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How do tumor cells respond to HDAC inhibition?

Andrea Newbold[1,2], Katrina J Falkenberg[3], H. Miles Prince[2,4], and Ricky W Johnstone[1,2]

[1]Cancer Therapeutics Program, The Peter MacCallum Cancer Centre, St Andrews Place, East Melbourne 3002, Victoria, Australia

[2]Sir Peter MacCallum Department of Oncology, University of Melbourne, Parkville 3052, Victoria, Australia.

[3] IMP - Research Institute of Molecular Pathology, Dr. Bohr-Gasse 7, 1030 Vienna, Austria

[4]Division of Cancer Medicine, The Peter MacCallum Cancer Centre, St Andrews Place, East Melbourne 3002, Victoria, Australia

Corresponding author

Prof Ricky W Johnstone

Postal Address: Locked Bag 1, A'Beckett St, Melbourne, Vic, Australia, 8006

Telephone: +61-3-9656-3727

Fax: +61-3-9656-1411

Email: ricky.johnstone@petermac.org

Key words

Histone Deacetylase inhibitor (HDACi), apoptosis, cell cycle arrest, senescence, differentiation, tumor immunogenicity.

Abbreviations

Trichostatin A (TSA)

Histone deacetylase inhibitor (HDACi)

Chronic lymphocytic leukemia (CLL)

Reactive oxygen species (ROS)

Homology-directed repair (HRR)

Non-homologous end joining (NHEJ)

Valproic acid (VPA)

Senescence-associated β-galactosidase (SA-β-Gal)

Acute myeloid leukemia (AML)

Acute promyeloctyic leukemia (APL)

NUT midline carcinoma (NMC)

Patient derived xenografts (PDX)

Breast cancer cell lines (BCLs)

Long non-coding RNA (lncRNA)

Cancer stem cell (CSC)

Leukaemia stem cell (LSC)

Chronic myeloid leukemia (CML),

Imatinib mesylate (IM)

Head and neck squamous cell carcinoma (HNSCC)

Down syndrome-associated myeloid leukemia (DS-AML)

Natural killer cell (NK cell)

Dendritic cell (DC)

Cytotoxic T lymphocyte (CTL)

ABSTRACT

It is now well recognised that mutations, de-regulated expression and aberrant recruitment of epigenetic readers, writers and erasers is a fundamentally important process in the onset and maintenance of many human tumors. The molecular, biological and biochemical characteristics of a particular class of epigenetic erasers, the histone deacetylases (HDACs), have been extensively studied and small molecule HDAC inhibitors (HDACi) have now been clinically approved for the treatment of human hemopoietic malignancies. This review explores our current understanding of the biological and molecular effects on tumor cells following HDACi-treatment. The predominant responses include induction of tumor cell death and inhibition of proliferation that in experimental models have been linked to therapeutic efficacy. However tumor cell-intrinsic responses to HDACi, including modulating tumor immunogenicity have also been described and may have substantial roles in mediating the anti-tumor effects of HDACi. We posit that the field has failed to fully reconcile the biological consequences of exposure to HDACis with the molecular events that underpin these responses, however progress is being made. Understanding the pleiotrophic activities of HDACis on tumor cells will hopefully fast track the development of more potent and selective HDACi that may be used alone or in combination to improve patient outcomes.

