma cell lines was seen at the same concentration. Phospho-histone variant H2AX (pH2AX) increased 2-fold in healthy donor PBMCs at 1 nmol/L, whereas the same effect in the melanoma cell line M229 required 10 nmol/L. pH2AX was inhibited slightly in the PBMCs of 3 patients with metastatic melanoma at 1 nmol/L and in the melanoma cell line M370 at 10 nmol/L. Panobinostat inhibited phospho-STAT1/3/5/6, -p38, -ERK, -p53, -cyclin D3, and -histone H3 in flow cytometry–gated healthy donor B and T cells, whereas it induced up to 6-fold activation in patients with metastatic melanoma and bone marrow samples. In human lymphocytes, panobinostat alters key lymphocyte activation signaling pathways and is cytotoxic at concentrations lower than those required for melanoma antitumor activity, resulting in an adverse therapeutic window. Cancer Immunol Res 2(5); 459–68. ©2014 AACR.

Introduction

Histone deacetylases (HDAC) are enzymes that play key regulatory roles in gene expression and cellular differentiation. HDAC functions include the removal of acetyl groups from transcription factors (e.g., p53; ref. 1) and signal transduction proteins (e.g., STAT3; 1), and the acetylation of the molecular chaperone Hsp90 (2) and α-tubulin (2, 3). Given the crucial role of HDAC as cellular epigenetic modifiers, inhibitors of HDAC proteins (HDACi) have been evaluated for clinical application as anticancer therapies. There are 18 HDAC enzymes in four subclasses (4): Classes I, II, and IV are zinc-dependent proteins, and class III are NAD+–dependent proteins.

HDACis inhibit the zinc-finger metalloproteinase HDAC by binding the zinc-finger domain in the catalytic site. The pan-HDACi vorinostat (Zolinza; Merck) and the class I HDACi romidepsin (Istodax; Celgene) were approved by the U.S. Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma in 2006 and 2009, respectively (5, 6). Several other HDACis, including the pan-HDACi panobinostat (LBH589), are being investigated in clinical trials. Among the hydroxamic acid HDACis, panobinostat has the most potent inhibitory activity (7). Panobinostat is a second-generation HDACi; it has potent antitumor activity in vitro in T-cell lymphoma, Hodgkin lymphoma, and chronic myelogenous leukemia. In phase I and II clinical trials, panobinostat has demonstrated the most efficacy in refractory cutaneous T-cell lymphoma, although studies are still ongoing in multiple myeloma, non–Hodgkin lymphoma, and gastric cancer (www.clinicaltrials.gov). As is the case with other HDACis, the most common side effects include nausea, diarrhea, fatigue, thrombocytopenia, and other cytopenias, all of which typically are managed easily (8). Panobinostat inhibits the growth of pancreatic, breast, prostate, and colon cancer cell lines; however, clinical data for panobinostat alone in solid tumors have been less robust than those for hematologic malignancies (6).

Several ongoing clinical trials are evaluating the safety and efficacy of adoptive cell therapy (ACT) for melanoma. In some of these studies, genetically engineered T cells expressing

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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tumor-specific T-cell receptors (TCR) are infused into patients after conditioning chemotherapy and lymphodepletion followed by the administration of interleukin (IL)-2 to stimulate T-cell proliferation in vivo. ACT can result in objective and durable responses in humans; however, the response rates tend not to be durable (9, 10). Therefore, agents that can be used safely as adjuncts to ACT are of interest (11).

