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# Targeting histone deacetylase 6 mediates a dual anti-melanoma effect: Enhanced antitumor immunity and impaired cell proliferation

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ABSTRACT

The median survival for metastatic melanoma is in the realm of 8–16 months and there are few therapies that offer significant improvement in overall survival. One of the recent advances in cancer treatment focuses on epigenetic modifiers to alter the survivability and immunogenicity of cancer cells. Our group and others have previously demonstrated that pan-HDAC inhibitors induce apoptosis, cell cycle arrest and changes in the immunogenicity of melanoma cells. Here we interrogated specific HDACs which may be responsible for this effect. We found that both genetic abrogation and pharmacologic inhibition of HDAC6 decreases *in vitro* proliferation and induces G1 arrest of melanoma cell lines without inducing apoptosis. Moreover, targeting this molecule led to an important upregulation in the expression of tumor associated antigens and MHC class I, suggesting a potential improvement in the immunogenicity of these cells. Of note, this anti-melanoma activity was operative regardless of mutational status of the cells. These effects translated into a pronounced delay of *in vivo* melanoma tumor growth which was, at least in part, dependent on intact immunity as evidenced by the restoration of tumor growth after CD4+ and CD8+ depletion. Given our findings, we provide the initial rationale for the further development of selective HDAC6 inhibitors as potential therapeutic anti-melanoma agents.



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## 1. Introduction

Cancer is the second leading cause of death in industrialized countries and the incidence of some cancers, including melanoma, continues to increase (Siegel et al., 2013; Watson et al., 2011; Rigel, 2010). There are few treatments that significantly improve overall survival in advanced melanoma. However, immunotherapeutic strategies that abrogate immunologic checkpoints have shown promise, especially in patients that lack a molecular target (Bhatia et al., 2009). For example, blocking antibodies against negative co-stimulatory receptors CTLA-4 and PD-1 have demonstrated significant clinical activity, achieving complete responses even in heavily pretreated populations; however, only approximately 20–30% of these are durable (O'Sullivan Coyne et al., 2014). This may be due to a multitude of immune evasive and suppressive strategies exploited by tumor cells. An effective immune response requires three signals: antigen presentation in the context of MHC, co-stimulation of T-cells, and regulatory cytokines. Tumor cells have been shown to be active participants in immune suppression through manipulation of these three axes via downregulation of MHC (Wu et al., 2012), upregulation of immune checkpoint blocking ligands, and expression of anti-inflammatory cytokines, respectively (Kamma et al., 1991; Ruiter et al., 1991; Daar and Fabre, 1983; Rabinovich et al., 2007; Neuner et al., 2001; Hsieh et al., 1997), as an example. Therefore, a better understanding of mechanisms that underlie immune tolerance versus activation may help improve the efficacy of these therapies.

Histone deacetylases (HDACs) are a group of enzymes that remove acetyl groups from lysine residues from diverse protein targets beyond histone proteins. In 2009, Choudhary et al., identified over 3000 potential sites on cytoplasmic and nuclear proteins with diverse roles including cellular signaling, protein turnover, and cell survival (Choudhary et al., 2009). The role of HDACs in oncogenesis has already led to the approval of three HDAC inhibitors (HDACi) for the treatment of refractory cutaneous T-cell lymphoma and peripheral T-cell lymphoma (Marks and Breslow, 2007; Piekarz et al., 2009; Thompson, 2014), and several others are currently under various stages of development from pre-clinical research to clinical trials (Woan et al., 2012).

HDACs have been reported to possess immunomodulatory activities (Chou et al., 2005; Khan et al., 2008; Magner et al., 2000; Buglio et al., 2011; Serrano et al., 2001). Our group and others have previously shown that HDACi possess antitumor activity, both through direct cytotoxicity and improved immune responses (Khan and Tomasi, 2008; Vo et al., 2009). In regards to the latter, treatment with some HDACi result in upregulation of MHC and co-stimulatory molecules, and modulation of cytokine expression (Woan et al., 2012). However, these prior studies have utilized pan-HDACi, which inhibit the 11 known HDACs with differing potencies. Therefore, it remains to be determined which HDACs are responsible for these anti-tumor effects. The idea of substrate specificity for individual HDACs is not new; however our group was the first to demonstrate opposing roles of two HDACs (HDAC6 and HDAC11) in regulating a single target, in antigen presenting cells, namely the potent anti-inflammatory cytokine IL-10 (Cheng et al., 2014). Moreover, identifying and targeting specific HDACs could improve the tolerability of treatment by limiting unwanted off target effects already observed in clinical trials involving pan-HDACi (Bishton et al., 2007; Duvic et al., 2007; Glaser et al., 2003).

Since tumor cells are active immunologic participants and have been shown to exhibit many characteristics of professional APCs (Kamma et al., 1991; Ruiter et al., 1991; Daar and Fabre, 1983; Rabinovich et al., 2007; Neuner et al., 2001; Hsieh et al., 1997), we sought to determine if specific HDACs were responsible for the effects observed when using pan-HDACi. In this study we demonstrate that both pharmacologic inhibition and genetic abrogation of HDAC6 recapitulates many of the effects observed with pan-HDACi. Namely, HDAC6i resulted in cell cycle arrest in G1 and increased expression of tumor antigens *in vitro*, translating into delayed tumor growth *in vivo*, which was dependent on intact immunity. Taken together, our findings demonstrate an immunoregulatory role of HDAC6 in melanoma, providing rationale for the use of selective HDAC6i to improve antitumor immunity.

## 2. Materials and methods

### 2.1. Mice

All animal studies were performed in compliance with approved protocols by the IACUC at the University of South Florida. C57BL/6 mice were purchased from the NCI laboratories (Fredrick, Maryland, USA), and B6.CB17-Prkdc (scid)/SzJ immunodeficient mice were purchased from Jackson Laboratories (Bar Harbor, Massachusetts, USA) For *in vivo* tumor studies, mice were subcutaneously injected into the shaved flank with $1.3 \times 10^5$ B16–F10 melanoma cells suspended in 100 μL Hank's buffered salt solution (HBSS) 1×.

### 2.2. Patient samples

Patient-derived resected melanoma specimens were obtained from Dr. Sarnaik's Lab at Moffitt Cancer Center through a University of South Florida Institutional Review Board-approved regulatory protocol. The cells were extracted directly from melanoma tumor and cultured in RPMI 1640 supplemented with L-glutamine, 10% FBS, 100 IU/mL Penicillin, 100 μg/mL Streptomycin, 1% sodium pyruvate, 1% non-essential amino acid, 0.05 mM of 2-mercaptoethanol and 1% gentamycin. The cells were grown under humidified conditions at 37 °C and 5% $CO_2$.

### 2.3. Cells

B16–F10-luc murine melanoma cell line was obtained from the ATCC and cultured in RPMI 1640 supplemented with 10% FBS, 100 IU/mL Penicillin, and 100 μg/mL Streptomycin. The human melanocyte cell line HEMn-LP was obtained from Invitrogen and grow in Medium 254 supplemented with HMGS. Human melanoma cell lines were obtained from Dr. Smalley's

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