INTRODUCTION

A highly organised chromatin structure maintained and managed by 'epigenetic regulators' is crucial for the control of vital cellular processes including gene transcription, DNA replication and DNA repair [1, 2]. In simple terms, epigenetic regulators can be divided into different functional groups based on their molecular activities: epigenetic writers that lay down epigenetic marks on DNA or amino acid residues on histone tails (e.g. DNA methyltransferases and histone acetyltransferases respectively); epigenetic readers that bind to epigenetically modified DNA or histones and recruit other chromatin or transcriptional regulators (e.g. bromodomain-containing proteins); and epigenetic erasers, that catalyse the removal of epigenetic marks, such as histone deacetylases [3, 4]. It has recently become apparent that either mutations in genes encoding epigenetic regulators, or the aberrant recruitment of epigenetic regulators through direct physical interaction with mutant DNA binding proteins, can be key driver events in several human diseases, including cancer [1, 4]. Based in part on these findings, small molecules that target epigenetic readers, writers and erasers have been developed and many have progressed through pre-clinical development to testing in clinical trials. In fact, DNA methyltransferase inhibitors and HDAC inhibitors (HDACis) have been approved for clinical use by regulatory bodies such as the FDA [5]. This review will focus on HDACis in the treatment of cancer. Specifically, we will discuss the varied responses of tumor cells to these agents. A number of excellent reviews have been recently published that detail the chemical and molecular properties of a large number of HDACis and their current status in clinical development. The reader is directed towards these papers for background information [4, 6-9].

As a precursor to more detailed discussions on the anti-cancer activities of HDACis it is important to highlight two contextual issues regarding these agents. First, HDACis were not originally developed through a "rational" drug development program based on the identification of HDACs as important cancer targets. HDACis such as sodium butyrate, trichostatin A (TSA) and the now FDA-approved agent, vorinostat, were originally identified through empiric screens for compounds that induced tumor cell differentiation [10-12]. Subsequent studies [13] demonstrated that these agents targeted HDACs and this discovery led to more sophisticated analyses of the molecular mechanisms of action of these compounds. Second, while the focus has

traditionally been on histones as the major substrates of HDACs, it is well understood that more than 1700 cellular proteins can be acetylated [14] and hyperacetylation of non-histone proteins can have major effects on cell physiology [4]. This second point is particularly relevant to this review as the tumor cell-intrinsic responses to HDACis may result from histone hyperacetylation and altered transcriptional responses, or hyperactylation of non-histone proteins, or a combination of these effects (Fig 1). We posit that the field has failed to fully reconcile the biological consequences of exposure to HDACis with the molecular events that underpin these responses, however progress is being made. Herein we will explore the current understanding of how tumor cells respond to HDACis and how these responses may impact the anti-tumor effects of these anti-cancer agents.

BIOLOGICAL RESPONSES OF TUMOR CELLS TO HDACIS

1. Cell death

Cell death is one of the most well-studied anti-tumor activities of HDACis and has been directly linked to therapeutic efficacy in pre-clinical models [15-20]. There is unlikely to be a single molecular pathway or process that mediates HDACi-induced cell death and indeed both the intrinsic and extrinsic apoptotic pathways have been shown to be functionally important in different experimental systems (Fig 2). We and others have clearly demonstrated that activation of the intrinsic pathway through increased expression and/or activity of pro-apoptotic BH3-only genes such as *Bim, Bid, Bmf, Noxa* and *Puma* is essential for the potent apoptotic and therapeutic effects of diverse HDACis [20]. Moreover, using isogenic tumor and normal cells, we recently identified an HDACi-responsive, tumor cell-selective, pro-apoptotic transcriptional signature that was heavily weighted towards upregulation of pro-apoptotic Bcl-2-family genes and downregulation of pro-survival Bcl-2 genes [21]. The paradigm that HDACis can mediate tumor cell apoptosis through coordinated up- and down-regulation of Bcl-2 family proteins is supported by similar results from other studies using a variety of different HDACis and different tumor types including,

but not restricted to, chronic lymphocytic leukemia (CLL) [22] and other hematological malignancies [23], hepatocellular carcinoma [24] and breast carcinoma [25]. The hypothesis that altered expression of apoptotic genes following HDACi treatment occurs through direct histone hyperacetylation of promoters of these genes is experimentally supported for *Bmf* [26-28], *Bim* [29], *Puma* [30] and *Noxa* [29]. In addition, expression of pro- and anti-apoptosis genes can be regulated indirectly through HDACi-mediated activation and/or induction of transcription factors such as E2F1 [31], FoxO1 [32], p53 [33] and Sp1 [34], or altered expression of micro-RNAs such as miR-15a and miR-16 [35], miR-106b-93-25 [36], and miR-17-92 [37]. Collectively, these studies highlight how HDACis can trigger a common biological response (apoptosis) through different molecular mechanisms – direct and indirect regulation of apoptotic gene expression (Fig 1).

