Pharmacologic suppression of MITF expression via HDAC inhibitors in the melanocyte lineage

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Summary

Melanoma incidence continues to rise at an alarming rate while effective systemic therapies remain very limited. Microphthalmia-associated transcription factor (MITF) is required for development of melanocytes and is an amplified oncogene in a fraction of human melanomas. Microphthalmia-associated transcription factor also plays an oncogenic role in human clear cell sarcomas, which typically exhibit melanoma-like features. Although pharmacologic suppression of MITF is of potential interest in a variety of clinical settings, it is not known to contain intrinsic catalytic activity capable of direct small molecule inhibition. An alternative drug-targeting strategy is to identify and interfere with lineage-restricted mechanisms required for its expression. Here, we report that multiple histone deacetylase (HDAC)-inhibitor drugs potently suppress MITF expression in melanocytes, melanoma and clear cell sarcoma cells. Although HDAC inhibitors may affect numerous cellular targets, we observed suppression of skin pigmentation by topical drug application as well as evidence of anti-melanoma efficacy in vitro and in mouse xenografts. Consequently, HDAC inhibitor drugs are candidates to play therapeutic roles in targeting conditions affecting the melanocyte lineage.

Key words: HDAC inhibitor/MITF/melanoma/clear cell sarcoma/pigmentation

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Introduction

Microphthalmia-associated transcription factor (MITF) is a basic-helix/loop/helix-leucine-zipper transcription factor which plays an essential lineage-specific role during melanocyte development (Hodgkinson et al., 1993; Hughes et al., 1993; Steingrimsson et al., 1994). The MITF gene has a complex promoter organization, with at least nine distinct isoforms sharing exons 2 to 9. Some of these isoforms exhibit tissue-specific restriction such as M-MITF, which is expressed in melanocytes and melanoma but not most other lineages (Hershey and Fisher, 2005; Udo et al., 2000). The melanocyte-specific promoter is located most proximal to the common downstream exons and is known as the M-MITF promoter (Fuse et al., 1996). As a transcription factor, MITF regulates multiple genes related to melanin synthesis, control of apoptosis, and cell cycle progression (Goding, 2000; Levy et al., 2006; Shibahara et al., 2001). Appropriate regulation of MITF is required for cell growth/survival in melanocytes and recent work has implicated MITF as a melanoma oncogene due to its amplification in ~20% of human metastatic melanomas (Garraway et al., 2005). In addition, human clear cell sarcomas typically contain a chromosomal translocation which produces the EWS-ATF1 fusion protein (Brown et al., 1995; Fujimura et al., 1996; Lessnick et al., 1995). This chimeric oncoprotein directly triggers dysregulated expression of MITF, which in turn appears to play a vital role within this tumor (Davis et al., 2006). Thus, MITF is a potentially attractive molecular target for melanoma and clear cell sarcoma therapy. However being a transcription factor lacking ligand dependency, it is largely considered a difficult drug target by current approaches.

In contrast, histone deacetylase inhibitors (HDACi) have been shown to exhibit anti-cancer effects, and suberoylanilide hydroxamic acid (SAHA) has been FDA approved for treatment of cutaneous T-cell lymphoma (Kelly and Marks, 2005; Kelly et al., 2003, 2005; O’Connor et al., 2006). Cancer-related consequences of HDACi drugs include growth arrest and apoptosis as well as repression of angiogenesis (Liu et al., 2006). An effect of HDACi drugs on growth arrest and apoptosis has been shown in melanoma cells, but the mechanism has been poorly understood (Boyle et al., 2005). Recently, HDACi-treated embryos were observed to exhibit disrupted pigmentation in zebrafish and Xenopus
(Gurvich et al., 2005), suggesting that HDACi might have effects on melanocyte lineage-specific factor(s).

In this study, we provide evidence that multiple HDAC inhibitor drugs, including sodium butyrate (NaB), Trichostatin A (TSA), SAHA, and LBH589, repress M-MITF expression in melanoma and clear cell sarcoma cells. Although HDACi drugs have numerous effects, we observe this lineage-selective effect in melanocytes, which is associated with transcriptional downregulation of the M-MITF promoter. The resulting data suggest that HDACi drugs may antagonize MITF, with consequent therapeutic applications either alone or perhaps in combination with other MITF suppressing strategies, to control skin pigmentation and/or survival of MITF-dependent cancers.

