Enhanced Histone Deacetylase Activity in Malignant Melanoma Provokes RAD51 and FANCD2-Triggered Drug Resistance

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Abstract

DNA-damaging anticancer drugs remain a part of metastatic melanoma therapy. Epigenetic reprogramming caused by increased histone deacetylase (HDAC) activity arising during tumor formation may contribute to resistance of melanomas to the alkylating drugs temozolomide, dacarbazine, and fotemustine. Here, we report on the impact of class I HDACs on the response of malignant melanoma cells treated with alkylating agents. The data show that malignant melanomas in situ contain a high level of HDAC1/2 and malignant melanoma cells overexpress HDAC1/2/3 compared with noncancer cells. Furthermore, pharmacologic inhibition of class I HDACs sensitizes malignant melanoma cells to apoptosis following exposure to alkylating agents, while not affecting primary melanocytes. Inhibition of HDAC1/2/3 caused sensitization of melanoma cells to temozolomide in vitro and in melanoma xenografts in vivo. HDAC1/2/3 inhibition resulted in suppression of DNA double-strand break (DSB) repair by homologous recombination because of downregulation of RAD51 and FANCD2. This sensitized cells to the cytotoxic DNA lesion O6-methylguanine and caused a synthetic lethal interaction with the PARP-1 inhibitor olaparib. Furthermore, knockdown experiments identified HDAC2 as being responsible for the regulation of RAD51. The influence of class I HDACs on DSB repair by homologous recombination and the possible clinical implication on malignant melanoma therapy with temozolomide and other alkylating drugs suggests a combination approach where class I HDAC inhibitors such as valproic acid or MS-275 (entinostat) appear to counteract HDAC- and RAD51/FANCD2-mediated melanoma cell resistance.

Introduction

Patients suffering from melanoma in its metastatic stage have a dismal prognosis. Although newer therapies targeting mutant BRAF or immunotherapies with ipilimumab or anti-PD-1-antibody have shown promise (1-3), the cancer invariably becomes resistant, which necessitates the switch back to genotoxic-based therapies. These therapies include methylating agents such as dacarbazine and temozolomide. Methylating agents either require metabolic activation by cytochrome P450, in the case of dacarbazine (4), or spontaneously decompose, in the case of temozolomide (5), to the DNA reactive species 5-(3-methyltriazlen-1-yl)imidazole-4-carboxamide (MTIC). The clinically relevant cytotoxic DNA lesion for these drugs is O6-methylguanine (O6MeG). O6MeG is repaired by O6-methylguanine-DNA methyltransferase (MGMT) in a stoichiometric reaction, which restores guanine and inactivates MGMT (6). The resistance of melanoma cells to methylating agents is therefore proportional to the cells’ MGMT activity level (7). MGMT expression is governed by the methylation of CpG islands in its promoter (8) and the methylation status of MGMT has been shown to be predictive of temozolomide-based melanoma therapy (9).

As melanomas express low levels of MGMT (10, 11), O6MeG often persists in the DNA. Unrepaired O6MeG mismatches with thymine during replication and O6MeG/T mismatches are detected and bound by the mismatch repair (MMR) machinery. Futile MMR repairs cause persistent stretches of single-stranded DNA (ssDNA; ref. 12). If these stretches of ssDNA are not bypassed by homologous recombination (HR) during DNA synthesis, DNA double-strand breaks (DSB) arise (13, 14) that trigger apoptosis in melanoma cell lines (7). The protective role of RAD51-mediated HR in methylating agent–treated cells has been demonstrated in glioma and melanoma lines (15, 16). DNA repair by MGMT and HR is therefore central in protecting metastatic melanoma against methylating agent–based therapies.

Class I histone deacetylases (HDAC) have been described to influence the response of melanomas to temozolomide have shown promise (19, 20). For these reasons, we addressed the role of class I HDACs in melanoma and determined the molecular mechanism whereby these HDACs influence the response of melanomas to temozolomide. The class I HDACs are HDAC1, HDAC2, HDAC3, and HDAC4 as well as HDAC5, HDAC6, HDAC7, HDAC8, and HDAC9.