To this end, several published reports have demonstrated that HDACi sensitized cancer cells to immunotherapy. HDACis upregulate genes important in apoptosis (12–16) and increase the expression of proteins involved in antigen processing and presentation (17–19), of tumor antigens, and of ligands that can be recognized by natural killer cells (19–22). In mouse models of solid tumors, the antitumor effect of HDACi can be augmented by manipulating the immune system. Combining vorinostat or panobinostat with antibodies to CD40, which stimulates antigen-presenting cells, or to CD137, which costimulates cytotoxic T lymphocytes, led to enhanced tumor regression in mouse models of breast, renal, and colon carcinomas (23). Similar findings have been shown in mouse models of melanoma: Treatment of B16 murine melanoma with both the pan-HDACi LAQ824 (dacinostat) and ACT of pmel-1 T cells specific for the gp100 melanoma antigen resulted in increased antitumor activity (24). The ability of LAQ824 to augment the effects of immune therapy was also demonstrated in a prophylactic prime-boost vaccination mouse model of melanoma using the melanoma antigen tyrosinase-related protein-2 (25). The class I HDACi depsipeptide (romidepsin) increased the antitumor activity in the phylactic prime-boost vaccination mouse model of melanoma: Treatment of B16 murine melanoma with both the pan-HDACi LAQ824 (dacinostat) and ACT of pmel-1 T cells specific for the gp100 melanoma antigen resulted in increased antitumor activity (24). Although these mouse data are promising, HDACis have been shown to enhance the effects of regulatory T cells (Treg) and inhibit cell-cycle progression of lymphocytes (27–31). Furthermore, Song and colleagues (32) have demonstrated that treatment of myeloid dendritic cells (DC) from healthy donors with panobinostat impaired DC functions, decreased the expression of surface molecules associated with DC maturation, and reduced antigen presentation and T-cell costimulation. Therefore, it is unclear whether HDACi can be safe and effective as adjuncts to immunotherapy. To further evaluate the potential of HDACi in combination with immunotherapy, we studied the effects of panobinostat in human melanoma cell lines and human lymphocytes on growth inhibition, cell-cycle progression, and effects on mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling by phospho-flow analysis. In this study, we focus on the effects of panobinostat given its in vitro potency so that the effects in human cells could be directly compared with the results published by Song and colleagues (32).

Materials and Methods
Reagents and cell lines
Panobinostat (LBH589) was obtained from Novartis and reconstituted in dimethyl sulfoxide (DMSO) to a final stock concentration of 10 mmol/L. Peripheral blood mononuclear cells (PBMC) were obtained by leukapheresis from two healthy donors under University of California, Los Angeles (UCLA) Institutional Review Board (IRB) #04-07-063 and 12 patients with metastatic melanoma not on active therapy under UCLA IRB 10-000870, bone marrow from a patient with multiple myeloma (BM-1) or a patient with breast cancer (BM-2) obtained under UCLA IRB 08-08-062, or PBMCs from a healthy donor were transduced twice in retroenectin-coated plates with a retrovirus expressing a high-affinity Melan-A/MART-1 TCR (33). PBMCs were cultured in RPMI-1640 with l-glutamine (Mediatech Inc.) containing 5% human AB serum (Omega Ltd.).

Cell viability assays
Six PBMC and two melanoma cell lines were cultured in triplicate with 0, 0.1 nmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, 10 μmol/L, and 100 μmol/L panobinostat, or DMSO as vehicle control, for 72 hours. Viability was analyzed using an ATP-based luminescent cell proliferation assay kit following the manufacturer’s instructions (CellTiter-Glo Luminescent Cell Viability Assay; Promega). Data were analyzed using Microsoft Excel and the IC<sub>50</sub> value was then calculated using GraphPad Prism.

Cell-cycle analysis and assessment of apoptosis by flow cytometry
Six PBMC and two melanoma cell lines were incubated for 72 hours with 0.1 nmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, 10 μmol/L, and 100 μmol/L panobinostat, or DMSO as vehicle control, for 72 hours. Viability was analyzed using an ATP-based luminescent cell proliferation assay kit following the manufacturer’s instructions (CellTiter-Glo Luminescent Cell Viability Assay; Promega). Data were analyzed using FlowJo (Tree Star Inc.).

Assessment of DNA damage by flow cytometry
Phosphorylation of histone variant H2AX (pH2AX) as a marker of DNA damage was determined using the FlowCellec Multi-Color DNA Damage Response Kit (Millipore). Six PBMC and two melanoma cell lines were treated with 0.1 nmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, and 10 μmol/L of panobinostat or DMSO as vehicle control, fixed, permeabilized, washed using the buffers provided, and then stained with pHistone-H2AX-PerCP (Millipore) as per manufacturer’s
specifications. Events were collected by flow cytometry and data analysis was carried out using Cytobank (www.cytobank.org).