In certain circumstances, activation of the extrinsic pathway through increased expression of death receptors (e.g. DR4, DR5, Fas) and their cognate ligands (e.g. TRAIL, FasL) [20], and/or downregulation of intracellular regulatory molecules such as c-FLIP can play fundamentally important roles in HDACi-induced apoptosis [38, 39]. Blockade of these pathways can decrease the efficacy of HDACis in pre-clinical settings [15, 16]. Furthermore, the induction of death receptors and ligands has been demonstrated to be tumor cell-selective, providing an additional molecular mechanism for the tumor cell specificity of these agents [15, 16, 40]. As with the intrinsic pathway, a range of regulatory mechanisms have been demonstrated for death receptors and their cognate ligands, including both direct promoter hyperacetylation as well as more indirect effects [20] (Fig 1).

A third mechanism by which HDAC inhibition induces tumor cell death is the generation of reactive oxygen species (ROS) (Fig 2) and pre-exposure to antioxidants has been shown to protect from vorinostat-induced cell death [41-45]. HDACi-mediated ROS production, decreased expression of free radical scavengers [46, 47] or a combination of both leads to loss of mitochondrial membrane potential, release of cytochrome c and subsequent tumor cell death [42]. In a panel of prostate cancer cell lines, the most vorinostat-resistant cell line failed to show accumulation of ROS upon

vorinostat treatment, compared to large increases in ROS levels in sensitive cell lines [48]. Vorinostat and MS-275 treatment led to specific accumulation of ROS and caspase activation in transformed cells only, while normal cells exhibited increased levels of the reducing protein thioredoxin, thereby revealing a potential mechanism for tumor-cell selectivity of HDACis [46]. In addition, caspase inhibition did not prevent HDACi-induced cell death, suggesting a non-apoptotic ROS-mediated cell death.

HDACis can also induce cell death through accumulation of DNA damage, by down-regulating the expression or impairing the function of DNA repair proteins [45, 49] (Fig 2). HDACi treatment induces tumor cell-selective downregulation of proteins required for DNA damage sensing, homology-directed repair (HRR) and non-homologous end joining (NHEJ) including Ku70, Ku80, RAD50, RAD51, MRE11, DNA-PK, BRCA1, EXO1, CHK1 and CHK2 [49-52]. In addition, HDACis impair recruitment of DNA repair proteins such as RAD51 and BRCA1 to sites of DNA damage, correlating with decreased DNA repair efficiency and increased γH2AX foci [53]. Furthermore, HDACi treatment was shown to induce hyperacetylation of Ku70 in prostate cancer cells, reducing Ku70 DNA binding and thereby sensitising to DNA damaging agents, etoposide and doxorubicin [54]. Acetylation of Ku70 also disrupted its interaction with Bax, resulting in release of Bax and induction of apoptosis through the intrinsic pathway [54]. In addition, DNA damage can result from HDACi-induced ROS accumulation [41, 55] as well as HDACi-induced inhibition of DNA replication [56]. HDAC3 deletion, or inhibition with the selective HDAC3 inhibitor RGFP966, leads to delayed cell cycle progression, increased DNA damage and apoptosis, indicating that HDAC3 is a key mediator of replication-mediated DNA damage [57, 58]. Based on these and other studies, the utilisation of HDACis as therapeutic radiosensitizers has been proposed [59, 60].