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

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HDAC8 (17, 18). They deacetylate histones and nonhistone proteins, thereby contributing to gene and protein regulation. In cancer cells, the inhibition of HDACs has been shown to influence the execution of the extrinsic and intrinsic apoptosis pathways as well as affecting DNA repair (21).

Our data reveal that melanoma specimens obtained from patients following surgery contain nuclear localized HDAC1/2 and that melanoma cell lines overexpress HDAC1/2/3 compared with primary melanocytes, peripheral blood lymphocytes (PBLC), and fibroblasts. Pharmacologic inhibition of class I HDACs sensitizes melanoma cells to temozolomide, fotemustine, and ionizing radiation (IR) and inhibits tumor growth in a xenograft melanoma tumor model more effectively than temozolomide alone. HDAC1/2/3 inhibition sensitizes melanoma cells to the temozolomide-induced DNA lesion O\textsuperscript{6}MeG due to suppression of HR-mediated DNA repair caused by downregulation of RAD51 and FANCD2 on gene and protein level. Finally, we identified HDAC2 as essential for RAD51 regulation. These findings are a sound basis for further exploring the targeting of class I HDACs during melanoma therapy with genotoxic agents.

Materials and Methods

Cell lines and culture conditions

D05 and A2058, SK-Mel187, and Mel537 were generous gifts from C.W. Schmidt (Queensland Institute of Medical Research, Queensland, Australia) in 2005 and W.K. Kaufmann (Department of Pathology and Laboratory Medicine, University North Carolina at Chapel Hill, Chapel Hill, NC) in 2012,
respectively. VH10tert was a gift from L.H.F. Mullenders (Department of Toxicogenetics, Leiden University Medical Centre, the Netherlands) in 2008. A375 and G361 were purchased from the ATCC in 2008. PBLCs were isolated from buffy-coat blood as described previously (22). Primary human epidermal melanocytes light pigmented (Hema-LP) were purchased from Gibco in 2012 and 2013. Upon receipt of the cell lines, they were cryopreserved in liquid-N2 and fresh cell stocks were used for every battery of tests. All lines were characterized in the laboratory of origin, displayed the expected phenotype, but were not reauthenticated in our laboratory. D05, Mel537, and PBLCs were cultured in RPMI-1640, while SK-Mel187, A2058, A375, and VH10tert were cultured in DMEM. RPMI1640 and DMEM were supplemented with 10% FBS and penicillin/streptomycin (PAA). Hema-LP was cultured in Medium 254 (Gibco) supplemented with human melanocyte growth supplement-2 (Gibco). Cells were maintained at 37°C in a humidified 5% CO2 atmosphere.

**Drugs, treatment, and irradiation**

The handling and preparation of temozolomide (Semaxo-Plough), fotemustine (Muphoran, Servier Research International), O6-benzylguanine (O6BG, Sigma-Aldrich), and olaparib (AZD2281, Selleck Chemicals) has been described previously (15, 23). To inhibit MGMT, O6BG was added to cells (final concentration 10 μmol/L) one hour before alkylating agent treatment. Irradiation was performed using a Cs-137 source. Valproic acid sodium salt (VPA, Sigma-Aldrich) was dissolved to 100 mmol/L in dH2O and stored at −80°C after sterile filtration. Cells were pretreated with VPA (1 mmol/L) for 168 hours with refreshment of VPA-containing medium every 48 hours and VPA removal before further treatment or irradiation. MS-275 (Entinostat, Selleck Chemicals) was dissolved in DMSO to a stock solution of 5 mmol/L and stored at −80°C, and before use it was diluted in PBS to 1 mmol/L. Cells were pretreated with MS-275 for 72 hours and then removing the compound by medium change.

