Histone Deacetylase Inhibitors Induce Growth Arrest and Differentiation in Uveal Melanoma

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Abstract

Purpose: Metastasis is responsible for the death of most cancer patients, yet few therapeutic agents are available which specifically target the molecular events that lead to metastasis. We recently showed that inactivating mutations in the tumor suppressor gene \textit{BAP1} are closely associated with loss of melanocytic differentiation in uveal melanoma (UM) and metastasis. The purpose of this study was to identify therapeutic agents that reverse the phenotypic effects of BAP1 loss in UM.

Experimental Design: \textit{In silico} screens were done to identify therapeutic compounds predicted to differentiate UM cells using Gene Set Enrichment Analysis and Connectivity Map databases. Valproic acid (VPA), trichostatin A, LBH-589, and suberoylanilide hydroxamic acid were evaluated for their effects on UM cells using morphologic evaluation, MTS viability assays, bromodeoxyuridine incorporation, flow cytometry, clonogenic assays, gene expression profiling, histone acetylation and ubiquitination assays, and a murine xenograft tumorigenicity model.

Results: Histone deacetylase (HDAC) inhibitors induced morphologic differentiation, cell-cycle exit, and a shift to a differentiated, melanocytic gene expression profile in cultured UM cells. VPA inhibited the growth of UM tumors \textit{in vivo}.

Conclusions: These findings suggest that HDAC inhibitors may have therapeutic potential for inducing differentiation and prolonged dormancy of micrometastatic disease in UM. \textit{Clin Cancer Res; 18(2); 408–16. ©2011 AACR.}

Introduction

Metastasis is responsible for the death of most cancer patients, yet there are few therapies available that effectively target the metastatic process. This is particularly true for metastatic melanoma, which is notoriously resistant to treatment. Because metastatic melanoma may remain asymptomatic in a state of dormancy for months to years before becoming clinically detectable, one therapeutic strategy is to prevent or delay micrometastatic disease from escaping dormancy by inducing senescence or differentiation (1). However, such a strategy has been hampered by an inadequate understanding of the genetic events driving metastasis and, consequently, a lack of rational drug targets.

Uveal melanoma (UM) is a highly aggressive form of melanoma that exhibits a strong tendency for lethal hematogenous metastasis to the liver and other sites (2). At the time the primary eye tumor is diagnosed, less than 4% of patients have detectable metastatic disease (3). Yet up to half of these individuals will eventually succumb to metastasis, despite successful treatment of the primary tumor, indicating that they harbored subclinical micrometastases at initial presentation. The most accurate method for identifying UM patients who are at high risk of metastasis is by gene expression profiling of the primary tumor, which can be done on a fine-needle biopsy using a validated 15-gene assay (4). This method separates UMs into 2 classes. Class 1 tumors, which have a very low metastatic risk, are composed of more differentiated tumor cells, and their gene expression profile is highly similar to that of normal differentiated melanocytes (5). In contrast, class 2 tumors, which have a high risk of metastasis, contain cells that have lost morphologic features of melanocytic differentiation, and their gene expression signature is enriched for genes expressed in primitive neuroectodermal cells (5).

We recently showed that inactivating somatic mutations in the tumor suppressor gene \textit{BAP1}, located at chromosome 3p21, and concomitant loss of the other copy of chromosome 3 are present in the vast majority of class 2 tumors but not class 1 tumors (6). Depletion of BAP1 in cultured class 1
Uveal melanoma (UM) is highly metastatic and, once it has disseminated, it is highly recalcitrant to available therapies. Recently, we discovered that inactivating mutations in the histone H2A ubiquitin hydrolase BAP1 are strongly associated with metastasis in UM. In this study, we show that histone deacetylase (HDAC) inhibitors reverse the biochemical effects of BAP1 loss, induce melanocytic differentiation and cell-cycle arrest, and inhibit the growth of UM tumors in vivo in a xenograft model. These findings suggest that HDAC inhibitors may be effective in an adjuvant setting for inducing differentiation and prolonging the dormancy of micrometastatic disease in UM.