**Results and discussion**

**HDACi drugs repress M-MITF levels in melanoma and clear cell sarcoma cell lines**

Microphthalmia-associated transcription factor is a lineage-survival factor as well as amplified oncogene in ~20% of metastatic melanomas. Because of its vital role in the melanocyte lineage as well as in clear cell sarcoma (Davis et al., 2006), MITF is an attractive molecular target for melanoma and clear cell sarcoma therapy. In searching for small molecules which affect MITF expression, we wanted to explore if HDACi drugs could exhibit a role as MITF antagonists given the observed disruption of pigmentation in zebrafish and Xenopus (Gurvich et al., 2005). The melanocyte-specific isoform of MITF (M-MITF) migrates as a doublet (Figure 1A, B, arrows) due to serine 73 phosphorylation by MAPK (Hemesath et al., 1998), whereas higher migrating MITF isoforms (asterisk) reflect non-melanocyte-specific forms of MITF derived from distinct promoters (Hershey and Fisher, 2005; Udono et al., 2000). We subjected several established melanoma and clear cell sarcoma cell lines to HDACi treatments and reproducibly observed suppressed expression of the M-MITF doublet protein species, but not the non-melanocyte-specific forms of MITF (Figure 1A, B, and data not shown). The acetylation status of histone H3 by HDACi in human melanoma cells correlated with activity of the HDACi drugs. Other HDACi drugs, suberoylanilide hydroxamic acid (SAHA) and LBH589 (George et al., 2005), also repressed M-MITF expression in melanoma cells (Figure 1A). We detected diminished RNA levels of the M-MITF isoform in UACC62 melanoma cells with NaB and TSA (Figure 2A and data not shown), suggesting that the decreased M-MITF protein levels result from reduced mRNA levels. To further examine whether stability of the MITF mRNA may contribute to diminished protein levels following HDACi exposure, MITF mRNA decay kinetics were tested following actinomycin D treatment, in the presence or absence of HDACi. No significant difference was observed in the rate of MITF mRNA decay by this assay (Figure S1A), suggesting that HDACi exposure is unlikely to affect MITF expression via altered RNA stability.

As HDACi drugs led to M-MITF-specific mRNA downregulation and SOX10 and LEF1 are key upstream transcriptional regulators of the M-MITF promoter, we also examined the expression of these upstream regulators after the same treatments. Diminished protein and mRNA levels of SOX10 and LEF1 were observed in a time-dependent manner (Figure 2A, B) which correlated closely with, or slightly preceded MITF's decreases. Moreover, SOX10 overexpression was able to significantly rescue M-MITF expression and growth upon HDACi treatment in UACC62/SOX10 cells but not in UACC62/GFP (control) cells or UACC62/SOX10/Dom cells (Figure 2C and Figure S1B). Collectively these data suggest that the potent suppression of M-MITF expression by HDACi drugs likely occurs via downregulation of transcription of the MITF gene and might be
mediated by suppression of the upstream factors SOX10 or LEF1.

Skin-lightening effects of topical HDACi treatment

Multiple human pathologic hyperpigmentation conditions exist, including melasma, aging spots (lentigo senilis), post-inflammatory hyperpigmentation, and others. Aside from MITF’s requirement for melanocyte viability, it also plays a central role in control of pigmentation. Multiple pathways control expression and activity of MITF, including the action of α-melanocyte stimulating hormone (e.g. in sun/UV-induced pigmentation) where it is thought to activate expression of multiple enzymes in the pigment synthesis cascade and related factors involved in melanin transport/secrection (Levy et al., 2006). Moreover, mutants of MITF exist which alter pigment color, without absence of melanocytes (Steingrimsson et al., 1996) suggesting that suppression of MITF might potentially offer a topical therapeutic strategy for treatment of hyperpigmentation, if a dose were utilized which affects pigmentation without loss of melanocyte viability. To test this possibility, we used a mouse model of forskolin-induced hyperpigmentation (D’Orazio et al., 2006). The congenic C57BL/6 K14-SCF Mc1r<sup>e/e</sup> mouse strain contains epidermal melanocytes due to the keratin 14 (K14) promoter driving expression of stem cell factor (Scf; also known as Kitl) (humanized skin model) (Kunisada et al., 1998). Mc1r<sup>e/e</sup> encodes a frameshift mutation in the melano-cortin receptor gene, which produces the redhair/fairskin phenotype, and which is susceptible to forskolin-induced hyperpigmentation (D’Orazio et al., 2006). As shown in Figure 3A, dorsal pigmentation induced by topical treatment with forskolin was potently repressed by topical treatment with the HDAC inhibitor TSA. Using Fontana-Masson staining, which detects melanin in tissue sections, substantial suppression of skin pigmentation was
observed in the TSA-treated skin (Figure 3B). Direct assessment of MITF expression in skin upon TSA treatment was carried out by co-staining with a monoclonal antibody against MITF as well as staining for S100-antigen (melanocytic marker) as MITF and S100-antigen are co-expressed in epidermal melanocytes (Figure 3C; EtOH and FK). Treatment of mice with topical HDACi drug produced a significant decrease in MITF-staining, whereas S100 appeared unaffected by TSA treatment (Figure 3C; TSA and TSA + FK). As M-MITF appears to be the most abundantly expressed MITF isoform in melanocytes (Figure 1A), decreasing levels of MITF-staining in S100-antigen positive cells likely reflected M-MITF reduction although the MITF antibody could detect other MITF isoforms as well. Importantly using this topical treatment regimen, melanocyte viability did not appear to be affected because the ratio of melanocytes/number of keratinocytes/field) among each group did not significantly change (EtOH; 5.49 ± 1.03, TSA; 6.47 ± 1.07, FK; 5.81 ± 1.97, FK + TSA; 5.67 ± 0.87). These data demonstrate that HDACi could repress MITF expression as well as pigmentation within skin of topically treated mice under conditions in which melanocyte viability was not compromised.