The p53 tumor suppressor protein is an important mediator of apoptosis and is itself functionally regulated through acetylation [61]. Hyperacetylated p53 is more stable than the unmodified form and has enhanced DNA binding activity leading to

increased expression of apoptotic genes such as NOXA[61]. The importance of p53 in mediating HDAC-induced cell death is somewhat unclear and likely to be context dependent. We have demonstrated that knockout of p53 does not affect the ability of different HDACi to kill tumor cells [17, 19, 62] however there is evidence that acetylated p53 may contribute to HDAC-induced cell death. In one study, HDACis and γ-irradiation synergistically triggered cell death in human non-small cell lung cancer cells, and p53 acetylation at lysine 382 was significantly increased [63]. Interestingly, the apoptotic cell death seen by the combination was suppressed in cells transfected with mutant K382R p53 and C135Y p53 which also resulted in the loss of the acetylation at lysine 382. In another study, HDACis induced cell death via the mitochondrial apoptotic pathway and activated p53 via hyperacetylation and nuclear re-localization, without affecting its protein expression. HDACi-induced cell-killing and $p21^{WAF1/CIP1}$ upregulation was impaired in p53 inactive-cells indicating that functional p53 may be important for the molecular activities of HDACi in this experimental system [64].

2. Cell cycle arrest

HDACis can induce cell cycle arrest, often concomitantly with other effects such as apoptosis, senescence and differentiation (Fig 2). HDACi-induced cell cycle arrest commonly occurs at the G1/S cell cycle checkpoint however arrest at the G2/M boundary is also observed [65]. Blocking apoptosis results in cell cycle arrest at either the G1/S or G2/M checkpoints suggesting a "hierarchy" of HDACi-induced biological responses where apoptosis is dominant over cell cycle arrest [65]. Mechanistically it is believed that HDACi-induced G1/S arrest occurs primarily through transcriptional changes in cell cycle regulatory genes [65]. A prominent negative regulator of G1/S transition is the CDK inhibitor $p21^{WAF1/CIP1}$ encoded by *CDKN1A* and HDACi treatment commonly leads to p53-independent induction of *CDKN1A* transcription [65, 66]. Using a t(8;21) acute myeloid leukemia murine xenograft model to analyse the cell cycle effects of HDACis, valproic acid (VPA) induced hyperacetylation of the *CDKN1A* promoter, enhanced $p21^{WAF1/CIP1}$ expression, suppressed the phosphorylation of retinoblastoma protein and blocked transcription

activated by E2F, thus inducing a G0/G1 arrest. In addition, VPA induced direct downregulation of gene/protein expression of cyclin D1, cyclin E1, CDK4, and CDK6 [67]. In a similar study using a model of colon carcinoma, vorinostat decreased tumor volume in a nude mouse model via decreased expression of survivin and cyclin D1 [68]. Other CDK inhibitors including $p15^{INK4B}$, $p19^{INK4D}$ and $p57^{KIP2}$ that inhibit entry into S phase are also up-regulated by HDACi treatment [69].

HDACi-induced G2/M cell cycle arrest appears to occur independently of DNA damage-induced G2/M arrest [70]. HDAC inhibition blocks activation of G2/M regulatory complexes such as Cyclin A/CDK2 and Cyclin B/CDK1, through decreased levels of Cyclin A and Cyclin B1, thereby leading to G2/M arrest [70]. In the case of Cyclin A, HDACi treatment induces its hyperacetylation resulting in subsequent ubiquitination and degradation [71]. HDACis also downregulate levels of Aurora A and PLK1, both involved in G2/M transition, and induce expression of Gadd45 which can induce G2/M arrest through inhibiting activity of cdc2/Cyclin B [69].

We recently demonstrated that mouse lymphoma cells made resistant to HDACi-induced apoptosis through overexpression of Bcl-2 underwent G1 arrest concomitant with increased expression of *Cdkn1a* and *Cdkn1b* (encoding $p27^{Kip1}$) following HDACi treatment. Knockout of *Cdkn1a* together with knockdown of *Cdkn1b* in these cells relieved the HDACi-induced block at G1/S, however the cells then arrested in G2/M [72]. This provides evidence for an additional biological hierarchy where HDACi-induced apoptosis is dominant over G1/S arrest and G1/S arrest is dominant over G2/M arrest.