Materials and Methods

Tumor samples

This study was approved by the Institutional Review Board of Washington University in St. Louis and adhered to the tenets of the Declaration of Helsinki. Primary UM samples were collected at the time of enucleation as previously described (12). Informed consent was obtained for each patient. All samples were confirmed to be UM by pathologic evaluation. Tumor samples were snap-frozen for DNA and RNA isolation or collected in HAM’S F-12 medium for tissue culture. Primary UM cells were grown in serum-free MDMF medium on collagen-covered tissue culture plates in 5% CO₂ and 4% O₂ (13). Elimination of contaminating cells was confirmed by microscopic inspection and Melan-A staining. The multigene prognostic assay for assignment of tumors to class 1 or class 2 groups was done as described elsewhere (4). To determine BAP1 status of primary tumors, genomic DNA was subjected to Sanger sequencing of all BAP1 exons as described elsewhere (14). The UM cell lines 92.1, OCM1A, and Mel202 (kindly provided by Drs. M. Jager, J. Kan-Mitchell and B. Ksander, respectively) were grown in RPMI-1640 supplemented with 10% FBS, l-glutamine, and antibiotics at 5% CO₂. These UM cell lines are all BAP1 wildtype with a gene expression profile similar to class 1 tumors.

Gene set enrichment analysis and connectivity mapping

Gene expression profiles from 14 class 1 and 10 class 2 UM were obtained on the Affymetrix U133A platform, as previously described (15). Curated gene sets enriched in class 1 UM tumors versus class 2 UM tumors were identified with gene set enrichment analysis (GSEA; version 2.0.4; Broad Institute), using a significance threshold set at a nominal P value of less than 0.01 (16), as we previously described (5). To identify compounds that could potentially induce differentiation of class 2 UM cells, we interrogated the connectivity map database (Broad Institute; ref. 17) for compounds that caused gene expression changes that closely resembled the gene expression differences between normal uveal melanocytes versus class 2 UM cells, as well as class 1 UM cells versus class 2 UM cells. The 100 most differentially expressed genes were chosen using Student t test. This gene set was compared with those from the connectivity map database, and potential compounds were chosen using the following parameters (e.g., positive enrichment score with a P < 0.05).

Chemosensitivity assays

The effects of 4 HDAC inhibitors, VPA (Sigma), TSA (Sigma), LBH-589 (Selleck), and SAHA (Selleck) were studied in UM cell lines and primary UM cells. Drug concentrations ranged from 0.5 to 2 mmol/L for VPA, 50 to 250 nmol/L for TSA, 5 to 60 nmol/L for LBH-589, and 0.5 to 2.5 μmol/L for SAHA. Dimethyl sulfoxide was added as vehicle in TSA, LBH-589, and SAHA untreated controls. MTS assays were done according to manufacturer’s instructions (CellTiter 96 AQueous Assay reagent; Promega). Bromodeoxyuridine (BrdU; 1:1,000; Amersham Biosciences) incorporation assays were done using an anti-BrdU-peroxidase–conjugated antibody according to the manufacturer’s instructions (Roche; dilution 1:75). Tetramethylbenzidine (Sigma) was added as substrate for signal detection, and colorimetric changes were measured at 370 nm using a Microplate spectrophotometer (SpectraMax 190; Molecular Devices). Flow cytometry was done.
after treatment of cells for 48 hours using a standard propidium iodide staining protocol as previously described (18) using a FACScan analyzer (BD Biosciences). The percentage of cells in each phase was determined with the VenturiOne software (Applied Cytometry). For clonogenic assays, flow cytometry (MoFlo; Cytomation) was used to seed one viable cell per well in ultralow attachment 96-well plates containing MDMF medium only or MDMF with drug. Cells were monitored with phase contrast microscopy, and cell number was assessed for each well at 7 days. Cell morphology was assessed by phase contrast microscopy and morphologic changes were quantified by counting the number of dendritic arborizations per cell in primary UM cells or by determining the maximal cell length/width ratio (spindle morphology index) in UM cell lines using ImageJ. Higher values are consistent with increasing melanocytic differentiation.