**Melanoma growth suppression in vitro and in vivo**

Depletion of MITF induces cell cycle arrest or loss of survival (Carreira et al., 2006; Garraway et al., 2005; Widlund et al., 2002) likely via a variety of mechanisms including induction of p27kip1 or alterations of other cell cycle or apoptotic factors in specific settings. In order to examine if HDAC inhibitors (HDACi) influence melanoma cell growth or survival, WST-1 assays were carried out using human melanoma cells treated with HDACi drugs sodium butyrate (NaB) or trichostatin A (TSA) (Figure 4A). Both NaB (2.5 mM) and TSA (1 μM) repressed cell growth at 48 h in UACC62 melanoma cells and UACC257 melanoma cells. We also detected apoptosis [propidium iodide (PI) negative/Annexin V positive cells] in UACC62 cells treated with TSA (12.2%) as compared with vehicle (1.7%) by FACS analysis (Figure 4B). To determine whether HDAC inhibition would attenuate melanoma growth in vivo, we assessed the effects
of systemic administration of LBH589 on the growth of UACC62 xenograft tumors. Mice with established tumors were treated with either vehicle or LBH589 at a dose of 10 mg/kg administered daily via intraperitoneal injection. Treatment with LBH589 significantly (P < 0.05) suppressed tumor growth, such that the average tumor volume of LBH589-treated animals was 22% of vehicle-treated animals after 17 days of treatment (Figure 4C). There was no appreciable difference in body weights between the two treatment groups throughout the study (Figure 4D), demonstrating that treatment with LBH589 was well tolerated and not overtly toxic. To determine whether LBH589 treatment altered MITF expression, RNA was isolated from tumors and isoform-specific quantitative RT-PCR was used to determine M-MITF expression. Tumors from animals treated with LBH589 had reduced abundance of M-MITF mRNA compared with vehicle-treated tumors (Figure 4E, P < 0.05).

In this study, we provide evidence that the HDACi drugs NaB, TSA, SAHA, and LBH589 repress MITF expression in melanoma and clear cell sarcoma cells. Moreover, the repression of MITF with HDACi was also observed in vivo using a humanized mouse skin model in which HDACi topical treatment inhibited forskolin-induced pigmentation without loss of melanocyte viability. In addition, systemic HDACi treatment exhibited significant growth suppression in a human melanoma xenograft model. The mechanism of MITF suppression by HDACi is incompletely understood, but appears likely to involve suppression of one or several required upstream transcriptional regulators of MITF. The mechanism through which HDACs affect their expression awaits further analysis.
Collectively these data identify HDACi drugs as small molecule inhibitors of MITF expression, which may be worthy of clinical study in a variety of settings associated with MITF over-activity, including aberrant pigmentation or melanocytic neoplasia. As MITF small molecule targeting strategies are identified, it is plausible that combinations of such agents (including HDACi) may hold therapeutic promise for systemic treatment of melanoma.

Materials and methods

Cell cultures

UACC62, UACC257, SK-MEL-2, and SK-MEL-28 human melanoma cells were cultured in RPMI-1640 medium (Mediatech Inc., Herndon, VA, USA) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin/glutamine. MeWo and SK-MEL-5 human melanoma cells were cultured in DMEM medium (Mediatech Inc.) containing 10% FBS and penicillin/streptomycin/glutamine. 501mel human melanoma cells were cultured in Ham’s F-10 medium (Mediatech Inc.) containing 10% FBS and penicillin/streptomycin/glutamine. Primary human melanocytes between passages 2 and 5 were derived from neonatal foreskins and grown in TIVA media (Ham’s F12; Mediatech Inc.), 7% fetal bovine serum, penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA, USA), 1 x 10^{-4} M 3-isobutyl-1-methyl xanthine (IBMX; Sigma), St Louis, MO, USA), 50 ng/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA; Sigma), 1 μM Na2VO4, and 1 x 10^{-3} M N\textsuperscript{6},\textsuperscript{2}-O-dibutyryladenosine 3:5-cyclic monophosphate (dbcAMP; Sigma). Clear cell sarcoma cell lines, DTC-1, SU-CCCS-1, and CCS292, were cultured in DMEM medium containing 15% FBS and penicillin/streptomycin/glutamine. The stable UACC62 transformed cells containing pLNCTX-GFP, pLNCTX-SOX10 (full-length SOX10), and pLNCTX-SOX10/Dom (truncated SOX10) were selected with G418 (500 μg/ml) (Mediatech Inc.), and referred to as UACC62/GFP (control), UACC62/SOX10, UACC62/SOX10/Dom. These stable transformed lines were grown in RPMI-1640 medium containing 10% FBS, 500 μg/ml G418, and penicillin/streptomycin/glutamine. The cells were treated with sodium butyrate (NaB) (2.5 mM) (Sigma-Aldrich, St Louis, MO, USA), Trichostatin A (TSA) (1 μM) (Sigma-Aldrich), suberoylanilide hydroxamic acid (SAHA) (10 μM) (BIOMOL, Plymouth Meeting, PA, USA), or LBH589 (10 μM) (Novartis, New York, NY, USA) for various times as indicated.