3. Senescence

Treatment of tumor cell lines with HDACis, or dual knockout of HDACs 1 and 2, can induce senescent-like cell morphological changes and biochemical markers such as senescence-associated β-galactosidase (SA-β-Gal) staining, concomitant with increased expression of $p21^{CIP1/WAF1}$ [73]. Interestingly, functional *CDKN1A* and *TP53* were dispensable for vorinostat-induced senescence of HCT116 colon

carcinoma cells [74]. More recently, polycomb repressor group proteins such as Bmi1 and EZH2 have been shown to mediate HDACi-induced senescence. Two studies implicated the downregulation of PRC1 component, Bmi1, as a key effector mechanism in HDACi-induced senescence [75, 76]. Cho and colleagues demonstrated that HDACi-induced Bmi1 downregulation was an indirect consequence of enhanced miR-31 expression [75]. Moreover, the PRC2 component and senescence inhibitor protein, EZH2, was similarly downregulated following HDACi treatment, also through activation of miR-31 [75, 77].

4. Differentiation

HDACis such as butyrate were first identified as potential anti-cancer therapies based on their ability to induce differentiation [10], another key anti-tumor effect. Oncogenic fusion proteins such as PML-RARα and AML1-ETO initiate tumorigenesis at least in part by inhibiting cellular differentiation [78, 79]. These oncoproteins recruit HDACs and in the case of PML-RARα-driven acute myeloid leukemia (AML), namely acute promyeloctyic leukemia (APL), HDAC inhibition can induce a differentiation-mediated anti-tumor response. Repression of RARα-target genes by PML-RARα results in a myeloid differentiation block that can be reversed following treatment with HDACis [80], leading to therapeutic benefit in mouse models of PML-RARα-driven AML [81]. Understanding the mechanistic drivers of this form of AML provided a strong molecular rationale to combine HDACis with ATRA to excellent effect in pre-clinical studies [79]. HDACi treatment significantly enhanced responses to retinoic acid in sensitive APL cell lines and restored retinoid sensitivity in resistant APL lines [82]. Clinical trials demonstrated impressive response rates [83, 84], with one clinical study showing monomyelocytic differentiation in patients with stable disease following treatment with VPA and ATRA [84].

Using a genetically engineered mouse model of AML1-ETO-driven t(8;21) AML, we have recently demonstrated that HDACis induce terminal differentiation of AML1-ETO cells *in vitro* and *in vivo,* resulting in prolonged therapeutic benefit in mice bearing these leukemias [85]. Induction of myeloid differentiation occurred concomitantly with HDACi-mediated induction of tumor cell cycle arrest, decreased

expression of c-Kit and an increase in the pro-myeloid differentiation genes *PU-1*, *GATA-2*, *SCL* and *C/EBPa* [85]. Additionally, *in vitro* HDACi-treatment of AML1-ETO-positive Kasumi-1 cells led to tumor cell differentiation, sometimes accompanied by apoptosis [86, 87].

HDAC inhibition also functions as a differentiation therapy in NUT midline carcinoma (NMC), a lethal paediatric tumor driven by the BRD4-NUT oncogenic fusion protein, which prevents squamous cell differentiation. Vorinostat treatment of NMC cell lines restored normal histone acetylation levels and squamous differentiation transcriptional programs *in vitro*, resulting in tumor growth inhibition and increased survival of mice transplanted with BRD4-NUT NMC tumors [88]. In a direct bench-to-bedside approach, a patient with NMC received vorinostat treatment after differentiation of their primary tumor cells was achieved *ex vivo*. The patient showed an anti-tumor response by FDG-PET, as did xenografted tumors from the same patient, providing matching pre-clinical and clinical evidence for HDACi differentiation therapy for NMC. More broadly, HDACis have been shown to induce differentiation of primary sarcoma cell lines [89] and human tumor cells representative of liver [90], lung [91] and breast cancer [92]. Finally, a study evaluating the effect of the HDACi, abexinostat, on cancer stem cells showed differentiation of putative cancer stem cells in patient derived xenografts (PDX) and breast cancer cell lines (BCLs) [93]. Gene expression profiling of BCLs sensitive and resistant to HDACi-induced differentiation identified expression of the long non-coding RNA (lncRNA) *Xist* as a predictor of the differentiation response, with resistant PDX and BCLs expressing significantly higher levels of *Xist* than sensitive cells [93].