**Figure 2.** Inhibition of HDAC1/2/3 sensitizes melanoma cells to genotoxic-based therapeutics. A, melanoma cells (D05 and A375) and primary human melanocytes (Hema-LP) were pretreated with VPA, then cells were exposed to 50 μmol/L temozolomide (TMZ), 32 μmol/L fotemustine (FM), or 5 Gy IR and the apoptosis response was determined 120 and 72 hours after alkylating agents and IR exposure, respectively. B, VPA pretreatment sensitizes to temozolomide concentration dependently. Apoptosis was assayed 120 hours after temozolomide addition. C, VPA pretreatment sensitizes to temozolomide time dependently. Apoptosis was assayed following 50 μmol/L temozolomide exposure at the indicated time points. D, HDAC inhibition sensitizes to O6MeG. VPA pretreatment followed by 50 μmol/L temozolomide in the presence and absence of MGMT inhibition by O6BG (10 μmol/L). Apoptosis was assayed 120 hours after temozolomide exposure. E, MS-275 pretreatment for 72 hours at indicated concentrations sensitizes D05 and A375 cells to temozolomide (50 μmol/L). Apoptosis was determined 120 hours after temozolomide addition. F, MGMT repair activity in D05 and A375 cells with and without VPA pretreatment. Data are presented as mean ± SEM. ***, P < 0.05; ****, P < 0.005; and , P < 0.0001. For A, B, D, and E, the apoptotic response was determined by flow cytometric analysis of Annexin V-FITC/PI double-stained cells, and for C by sub-G1 analysis.
Tumor biopsies and IHC

Samples of malignant melanoma were obtained from patients following surgery. Patient material was obtained with informed consent and approval from the Institutional ethics committee of the University Medical Center Mainz (Mainz, Germany). Slices were obtained from paraffinized sections, which were labeled as to the tumor area. Immunohistochemical analysis of HDAC1/2 levels was performed as described previously (23). For antibodies, see Supplementary Table S1. Nuclei were stained with TO-PRO-3. Microphotographs were acquired by laser scanning microscopy (LSM710, Carl Zeiss MicroImaging). Three different tumors were analyzed.

Preparation of protein extracts and Western blot analysis

Preparation of whole cell (24) and nuclear (25) protein extracts were described previously. Protein concentrations were determined using the Bradford method (26). For antibodies, see Supplementary Table S1.

Determination of apoptosis and cell cycle

Analysis of cell cycle, sub-diploid DNA content (sub-G1; ref. 27) and Annexin V-FITC/propidium iodide (PI) double-stained cells have been described previously (7). Flow cytometry was performed using FACSCanto II (BD Biosciences) and the data were analyzed with WinMDI (sub-G1, Annexin V/PI) or ModFit (cell cycle).

Determination of MGMT activity

MGMT activity assay was performed as described previously (28).

Colony survival assay

G361, SK-Mel187, Mel537, A2058, and A375 melanoma cells were pretreated with VPA for 168 hours. Following VPA removal, logarithmic growing cells were seeded and allowed to attach for 6 hours before O6BG addition and 1 hour later cells were exposed to temozolomide. Colonies were fixed in acetic acid:methanol: 

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA isolation, cDNA synthesis, and real-time PCR were performed as described previously (29). RNA levels were normalized to GAPDH and ACTB.

Homologous recombination repair assay

HR repair capacity was assessed with a stably genomic integrated reporter plasmid as described previously (30). The pDR-GFP reporter plasmid (Addgene plasmid 26475; ref. 30) and the SceI-expressing plasmid pCBASceI (Addgene plasmid 26477) were used (31). The cells were analyzed 48 hours (A375) or 72 hours (D05) after pCBASceI transfection, using Effectene (Qiagen), by flow cytometry.

Figure 3.

Class I HDACs protect melanoma cells in vitro and in vivo. A, colony survival in G361, SK-Mel187, Mel537, A2058, and A375 melanoma cells in the presence and absence of VPA pretreatment exposed to the indicated concentrations of temozolomide (TMZ). B, compilation of the survival data obtained in A for G361, SK-Mel187, Mel537, A2058, and A375. C, Western blot analysis of acetylated-H3 in A375 xenografts of mice treated with VPA. H3 served as a loading control. D, effect of HDAC inhibition on growth inhibition of A375 xenografts following temozolomide treatment. Relative tumor volume is shown as a function of time. See Materials and Methods for detailed treatment conditions. Data are presented as mean ± SEM. *; P < 0.05; **; P < 0.005; and ***; P < 0.0001.
**Immunofluorescence**

Immunofluorescent labeling of γH2AX-foci was performed as described previously (15). For antibodies, see Supplementary Table S1.