**Multigene prognostic assay and qPCR**

Total RNA extracted with TRIzol (Invitrogen) was DNase treated and reverse transcription was done using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was preamplified for 14 cycles with pooled primers according to the manufacturer’s protocol (Applied Biosystems). The multigene prognostic assay was done with RNA from primary tumor cells treated with VPA, LBH-589, and SAHA, and molecular class was determined using support vector machine (SVM), as described elsewhere (4). BAP1 mRNA levels were measured by qPCR as previously described (6).

**BAP1 knockdown**

BAP1 was depleted in the 92.1 UM cell line by using a lentiviral-based short hairpin RNA (shRNA). Lentiviral pLKO.1 shRNA vectors for GFP (clonetechGfp-438s1c1) and BAP1 [NM_004656.2-321s1c1; developed by the RNAi Consortium (TRC)] were purchased from the Children's Discovery Institute/Genome Sequencing Center at Washington University in St. Louis Animal Studies Committee. A total of $1 \times 10^6$ 92.1 UM cells were resuspended in Cultrex (Trevigen) and injected subcutaneously into the flanks of NOD.Cg-Pkdcscid I2rgtm1Wjl/J [nonlyse diabetic severe-combined immunodeficient (NOD SCID) gamma] mice (Jackson Laboratory). Mice were then given intraperitoneal injection of VPA (0.1 mg/g of body weight) every other day, beginning at day 7. Tumor volume was monitored once a week and the mice were euthanized after 42 days. Tumors were collected and volume measured using the ellipsoid volume formula ($\pi xyz/6 \text{ mm}^3$).

**Statistical analysis**

Except where otherwise noted, data were analyzed for statistical significance using MedCalc software, version 9.5.1.0.

**Western blotting and immunofluorescence**

Western blotting was done as previously described (18) using anti-BAP1 (1:250; Santa Cruz), anti–α-tubulin (loading control, 1:1000; Sigma), anti-acetyl-histone H3lys9 (1:200; Cell signaling), anti-histone H3 (1:200; Cell signaling), anti–ubiquityl-histone H2A (1:100; Millipore), and anti-histone H2A (1:200; Millipore) antibodies. Histone proteins for Western blotting were extracted from cells according to previously published protocol (20). Western blotting densitometry was carried out with the AlphaEaseFC software using the Spot Denso Analysis Tool. Intensity of each Ub-H2A and total H2A bands was measured using a rectangular spot and Ub-H2A was normalized to total H2A after background subtraction. Immunofluorescence was done by plating $5 \times 10^3$ 92.1 cells on Lab-Tek 8-well Permanox chamber slides (Nunc) and using anti-BAP1, anti–acetyl-histone H3lys9 and anti–ubiquityl-histone H2A antibodies.
Results

In silico screening for compounds that reverse the effects of BAP1 loss

BAP1 loss in UM cells results in morphologic and transcriptomic changes consistent with a loss of melanocytic differentiation and a shift from class 1 to class 2 transcriptomic profile (6). Thus, we sought to identify therapeutic compounds that may reverse the effects of BAP1 loss by restoring a more differentiated, class 1-like transcriptomic profile. We used 2 complementary in silico approaches—GSEA and Connectivity Mapping—to compare genes that are...
differentially expressed between class 1 and class 2 UMs to curated gene sets associated with perturbation of cancer cells with therapeutic compounds. Using GSEA, the gene set that was most similar to the genes upregulated in class 1 UMs (relative to class 2) was PEART_HISTONE_UP (Fig. 1), which consisted of genes upregulated by the HDAC inhibitors SAHA and depsipeptide (21). We obtained similar results with the Connectivity Mapping, which identified 3 HDAC inhibitors (VPA, TSA, and SAHA) among its top matches (Supplementary Table S1).

**HDAC inhibition blocks proliferation of UM cells**

Initially, we chose VPA to test the effects of HDAC inhibition in UM cells. As expected, VPA caused a dose-dependent increase in histone H3 acetylation (Supplementary Fig. S1). In all 3 UM cell lines (92.1, OCM1A, and Mel202), VPA inhibited proliferation but did not significantly reduce the fraction of viable cells, induced a G1 cell-cycle arrest, and markedly reduced the clonogenicity of UM cells (Fig. 2). The spindle morphology index increased after treatment with the HDAC inhibitors (Supplementary Fig. S2). Similar changes were seen with TSA and LBH-589, except that these agents significantly reduced the fraction of viable cells and increased the proportion of cells undergoing apoptosis (Fig. 2), consistent with increased cytotoxicity.