HDACi may be useful as inducers of cancer stem cell (CSC)/Leukaemia stem cell (LSC) apoptosis and differentiation [94-96] with studies in the leukemia setting the most compelling performed to date. In one study using a novel HDACi in acute myeloid leukemia (AML), HDAC inhibition abolished the chaperone function of heat shock protein 90 resulting in degradation of client proteins such as FLT3 and mediated selective loss of the LSC population [94]. In a separate model of Philadelphia chromosome (Ph+) chronic myeloid leukemia (CML), the BCR-ABL inhibitor imatinib (IM) failed to eliminate LSCs alone, however combined with an

HDACi induced apoptosis in quiescent CML LSCs [96]. Finally, the *in vivo* self-renewal activities of LSCs in AML1-ETO and PLZF-RARα-driven leukemia models were substantially suppressed by HDACi treatment [97]. However, it is clear that not all LSCs will be affected by HDACi as treatment of mice bearing PML-RARα acute promyelocytic leukemia (APL) with VPA reduced the bulk leukemia population but had no effect on the LSCs [81]. Interestingly, VPA has been recently shown to suppress the self-renewal properties of head and neck squamous cell carcinoma (HNSCC) CSCs [98] and other studies have suggested that HDACi can suppress CSC function in models of breast cancer [93], glioblastoma [99] and sarcoma [100]. In most cases the molecular events underpinning the putative effects on solid CSC function have yet to be revealed and whether these responses translate from *in vitro* to *in vivo* effects remains unclear.

5. Autophagy

HDACis can induce autophagy in tumor cells [101-103] (Fig 2), however whether this plays any role in mediating the therapeutic effects of HDACis is debatable. *In vitro* studies have shown that autophagy was required for vorinostat-induced death of hepatocellular carcinoma cell lines [104], however induction of autophagy suppressed vorinostat-induced apoptosis in glioblastoma cell lines [105]. Autophagy can also play opposing roles in matched HDACi sensitive and resistant cells. Activation of autophagy promoted apoptosis in vorinostat-treated U937 parental cells, while intriguingly, U937 cells with acquired resistance to vorinostat demonstrated high basal levels of autophagy that was necessary to protect the cells from vorinostat-induced apoptosis and maintain the resistant phenotype [106]. Exactly how HDAC inhibition activates an autophagic response remains unclear however a recent study demonstrated that activation of the FOXO1 transcription factor by HDACis as an important mediator of this response [107]. Knockdown of key autophagy genes was used to address the functional importance of autophagy in mediating HDACi-induced cell death and therapeutic benefit in an *Apaf1*-null, apoptosis-defective model of B cell lymphoma [108]. Eμ-myc/*Apaf-1*$^{-/-}$ lymphomas treated with vorinostat underwent

cell death in the absence of caspase activation but with hallmark biochemical and morphological changes associated with autophagy. shRNA-mediated knockdown of autophagy genes *Atg5* and *Atg7* resulted in a loss of vorinostat-mediated autophagy changes but had no effect on vorinostat-mediated tumor cell death *in vitro* and *in vivo.* Furthermore, the therapeutic benefit of vorinostat treatment *in vivo* was unaffected, indicating that autophagy is dispensable for vorinostat's anti-tumor activity in this model.