**Phospho-proteomic platform at a single-cell level**

Phosphorylated intracellular signaling molecules at single-cell level were detected as described (33). Briefly, two PBMC samples and a bone marrow sample from a patient with multiple myeloma (BM-1) were cultured in 0.1 nmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L, and 1 μmol/L panobinostat for 24 hours and then stimulated for 15 minutes with 10,000 IU/mL IFN-α or 400 IU/mL of IL-2. After stimulation, cells were surface stained, then fixed with formaldehyde to a final concentration of 1.6%, and permeabilized using methanol (90%). Fluorescent barcoding of PBMC was carried out with a combination of 0, 3, or 8 mg/mL of Ax350-NHS and 0, 3, or 8 mg/mL of Ax750-NHS to allow the simultaneous analysis of six different populations in one sample. After two washes, barcoded samples were incubated for 30 minutes with cocktails of antibodies to simultaneously stain intracellular proteins. In total, five different antibody cocktails were used per condition (Supplementary Table S1). All antibodies were used at saturating concentrations. Flow cytometric compensation was carried out using anti-mouse IgG/Negative Control FBS compensation particles (BD Biosciences). A total of 100,000 to 300,000 lymphocyte events were collected by flow cytometry and analyzed using Cytobank (www.cytobank.org).

**Statistical analysis**

Because of the small size of the population, statistical analyses are essentially descriptive. Each experiment was performed at a minimum in triplicate independent studies, with each sample in duplicate or triplicate for each experiment. A descriptive data analysis was performed with Microsoft Excel and GraphPad Prism (v4) and paired t test was applied. All flow cytometry experiments were carried out using an LSRII (BD Biosciences), using a biexponential axis.

**Results**

**Effects of panobinostat on melanoma cell lines and human PBMCs**

To determine the susceptibility of human cells to panobinostat, 12 melanoma cell lines, resting PBMCs, and activated PBMCs from multiple donor sources were treated with increasing concentrations of panobinostat for 72 hours and the IC50 was determined. In addition to PBMCs from melanoma subjects, specimens from healthy subjects or subjects with non-melanoma solid tumor (breast cancer) and hematologic malignancy (multiple myeloma) were chosen to determine whether the source of lymphocytes would affect the results. As shown in Fig. 1, the human PBMC and melanoma cell lines tested fell into three categories with respect to sensitivity to panobinostat: IC50 of 20 nmol/L or less (defined as sensitive), IC50 of 21 to 50 nmol/L (intermediately sensitive), and IC50 of greater than 50 nmol/L (resistant), consistent with previous published reports (7). Thirteen of 15 resting PBMC specimens (from both healthy donors and patients with metastatic melanoma) were sensitive to panobinostat; MD8 was intermediately sensitive with an IC50 of 26 nmol/L. Twelve of 17 anti-CD3 and IL-2–activated PBMCs were sensitive, with an additional three activated PBMC samples showing intermediate sensitivity. Of the 10 from 11 patient samples for which both resting and activated PBMCs were evaluated, the activated PBMCs were generally less sensitive to panobinostat. However, with the
exception of the activated MD7 (IC$_{50}$: 22.4 nmol/L), all 10 of these activated samples remained sensitive to panobinostat. For MD9, the IC$_{50}$ of the resting PBMCs was less than two times greater than that of the activated PBMCs, though both samples were highly sensitive to panobinostat (16 and 12 nmol/L, respectively). To evaluate the potential utility of panobinostat for immunotherapy, we evaluated the effects of panobinostat on PBMCs from a healthy donor (T-HD1) and a patient with metastatic melanoma (T-MD5); both PBMC samples had been transduced to express the TCR for melanoma antigen MART-1. Although T-MD5 was sensitive, with an IC$_{50}$ of 14 nmol/L, the IC$_{50}$ of T-HD1 was 80 nmol/L. The bone marrow from a patient with multiple myeloma (BM-1) was resistant to panobinostat, with an IC$_{50}$ of 90 nmol/L. In all, 24 of 30 PBMC samples tested were sensitive to panobinostat.

Of the 12 melanoma cell lines studied, seven had mutations at $\text{BRAF}^{\text{V600E}}$, 2 had mutations at $\text{NRAS}^{\text{Q61}}$, one had both $\text{BRAF}^{\text{V600E}}/\text{NRAS}^{\text{Q61K}}$ mutations, and one was wild-type for the $\text{BRAF}$ and $\text{NRAS}$ genes. Table 1 lists the characteristics of the 12 melanoma cell lines and their sensitivity to vemurafenib, an FDA-approved targeted therapy for metastatic melanoma (34–36). In contrast with results for PBMCs, only 2 of 12 human melanoma cells were sensitive to panobinostat, including M308, a $\text{BRAF}^{\text{V600E}}$-mutant melanoma cell line highly resistant to vemurafenib. Four melanoma cell lines had intermediate sensitivity to panobinostat, whereas the remaining six were resistant to panobinostat, including three with IC$_{50}$ of greater than 100 nmol/L (IC$_{50}$ ≥ 125 nmol/L). Neither the BRAF wild-type (M285) nor the NRAS-mutated melanoma cell lines (M202, M408, and M376) were sensitive to panobinostat.