**siRNA, plasmids, and stable knockdown**

siRNA targeting the mRNA of HDAC1 (Dharmacon), HDAC2 (Santa Cruz Biotechnologies Inc.), and HDAC 3 (Ambion, Life Technologies) was transfected into A375 and D05 cells using Lipofectamine RNAiMax (Invitrogen). A pSuper (OligoEngine) construct was generated to express shRNA targeting RAD51 mRNA using the previously described sequence (5'-GAAGAAAUUGGAA-GAAGCU-3'; ref. 32). Plasmid DNA was transfected using Effectene (Qiagen). Transfected cells were selected with 0.75 mg/mL G418 (Invitrogen).

**Animal experiments**

Immunodeficient mice (NOD.CB17-Prkdcsid/J) were housed in a sterile environment and allowed free access to food and water. The animal experiments were approved by the government of Rhineland-Palatinate and the Animal Care and Use Committee of the University Medical Center Mainz (Mainz, Germany) and performed according to the German federal law and the guidelines for the protection of animals. A375 human melanoma xenografts were initiated by injecting $8 \times 10^6$ cells in the right and left flank and the treatments began when tumor volumes $[(L \times W \times H \times \pi)/6]$ reached a suitable size ($50–100 \text{ mm}^3$). Four animals each were assigned to the different groups and tumor volume was standardized across the groups. The groups that received VPA pretreatment were treated as follows. Six days before O6BG and temozolomide injection, mice were injected intraperitoneally once daily with 500 mg/kg VPA. The mice received one dose intraperitoneal of temozolomide (150 mg/kg). One hour prior to temozolomide injection, all mice received O6BG 30 mg/kg intraperitoneally. Animal weights and tumor volumes were monitored following temozolomide injection. The in vivo experiments were performed twice, once with female mice (4 per group) and once with male mice (4 per group). The presented data show the results from both groups.

**Results**

**Melanoma cells overexpress HDAC1/2/3**

Class I HDACs are often overexpressed in cancer. We addressed whether this is the case for melanomas. Making use of the publicly accessible microarray dataset E-GEOD-3189 from ArrayExpress and published in ref. 33, the expressions of HDAC1, HDAC2, and HDAC3 in 45 primary melanoma samples were compared with 18 benign skin nevi and 7 normal skin samples. The primary melanoma samples showed a significant overexpression of HDAC1 and HDAC2 compared with the benign nevi and the normal skin tissue (Supplementary Fig. S1A). Furthermore, melanoma tumor samples analyzed by IHC showed strong HDAC1/2 nuclear staining compared with the cytoplasmic staining (Fig. 1A), whereas melanoma cell lines express significantly more HDAC1/2/3 proteins compared with primary human epidermal melanocytes (Hema-LP), peripheral blood lymphocytes (PBLC), and diploid human fibroblasts (VH10tert; Fig. 1B and C). PBLCs did express class I HDACs (Supplementary Fig. S1B), but were excluded from further analysis because of their very low expression, but the significance remained when they were included in the analysis (Supplementary Fig. S1C). As class I HDACs can deacetylate histones 3 (H3) and 4 (H4; ref. 34), we determined whether inhibition of class I HDACs with valproic acid (VPA; ref. 35) influences the acetylation level of H3 and H4 in melanoma lines (Fig. 1D). Upon VPA treatment, all the melanoma cell lines showed increased H3 and H4 acetylation levels, decreased HDAC2 levels in A375, G361, SK-Mel187, Mel537, and D05, which is consistent with what has been reported previously.