**VPA inhibits UM tumor growth in vivo**

Because VPA induced cell-cycle arrest rather than cell death, we predicted that it may have therapeutic potential in an adjuvant setting to block the growth of micrometastatic disease. To model this situation, we established small subcutaneous flank tumors in NOD SCID gamma mice and tested whether VPA could prevent the growth of these tumors. Treatment with VPA or control was initiated when the tumors became barely palpable (usually about 7 days after injection). VPA markedly reduced the growth and final volume of these tumor deposits compared with control (Fig. 3).

**BAP1 loss sensitizes UM cells to HDAC inhibition**

BAP1 is the catalytic subunit of the PR-DUB complex that deubiquitinates histone H2A (22). As expected, RNAi-mediated knockdown of BAP1 in UM cells (Fig. 4A) induced a marked increase in H2A ubiquitination (Fig. 4B, top panel). Because a recent report showed that HDAC inhibitors decrease histone H2A ubiquitination through transcriptional repression of the PRC1 component BMI1 (23), we wished to determine whether HDAC inhibition may reverse this H2A hyperubiquitination in BAP1-deficient UM cells. Indeed, VPA markedly reduced the levels of histone H2A ubiquitination in BAP1-depleted cells (Fig. 4B, bottom panel and Fig. 4C top panel), whereas total histone H2A level remained unchanged (Fig. 4C, bottom panel). We showed in Fig. 2A that VPA inhibited proliferation of BAP1 wildtype UM cell lines, but it did not reduce the fraction of viable cells. However, when we stably depleted BAP1 in UM cells using lentiviral shRNA, VPA significantly reduced the fraction of viable cells compared with control cells (Fig. 4D), indicating that BAP1 loss sensitized the UM cells to HDAC inhibition.

We then investigated whether HDAC inhibition could reverse the phenotypic consequences of BAP1 loss: loss of melanocytic differentiation and acquisition of the class 2 gene expression profile (6). Because UM cell lines undergo genetic and epigenetic artifacts in long-term culture, for these experiments we used primary UM cells from 5 class 2 tumors (MM137, MM138, MM151, MM161, and MM162) and 1 class 1 tumor (MM131) which were obtained immediately after surgical resection and placed in short-term culture. Although only 4 of the class 2 samples contained detectable BAP1 mutations, all 5 showed low BAP1 RNA levels compared with the class 1 tumors (Supplementary Table S2 and Supplementary Fig. S3). In cells treated with VPA for 3 and 7 days, cell bodies became enlarged and flattened, and there was a marked increased in the number of dendritic arborizations, consistent with melanocytic differentiation (Fig. 5A).
and B). Untreated or VPA-treated cells were subjected to a multigene expression profile that classifies UMAs as class 1 or class 2 (4). The results of this assay were reported as a SVM discriminant score; the more negative the score, the more class 1 the expression profile, the more positive the more class 2. VPA shifted all of the class 2 UM cells toward a class 1 profile, and it even shifted the class 1 UM cells toward a more class 1 profile (Fig. 5C and Supplementary Fig. S4). This effect was dose dependent, and similar results were observed with LBH-589 (Supplementary Figs. S4 and S5C). The genes most affected by VPA were HTR2B (downregulated by VPA) and LMCD1 (upregulated by VPA; Supplementary Fig. S5), both strong indicators of metastasizing UMAs (4). A fourth HDAC inhibitor, SAHA, induced effects similar to VPA in UM cell lines and primary UM cells (Supplementary Fig. S6).

Discussion

Here, we show that clinically achievable concentrations of several HDAC inhibitors can reprogram highly aggressive UM cells to a low-grade, differentiated state. This is consistent with similar findings in other cancers (24). These findings suggest a potential role for HDAC inhibitors in patients with high-risk class 2 UMAs. Most of these patients harbor undetectable micrometastases at the time of primary eye tumor diagnosis, and the goal of HDAC inhibitor therapy would be to induce prolonged clinical dormancy (and longer patient survival) by driving these micrometastatic cells into a differentiated, quiescent state.