### Effects of panobinostat on cell-cycle progression and apoptosis

Cell-cycle analysis by flow cytometry was performed to determine the effects of panobinostat in melanoma or activated PBMCs. Four sensitive (HD1, MD1, MD2, and MD3), one intermediately sensitive (MD4), and three resistant samples (T-HD1, M229, and M370) were exposed to 0 to 10 μmol/L panobinostat for 24 hours and then analyzed by DAPI staining combined with intracellular staining for cleaved PARP. As shown in Fig. 2, panobinostat increased the sub-G0 population of cells for all samples, though the most profound effect was seen in the sensitive cells HD1, MD1, MD2, MD3, and MD4. Furthermore, cleaved PARP staining increased with panobinostat treatment. Indeed, in all lymphocyte samples, nearly all cells in sub-G0,G0–G1, and G2–M were positive for cleaved PARP.

### Table 1. Characteristics of melanoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$\text{BRAF}$ or $\text{NRAS}$ mutational status</th>
<th>Other oncogenic events or tumor-suppressor deletions</th>
<th>Sensitive (S) or resistant (R) to vemurafenib$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M285</td>
<td>Wild-type (36)</td>
<td>MIHF amplification</td>
<td>R</td>
</tr>
<tr>
<td>M229</td>
<td>$\text{BRAF}^{\text{V600E}}$</td>
<td>AKT1 amplification</td>
<td>S (34, 35, 45)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTEN heterozygous</td>
<td></td>
</tr>
<tr>
<td>M233</td>
<td>$\text{BRAF}^{\text{V600E}}$</td>
<td>AKT1 amplification</td>
<td>R (35)</td>
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<tr>
<td></td>
<td></td>
<td>CCND1 amplification</td>
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<tr>
<td></td>
<td></td>
<td>EGFR amplification</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>CDKN2A heterozygous</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>PTEN heterozygous</td>
<td></td>
</tr>
<tr>
<td>M249</td>
<td>$\text{BRAF}^{\text{V600E}}$</td>
<td>MIHF amplification</td>
<td>S (34, 35, 45)</td>
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<tr>
<td></td>
<td></td>
<td>AKT2 amplification</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTEN homozgyous</td>
<td></td>
</tr>
<tr>
<td>M263</td>
<td>$\text{BRAF}^{\text{V600E}}$</td>
<td>CDKN2A heterozygous</td>
<td>R (34, 35)</td>
</tr>
<tr>
<td>M308</td>
<td>$\text{BRAF}^{\text{V600E}}$</td>
<td>MIHF amplification</td>
<td>R (35)</td>
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<td></td>
<td></td>
<td>AKT2 amplification</td>
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<td>EGFR amplification</td>
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<td></td>
<td></td>
<td>CDKN2A homozgyous</td>
<td></td>
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<tr>
<td>M370</td>
<td>$\text{BRAF}^{\text{V600E}}$</td>
<td></td>
<td>R (34)</td>
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<tr>
<td>M395</td>
<td>$\text{BRAF}^{\text{V600E}}$</td>
<td></td>
<td>S (34, 45)</td>
</tr>
<tr>
<td>M417</td>
<td>$\text{BRAF}^{\text{V600E}}$</td>
<td></td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\text{ABL1 E255K, Y253H}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGFR P753S</td>
<td></td>
</tr>
<tr>
<td>M202</td>
<td>$\text{NRAS}^{\text{Q61L}}$</td>
<td></td>
<td>R (35)</td>
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<tr>
<td>M408</td>
<td>$\text{NRAS}^{\text{Q61K}}$</td>
<td></td>
<td>R</td>
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<tr>
<td>M376</td>
<td>$\text{BRAF}^{\text{V600E}}/\text{NRAS}^{\text{Q61K}}$</td>
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$^a$Sensitive, <1 μmol/L; resistant, >1 μmol/L.
PARP (Fig. 2A). Although the G_0–G_1 population of cells increased at 1 nmol/L for MD1 and MD2 lymphocytes, no other statistically significant trends in the G_0–G_1, G_2–M, or S-cell populations were seen with increasing panobinostat concentration. Instead, with the exception of M370, a dramatic cytotoxic effect was observed for all samples (Fig. 2B). Panobinostat induced 30% or greater apoptotic cell death demonstrated by cleaved PARP in PBMC samples, whereas it was about 10% in M229 and 8% in M370 at 100 nmol/L. Only at 10 μmol/L was cleaved PARP about 30% for M229, whereas approximately 10% cleaved PARP was observed for M370 at this concentration (Fig. 2C). We stained for pH2A.X by flow cytometry as a marker of DNA damage (37). With the exception of M370, there was an up to 3-fold increase in pH2A.X in all PBMCs at 10 μmol/L (Fig. 3). These studies support the notion that panobinostat has greater cytostatic and cytotoxic effects on human T cells than on melanoma cells.