![Figure 4](https://www.aacrjournals.org/cancerres/76/10/2016/OF5/cancerres_aacrjournals.png)

**Figure 4.** Inhibition of class I HDACs downregulates RAD51 and FANCD2 on RNA and protein level. A, real-time PCR array results showing the down- and upregulation of the listed genes. HDACs were inhibited by VPA in D05 cells and fold change in gene expression was calculated compared with untreated controls. B, validation of gene regulation using real-time PCR. HDACs were inhibited by VPA pretreatment in D05 and A375 cells and fold change in gene expression was calculated compared with untreated controls. C, Western blot analysis of RAD51, FANCD2, RAD51D, ATR, KU80, BAX, NOXA, and BCL2 protein levels. β-Actin served as a loading control and relative expression (R.E.) levels are indicated.
while HDAC1/3 levels were not influenced significantly. For the noncancer cells Hema-LP, PBLCs and VH10tert VPA pretreatment also caused an increase in acetylated-H3 levels (Supplementary Fig. S1D). Collectively, the data show that malignant melanomas express HDACs and that the overexpressed class I HDACs in melanoma cell lines can be inhibited by VPA.

**HDAC1/2/3 protect malignant melanoma cells against genotoxin-induced cell death**

Having shown that HDAC1/2/3 are overexpressed in melanoma cells, the influence of these HDACs on genotoxin-induced cell death was determined. Two melanoma cell lines, D05 and A375, as well as primary human melanocytes, Hema-LP, were pretreated with the HDAC inhibitor (HDACi) VPA, a fatty acid inhibiting HDAC1/2/3/8 and clinically used for the treatment of epilepsy (35, 38), and exposed to temozolomide, fotemustine, or IR (Fig. 2A). It is important to note that at the administered concentration of temozolomide and fotemustine, O6MeG and O6-chloroethylguanine (O6ClEtG) are the clinically relevant apoptosis-inducing DNA lesions in melanoma cells (7). For this reason, we performed all experiments, unless stated otherwise, by inhibiting MGMT with O6BG before temozolomide or fotemustine treatment. As shown in Fig. 2A, VPA significantly sensitized the melanoma cells to temozolomide-, fotemustine-, and IR-induced apoptosis while not influencing the response of primary human melanocytes.

Furthermore, VPA sensitized melanoma cells to temozolomide in a concentration (Fig. 2B) and pretreatment time-dependent (Fig. 2C) manner. Because temozolomide exerts its cytotoxicity through O6MeG and this lesion is repaired by MGMT (7, 9), the sensitization of melanoma cells by HDAC inhibition was determined in the absence and presence of MGMT activity using O6BG (Fig. 2D). MGMT protects not only against temozolomide-induced apoptosis, but also against the sensitization caused by VPA (Fig. 2D), indicating that VPA sensitizes melanoma cells to O6MeG DNA lesions. As HDAC8 was not overexpressed in melanomas (Fig. 1C), we determined whether it contributes to the sensitization of melanoma cells to temozolomide. This was accomplished by using the benzamide HDACi MS-275, which specifically inhibits HDAC1/2/3, but not HDAC8 (38). MS-275 sensitized D05 and A375 melanoma cells to temozolomide in a concentration-dependent manner (Fig. 2E), showing that the catalytic activity of HDAC1/2/3, but not HDAC8, contributes to the resistance of melanoma cells to temozolomide.

As MGMT protects cells against alkylating agents, we determined whether HDAC inhibition affects this enzyme. HDAC inhibition only marginally influenced MGMT activity in one cell line tested (A375), while not affecting the other (D05; Fig. 2F). Seeing that HDAC inhibition did not influence MGMT activity in D05 cells (Fig. 2F) and HDAC inhibition sensitizes cells to IR-induced DNA damage (Fig. 2A), which are not subject to repair by MGMT, we conclude that the sensitization of melanoma cells to temozolomide is not due to an effect on MGMT. Furthermore, cellular replication rate significantly influences the response of cells to methylating agents (22). To exclude this as a reason for HDACi-mediated sensitization to temozolomide, analysis of...
cell-cycle distribution (Supplementary Fig. S2A) and cellular growth rates (Supplementary Fig. S2B) were performed following VPA pretreatment. VPA neither changed the cell-cycle distribution nor the growth rate of melanoma cells. Collectively, the data indicate that overexpression of HDAC1/2/3 protects melanoma cell lines against DNA damage triggered cell death.