Western blotting analysis

Whole cell extracts (10 μg/lane) were prepared using the method of Schreiber et al. (1989) and then subjected to Western blotting analysis using anti-MITF monoclonal antibody (Weilbaecher et al., 1998), anti-acetylated histone 3 (Ac-H3) polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA), anti-SOX10 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LEF1 antibody (REMB6) (Exalpha Biologicals, Maynard, MA, USA), anti-HA monoclonal antibody (Roche, Indianapolis, IN, USA) or anti-α-tubulin monoclonal antibody (Sigma).

Real-time RT-PCR

The total RNAs were prepared by using Trizol (Invitrogen) from UACC62 and 501mel human melanoma cells with TSA for each indicated time. Real-time RT-PCR was performed by using iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA) from cDNAs. The primers used were 5’-cat tgt tat gct gga aat gct aag a-3’ (sense) and 5’-ggc ttc tgt tat ggt gta ctt gg-3’ (antisense) for M-MITF, 5’-gct gct gaa cga aag tga ca-3’ (sense) and 5’-ggc ttc tgt tat ggt gta ctt gg-3’ (antisense) for LEF1. All reactions were run in triplicate on an iCycler instrument (Bio-Rad), and MITF-M message levels were normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

In vivo de-pigmentation assay

C57BL/6JJ Mc1r<sup>dom</sup> mice were crossed with K14-Scf transgenic mice (D’Orazio et al., 2006). Depleted animals were exposed to topical forskolin, TSA, or vehicle control. Skin biopsies were harvested and processed for Fontana-Masson (eumelanin) staining and immunostaining by S100 antibody (DakoCytomation, Carpinteria, CA, USA), or MITF antibody (23).

In vitro growth assays

Cell metabolic activity was assessed by Cell Proliferation Reagent WST-1 (Roche Diagnostics) according to the manufacturer’s instruction. Briefly, the UACC62, UACC257, SK-MEL-2, SK-MEL-28, and primary human melanocytes (1 x 10^{3} cells/well) were cultured for 24 h after plating in 96-well dishes and then the HDAC inhibitors, NaB (2.5 mM) or TSA (1 μM), were added to each well. After 48 h, the WST-1 solution was added. The cells were incubated for an additional 1 h with the WST-1 solution. Finally, the absorbance of 460 nm was measured using a microplate reader. Absorbance of cells treated with HDAC inhibitors was normalized to cells treated with the appropriate control, either PBS or ethanol vehicle.

Apoptosis analysis

UACC62 cells were incubated with TSA (1 μM) or EtOH for 24 h. After incubation, the cells were collected and washed by PBS twice. The cells were resuspended in Annexin V Binding buffer (10 mM Heps, 140 mM NaCl, and 2.5 mM CaCl\textsubscript{2}, pH 7.4). The suspensions were incubated with Annexin V Alexa647 (Invitrogen) conjugates and propidium iodide (PI).

In vivo melanoma growth assay

UACC62 xenografts were generated by subcutaneous injection of 5 x 10^5 cells into NCr/Nude mice. After 1 week of growth, mice with measurable tumors were segregated into groups that were treated with either LBHS89 10 mg/kg i.p. daily or vehicle control (n = 9 per group). Tumors were measured with calipers, and the tumor volume was calculated as 0.5 x length x width^2. For each animal, relative tumor volume over the treatment course was determined by normalizing to the tumor volume at the start of treatment (day 1 of treatment). Statistical significance between the two treatment arms was determined by two-tailed Student’s t-test. All animal studies were performed under the auspices of protocols approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.

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References


Supplementary material

The following supplementary material is available for this article online from http://www.blackwellsynergy.com/doi/full/10.1111/j.1755-148X.2008.00480.x

Figure S1. M-MITF mRNA decay and rescue of growth suppression by SOX10 overexpression.

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