**Single-cell phospho-proteomic analysis of lymphocytes after exposure to panobinostat**

Using phospho-flow, we studied the effects of panobinostat on lymphocyte signaling proteins, including key MAPK and PI3K/AKT signaling proteins and those downstream of the TCR, B-cell receptor, and cytokine receptors in one sample each of healthy, melanoma donor, and bone marrow specimens. Two of them were sensitive (HD1, IC_{50} < 10 nmol/L; MD2, IC_{50} > 10 nmol/L) and one resistant (BM-1, IC_{50} < 50 nmol/L) to panobinostat. Given that IFN-α or IL-2 are key lymphocyte signaling cytokines and are often used clinically to treat advanced melanoma, activated lymphocytes stimulated with IFN-α or IL-2 were studied after 24-hour exposure to panobinostat and stained for surface and intracellular proteins. Figure 4 shows the gating strategy used for these analyses.

Panobinostat slightly inhibited the phosphorylation of STAT5, ERK1/2, and H3 in flow-gated T cells from a healthy donor at concentrations of 10 and 100 nmol/L. At higher concentrations, there was a slight increase in these phospho-proteins. In contrast, CD8 and CD4 T cells from the patient with metastatic melanoma (MD2) demonstrated a uniform increase in all intracellular proteins with increasing concentrations of panobinostat. CD8 cells from MD2 had an approximately 4-fold increase in pSTAT1, pSTAT3, pSTAT5, pAKT, pERK1/2, pP38, pP53, CyD3, and pH3. Similarly, for MD2 CD4 T cells, pSTAT1, pSTAT3, pSTAT6, pAKT, pERK1/2, CyD3, and pH3 were increased by 4-fold compared with controls. pSTAT5, pP38, and pP53 were increased almost 8-fold in MD2 CD4 cells.
activated with IL-2 or vehicle control. Treatment with IFN blunted the effect of panobinostat as the levels of intracellular phosphoproteins were slightly less than those seen in vehicle control using the same concentration of panobinostat. Panobinostat treatment of CD8 and CD4 cells from the bone marrow of a patient with multiple myeloma (BM-1) increased pSTAT1, pSTAT5, pSTAT6, pP38, pP53, and CyD3 in vehicle control cells, whereas it induced a 6-fold increase in pH3. In these cells, treatment with IFN or IL-2 blunted this effect. These trends for each patient sample were consistent for CD20 cells (Fig. 5 and data not shown). These data indicate that panobinostat activates the MAPK and PI3K/AKT signaling in human lymphocytes, especially those from patients with melanoma and myeloma.

Discussion

In this in vitro study, the majority of the human lymphocytes were highly susceptible to treatment with panobinostat at 20 nmol/L or less, which were doses lower than that required to inhibit the melanoma cell lines. Most lymphocytes, resting or proliferating, are susceptible to panobinostat inhibition whether they were derived from the peripheral blood or the bone marrow, from healthy subjects, or patients with melanoma or multiple myeloma. Only a minority of the 12 melanoma cell lines tested were sensitive to panobinostat. Growth inhibition of lymphocytes was mediated primarily via increased apoptosis demonstrated by cleaved PARP and in the sub-G0 population. Increased DNA damage was induced in some lymphocyte cultures. Panobinostat upregulated MAPK and PI3K/AKT phosphoproteins by 4- to 8-fold compared with controls. This induction was even more pronounced in human lymphocytes from patients with myeloma or melanoma compared with a healthy donor.