Class I HDAC inhibitors promote temozolomide-induced cell growth inhibition in melanoma cell lines and a melanoma xenograft model

To clarify that the sensitization to temozolomide of melanoma cells by HDAC inhibition is a general effect, a panel of melanoma cell lines was subjected to the colony survival assay in the presence and absence of class I HDAC inhibition (Fig. 3A). All tested melanoma cell lines were sensitized to temozolomide following VPA. When combining the survival data of Fig. 3A, the sensitization caused by the HDACi in temozolomide-treated cells was still apparent (Fig. 3B), showing that this sensitization effect would very likely also be observed in heterogeneous melanoma tumors. To establish whether the findings obtained in cell lines in vitro can be translated to melanoma cell growth in vivo, we determined the effect of A375 cells grown subcutaneously in immunodeficient mice. HDAC inhibition with VPA pretreatment was able to increase the acetylated-H3 levels in melanoma xenografts (Fig. 3C), showing that VPA was able to inhibit class I HDACs in this model system. A single treatment with temozolomide (150 mg/kg) delayed tumor growth, HDAC inhibition by VPA pretreatment on its own had no effect on tumor growth, whereas temozolomide (150 mg/kg) in combination with VPA showed significant more tumor growth inhibition compared with temozolomide alone (Fig. 3D and Supplementary Fig. S3A). For the influence of this treatment schedule on the weight of the mice, see Supplementary Fig. S3B. The melanoma xenograft model results reflect the results obtained in vitro with cell lines and show that inhibition of class I HDACs enhances melanoma tumor growth inhibition by temozolomide.

Inhibition of HDAC1/2/3 downregulates RAD51 and FANCD2

To identify how class I HDAC inhibition causes sensitization to temozolomide, a real-time PCR array was performed. Class I HDACs were inhibited in D05 melanoma cells using VPA and changes in mRNA were monitored by real-time PCR (Fig. 4A and Supplementary Table S2). Genes involved in apoptosis, BCL2, NOXA, PUMA, and BAX, were either up- or downregulated while genes involved in DNA repair, RAD51, RAD51D, FANCD2, and KU80, were downregulated (Fig. 4A). To validate these results, specific real-time PCR experiments were performed with two melanoma cell lines, D05 and A375, following VPA pretreatment (Fig. 4B). Again, up- and downregulation of these genes was observed. Next, we tested whether changes in gene expression reflect changes in protein level. To this end, the influence of VPA on RAD51, FANCD2, RAD51D, ATR, KU80, BAX, NOXA, and BCL2 protein levels was analyzed (Fig. 4C). Following HDAC inhibition, only RAD51 and FANCD2 protein levels decreased, suggesting that decreased DNA repair capacity, and not increased apoptosis fidelity, is the cause for melanoma cell sensitization toward temozolomide upon HDACi.

Using RAD51 as a marker for downregulation of DNA repair following HDAC inhibition, we addressed the question of time and concentration dependence. The time of class I HDAC inhibition and the concentration of class I HDACi used for pretreatment showed an inverse relationship with the RAD51 protein level (Fig. 5A). This inverse relationship affects the sensitization to temozolomide, as temozolomide...
alone induced approximately 6% apoptosis in D05 cells while increasing VPA pretreatment time and concentration significantly increased temozolomide-induced apoptosis (Fig. 5B). Therefore, the decrease in RAD51 protein (Fig. 5A) mirrored the increase in melanoma cell sensitivity to temozolomide (Fig. 5B). Moreover, following class I HDACi removal, RAD51 protein levels stayed suppressed for 48 hours. Temozolomide caused a decrease in RAD51 protein level 72 hours after temozolomide addition, whereas pretreatment with VPA followed by temozolomide addition suppressed RAD51 protein levels for the duration of the experiment (up to 72 hours; Fig. 5C).