We have not yet found a UM cell line with naturally occurring BAP1 mutations (data not shown), despite many of these cell lines being derived from metastasizing UMAs that likely contained cells with mutant BAP1. This suggests that BAP1 mutant UM cells may not grow well in culture. This has hindered our ability to study the effects of HDAC inhibitors on BAP1 mutant UM cells in vivo. However, in short-term cultures, HDAC inhibitors did indeed reverse the biochemical, transcriptomic, and morphologic consequences of BAP1 loss in primary UM cells. Knockdown of BAP1 caused an increase in histone H2A ubiquitination, consistent with its H2A ubiquitin carboxy-terminal hydrolase activity (7, 22), which is critical for its tumor suppressor function (7, 8). HDAC inhibitors reversed the H2A hyperubiquitination caused by BAP1 loss, and they shifted the gene expression profile of class 2 cells toward class 1.
toward a class 1 profile. Furthermore, HDAC inhibitors induced morphologic changes consistent with melanocytic differentiation. These findings suggest that BAP1 normally functions, at least in part, to maintain melanocyte differentiation in UM cells and that this function can be at least partially restored in the absence of BAP1 by increasing histone H3 acetylation. HDAC inhibitors reverse the biochemical defect caused by BAP1 loss (i.e., H2A hyperubiquitination), which may explain why BAP1-deficient UM cells were more sensitive to HDAC inhibition than BAP1-competent cells. However, because HDAC inhibitors act through multiple mechanisms of action, it is not surprising that they also had a differentiating effect on BAP1 wildtype tumor cells.

Histones undergo extensive posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, and ribosylation, that comprise a complex combinatorial code that regulates gene expression (25). This system has been of particular interest in understanding the coordinated expression of transcriptional programs during development and differentiation. Among these modifications, acetylation has been one of the most thoroughly studied. The balance between acetylation and deacetylation is determined by the relative activities of histone acetyltransferases and HDACs, with increased acetylation promoting greater chromatin accessibility for gene expression. Histone ubiquitination is less well understood. Monoubiquitination of histone H2A on Lys119 plays an important role in X-chromosome inactivation, Polycomb group-dependent gene silencing, and other developmental processes (26, 27). Distinct histone modifications also can function together in a coordinated fashion to regulate
chromatin structure and gene expression. For example, the histone deubiquitinase 2A-DUB regulates transcription by coordinating histone acetylation and deubiquitination, and destabilizing the association of linker histone H1 with nucleosomes (28). Furthermore, inhibition of HDAC activity has been shown to decrease histone ubiquitination through transcriptional repression of the PRC1 component BMI1 (23). This may explain the efficacy of HDAC inhibitors in UM cells.

VPA and LBH-589 have both shown therapeutic potential as monotherapy or in combination with other anti-cancer drugs in solid and hematologic malignancies (29). As expected, on the basis of their mechanism of action, HDAC inhibitors have not been shown to be effective as a cytotoxic therapy in patients with advanced metastatic melanoma (30), but the findings herein suggest that they may have a role in preventing the progression of micrometastatic disease. We focused here on VPA because it had a more prominent effect on cell-cycle arrest and differentiation, compared with TSA and LBH-589, which were more cytotoxic. The effect of SAHA was similar to VPA. VPA is a well-characterized compound that has been used for almost 30 years in the treatment of epilepsy and, more recently, as an anticancer agent (29). The toxicity profile of VPA would allow its use as an adjuvant agent in high-risk cancer patients. Taken together, these factors suggest that a clinical trial of adjuvant VPA or a similar HDAC inhibitor in high-risk class 2 UM patients may be warranted. Gene expression profiling can allow enrollment of high-risk class 2 patients, about half of whom will develop overt metastatic disease within 3 years of eye tumor diagnosis (4).

Disclosure of Potential Conflicts of Interest

J.W. Harbour and Washington University may receive income based on a license of related technology by the University to Castle Biosciences, Inc. This work was not supported by Castle Biosciences, Inc.

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