This is the first report evaluating the inhibitory effect of panobinostat in a large panel of melanoma cell lines, which included several BRAF-mutant and wild-type melanoma cell lines inherently resistant to vemurafenib. These data are consistent with prior reports demonstrating that HDACis generally have only a modest inhibitory effect in solid tumors compared with hematologic malignancies (7, 38, 39). The melanoma cell lines tested in our study were derived from cutaneous melanoma, and not from uveal melanoma. A report had suggested that uveal melanoma cell lines may be more sensitive to panobinostat, with an IC50 of 60 nmol/L or lower.
networks within individual PBMC subpopulations (42). In gain insight into the effects of panobinostat on signaling cell phospho-proteomic analysis provides a powerful tool to 50 proteins or lymphocyte function at concentrations below with vemurafenib, which resulted in little change in phospho-contrast with treatment the changes in intracellular phosphoproteins at the level of affords a highly reproducible, quantitative evaluation of applicability of panobinostat in uveal melanoma may be (41). Therefore, additional studies to evaluate the clinical panobinostat in human lymphocytes should temper enthu-

siasm for combining HDACi with immunotherapy, given the panobinostat in combination with ACT therapy (24, 25), our data with processing (44). Despite promising mouse data demonstrat-
ing synergy of HDACi with anti-CD40 and anti-CD137 (23) or in combination with ACT therapy (24, 25), our data with panobinostat in human lymphocytes should temper enthusiasm for combining HDACi with immunotherapy, given the cytotoxic effect of panobinostat on lymphocytes at concentra-
tions lower than that required to inhibit most melanoma cell lines. Consistent with this, panobinostat had a detri-
tal effect on human DC viability and function (31). Panobino-
stat also decreased the expression of T-cell–activating costi-
mulatory receptor CD40, DC- and T-cell–activating receptor CD83 (23), and antigen-presenting molecules HLA-A/B/C (31). Our data in human lymphocytes are not surprising, given that pan-HDAC inhibitors have clinical application in the treatment of T-cell malignancies and are under active investigation in early-phase clinical trials for the treatment of other hematologic malignancies, such as multiple myeloma, Hodgkin lymphoma, and chronic myelogenous leukemia, and as immunomodulatory agents in inflammatory disorders such as rheumatoid arthritis. Because of the deleterious effects of panobinostat on human lymphocytes, we conclude that it is likely not a suitable adjunctive therapy after ACT. Instead, one potential application could be as an adjunct for lymphodepletion before ACT or as a useful immunosuppres-
sive agent, though additional investigations into this potential clinical application should be undertaken. Alternatively, the development of panobinostat as a local therapy via intratumoral injections or delivered via nanoparticles to minimize systemic toxicity may also be a consideration.

One strategy to augment responses to immunotherapy is combination therapy with agents that increase antigen presentation to T cells. HDACis have been reported to increase melanosomal antigen expression and improve combinatorial effect with immunotherapy in mouse models. However, exposure to a pan-HDACi resulted in both cytostatic and cyto-
toxic effects on human lymphocytes in vitro as it altered key lymphocyte signaling networks. These human in vitro data may support an argument against the use of panobinostat, a hydroxamic acid HDACi, in combination with immunothera-
pies in the clinic for patients with melanoma.

Figure 4. Single-cell phosphoprotein flow cytometry gating strategy. Cells were treated with 0 to 1 μmol/L panobinostat for 24 hours. Cell events were first identified by gating on morphology (SSC-A vs. FSC-A) and singlets (SSC-A vs. SSC-W). After that, the six barcoded cell populations with a combination of Ax-350-NHS 0, 3, or 8 μg/mL and Ax-750-NHS 0, 3, or 8 μg/mL were deconvoluted. Then, for each of the six populations, plotting CD20 versus CD3 identified B- and T-cell populations. Further plotting the CD3 population for CD4 and CD8 identified these T-cell subpopulations. Histograms of intracellular phosphorylated proteins such as pSTAT5 were then obtained. FSC-A, forward scatter area; SSC-A, side scatter area; W, width.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D.J.L. Wong, R.C. Koya, A. Ribas, B. Comin-Anduix

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Rao, E. Avramis, D.R. Matsunaga, K.M. Komatsubara, T. Chodon, B. Comin-Anduix

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.J.L. Wong, A. Rao, E. Avramis, K.M. Komatsubara, R.C. Koya, A. Ribas, B. Comin-Anduix

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Study supervision: A. Ribas, B. Comin-Anduix

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