Having demonstrated that the inhibition of HDAC1/2/3/8 causes downregulation of RAD51 and FANCD2 in the melanoma cell lines D05 and A375, we expanded our examination to additional melanoma cell lines: G361, SK-Mel187, Mel537, and A2058. Following HDAC1/2/3/8 inhibition, all these cell lines showed a decrease in RAD51 and FANCD2 protein (Fig. 5D). As VPA inhibits HDAC1/2/3/8, the contribution of the individual HDACs, HDAC1/2/3/8, to the expression of RAD51 and FANCD2 was addressed. First, HDAC8 was assessed by using the HDACi MS-275, which only inhibits HDAC1/2/3, in D05 and A375 melanoma cells. Upon MS-275, the downregulation of RAD51 and FANCD2 was still observed (Fig. 6A), showing that downregulation of these DNA repair proteins are not caused by the inhibition of HDAC8. To address the contribution of HDAC1, HDAC2, and HDAC3 to the expression of RAD51, the individual HDACs were knocked down using siRNA (Fig. 6B). Only the downregulation of HDAC2 corresponded to a simultaneous downregulation of RAD51, showing that of these three HDACs, only HDAC2 plays a role in the expression of RAD51.

Inhibition of class I HDACs decreases homologous recombination DNA repair, thereby sensitizing melanoma cells to temozolomide

As the inhibition of HDAC1/2/3 downregulates RAD51 and FANCD2, and both protect cancer cells against temozolomide (15, 39) because of their role in HR (13, 40), the effect of HDACi on HR was assayed. Inhibition of class I HDACs significantly decreased HR activity in both D05 and A375 melanoma cell lines upon DSB induction (Fig. 7A and B). Dysregulation of HR has been implicated in synthetic lethality caused by PARP-1 inhibition in cells and cancers defective for this repair pathway (41). Therefore, if downregulation of RAD51 mediated by HDACi influences HR repair then class I HDAC inhibition should sensitize melanoma cells to PARP-1 inhibitor olaparib. This was in fact the case, as VPA significantly sensitized D05 cells to olaparib (Fig. 7C). A decrease in DSBR repair caused by class I HDAC inhibition should increase the amount of unrepaired DSBs following temozolomide treatment. To this end, the amount of γH2AX foci, a marker for DSBs, was determined in temozolomide-treated melanoma cells following VPA. Pretreatment with VPA significantly increased the amount of unrepaired DSBs following temozolomide treatment (Fig. 7D and E), showing that class I HDAC inhibition reduces tolerance of melanoma cells to DSBs that arise during the processing of O\(^6\)MeG DNA lesions induced by temozolomide. Next, the question of whether RAD51, and consequently HR, protect melanoma cells against temozolomide was addressed. Following shRNA knockdown of RAD51, A375 cells were significantly sensitized to temozolomide-induced apoptosis (Fig. 7F). The data show that inhibition of class I HDACs suppresses DSB repair by HR in melanoma cells and that this results in their sensitization to temozolomide.

Discussion

Despite our growing understanding of how DNA lesions induced by chemotherapeutics such as dacarbazine or temozolomide trigger death (42), our grasp of the cellular context in melanomas that cause intrinsic resistance to these agents is still incomplete and may explain why melanoma patients face such a bleak prognosis. Examples of why metastatic melanomas are resistant to therapy are: they bypass cell-cycle checkpoints (43), they express low levels of critical apoptosis proteins (27, 44), they retain p53 wild-type status (45) allowing them to upregulate DNA repair genes (DDB2, XPC; ref. 29), and they express an oncogenic form of BRAF giving them a growth advantage (46). Here, we addressed the contribution of class I HDACs in malignant melanoma protection against methylating chemotherapeutics, used, despite serine/threonine kinase inhibitors and immune-stimulating biologics (1, 3), as genotoxic ‘gold standard’ in the therapy of this cancer.

First, we show that melanoma tumors obtained from pre-treatment patients express HDAC1/2, and that melanoma cell lines overexpress HDAC1/2/3. These findings point to possible roles of these HDACs in melanoma tumor formation and in the resistance of this cancer to therapy. Before we addressed whether HDAC1/2/3 contribute to the resistance of melanomas to methylating agents, we first had to show that these HDACs can be inhibited. To this end, an inhibitor of class I HDACs (35), namely VPA, was used and confirmed that this inhibitor is active in melanoma cells.

Having established the experimental system, we demonstrate that inhibition of class I HDACs sensitized melanoma cells to temozolomide-, fotemustine-, and IR-induced apoptosis, while not sensitizing primary melanocytes. Therefore, we conclude that class I HDAC activity protects melanoma cells against genotoxic interventions. Temozolomide, fotemustine, and IR used in the therapy of metastatic melanoma induce different spectrums of DNA lesions, repaired by different DNA repair pathways. Repair of temozolomide-induced DNA lesions is mediated by MGMT, base excision repair (BER) and HR, repair of fotemustine-induced DNA lesions is mediated by MGMT, nucleotide excision repair (NER) and interstrand crosslink (ICL) repair in which HR plays a role and IR-induced DNA lesions are repaired by BER, non-homologous end joining (NHEJ) and HR (6, 42). For temozolomide, our data show that inhibition of HDAC1/2/3 causes sensitization to the DNA lesion O\(^6\)MeG, and that this sensitization is likely not due to modulation of MGMT. Furthermore, the protective role of class I HDAC activity in melanomas was confirmed in a panel of cell lines in vitro and a melanoma xenograft model in vivo. Collectively the data revealed that class I HDAC activity protects melanoma cells against methylating agents and that this class of HDACs is a promising target for intervention during therapy with temozolomide.

HDACs are centrally involved in protein regulation and gene expression. Therefore, HDACs can theoretically play a role in the expression of any gene, which demands screening methodologies for identifying the factor causing the observed phenotype. To this end, we used a real-time PCR array. The array data showed that VPA affects factors involved in apoptosis and DNA repair on gene
expression level. The conclusions drawn from this are that VPA sensitizes melanoma cells to temozolomide either due to better apoptosis fidelity or less effective DNA repair. To test these hypotheses, the changes in these two groups of genes had to be validated by real-time PCR and Western blot analysis. RAD51 and FANCD2 passed validation. By employing different HDAC inhibitors and siRNA targeting the specific HDACs, we were able to identify HDAC2 as the class I HDAC responsible for the regulation of RAD51. Further support for the role of DNA repair in the observed sensitization is found in the results obtained with fotemustine- and IR-treated melanoma cells. Although temozolomide, fotemustine, and IR induce different spectrums of DNA lesions, only HR is involved in the repair of all these genotoxins (47). As RAD51 and FANCD2 play central roles in HR (48), the downregulation of these HR proteins may therefore be responsible for the observed sensitization.

Having identified decreased DNA repair as the possible mechanism for sensitization to temozolomide upon HDAC1/2/3 inhibition, we addressed the questions of whether class I HDAC inhibition affects HR activity, whether class I HDAC inhibition results in more unrepaired DSBs following temozolomide treatment, and whether RAD51 protects melanoma cells against temozolomide-induced apoptosis. Inhibition of class I HDACs decreased HR-mediated DSB repair activity by almost 50%. This decreased HR repair activity resulted in an increase in unrepaired DSBs showing that class I HDAC inhibition prevents melanoma cells from tolerating DSBs arising due to the processing of the temozolomide-induced O6MeG adducts. Furthermore, RAD51 protects melanoma cells treated with temozolomide as knockdown of RAD51 significantly sensitized the cells to temozolomide. We should state that this protective role of RAD51 was observed once MGMT was inhibited and that the DSBs arising from O6MeG is dependent on MMR. Attenuated
HR could sensitize melanoma cells to PARP-1 inhibition as synthetic lethality caused by PARP-1 inhibition is not influenced by MGMT or MMR. We show that HDAC inhibition by VPA exacerbates the killing effect of PARP-1 inhibition by olaparib in the absence of temozolomide, and that temozolomide could possibly increase this cytotoxicity.

The findings reported here suggest a model where HDAC1/2/3 are key nodes in the resistance of malignant melanoma cells to methylation-based therapies by ameliorating the HR-dependent repair of DSBs arising during the processing of O6MeG DNA lesions. It forms a firm basis for targeting these HDACs during genotoxic agent–based therapies in malignant melanoma and possibly also in other tumor types.

Disclosure of Potential Conflicts of Interest
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Authors' Contributions
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HDACs and Homologous Recombination in Melanoma

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Andrea Krumm, Christina Barckhausen, Pelin Kücük, et al.

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