In contrast to the many studies demonstrating that HDACi treatment induces autophagy, other studies report that autophagy can be repressed by HDACis [109-111]. HDACi treatment in Down syndrome-associated myeloid leukemia (DS-AML) cells repressed autophagy below a critical threshold to mediate cell death, likely through transcriptional downregulation of *ATG7* and ATG7 hyperacetylation resulting in functional suppression of this key autophagy protein [111].

6. Tumor immunogenicity and immunogenic cell death

HDACis can enhance immunogenicity and antigen-presenting capacity of tumor cells by increasing expression of putative tumor antigens [112], MHC class I and II molecules and associated antigen processing machinery [113-115], co-stimulatory molecules [116] and natural killer (NK) cell-activating ligands [117-119] (Fig 2). HDACis can increase NK cell-mediated anti-tumor responses through increased NKG2D ligand expression on the surface of HDACi-treated tumor cells, which led to increased NK cell cytotoxicity [120]. This increased cytotoxicity was abrogated by blocking the NKG2D receptor, suggesting that HDACi treatment activates NKG2D signaling pathways [121].

HDACis can also induce immunogenic tumor cell death, which is characterised by translocation of the endoplasmic reticulum protein calreticulin [122], release of the nuclear chaperone HMGB1 [123] and release of cellular ATP [124]. Immunogenic cell death can lead to enhanced tumor clearance by CTL killing [115] and dendritic cell (DC) phagocytosis [125]. We have established that immune-stimulating antibodies intended to augment APC and CTL activity strongly enhanced the *in vivo*

anti-tumor effects of HDACis and resulted in sustained adaptive anti-tumor immunity [125]. Moreover, we and others recently showed that an intact host immune system is critical for vorinostat and panobinostat to induce sustained anti-cancer responses against solid and haematological tumors [116, 126].

In contrast to these studies demonstrating that HDACis engage the immune system to mediate anti-tumor effects, others have reported that HDACi treatment and knockdown of HDAC2 and HDAC3 reduced levels of B7-H6, an NK cell activating ligand, reducing NK cell activation and tumor cell killing [127]. Moreover, HDACi treatment resulted in upregulation of the immune checkpoint molecules PDL-1 and PDL-2 on the surface of melanoma cells thereby providing a putative mechanism of immune escape for these tumors following exposure to HDACis.

HDACis can regulate the expression and stability of oncoproteins

As detailed above, HDACis induce a variety of biological responses that may ultimately be detrimental to tumor cell proliferation and survival and can regulate the expression, stability and function of oncogenic proteins to mediate these effects (Fig 1). Oncoproteins such as AKT, c-KIT, Her-2, Bcr-Abl, c-RAF and BRAF are chaperoned by Hsp90, a substrate of HDAC6, and HDACi-induced hyperacetylation of Hsp90 can result in their proteasome-mediated degradation [53, 128-132] (Fig 1). For example, Bcr-Abl drives t(9:22) chronic myeloid leukemia and treatment of Bcr-Abl-positive leukemia cells with the HDACi, LAQ824 or vorinostat, led to Hsp90 hyperacetylation and decreased association with Bcr-Abl resulting in its degradation and subsequent tumor cell apoptosis [131, 133]. Interestingly, tumors that developed resistance to small molecule Abl kinase inhibitors were still sensitive to HDACis via HDACi-induced degradation of Bcr-Abl [134]. It is this activity of HDACis that could prove hugely beneficial when treating patients who have failed to respond to ongoing treatment with small molecule kinase inhibitors.

The importance of the HDAC6/Hsp90 interaction for the therapeutic efficacy of HDACis has been the subject of considerable evaluation as it remains unclear how essential the selective inhibition of HDAC6 is for disruption of Hsp90 chaperone

protein function [132, 135]. While siRNA-mediated knockdown of HDAC6 resulted in Hsp90 hyperacetylation and Bcr-Abl degradation [131], romidepsin, a very weak HDAC6 inhibitor, also disrupted the Hsp90/Bcr-Abl interaction, leading to Bcr-Abl degradation [136]. In this case, romidepsin disrupted Hsp90 chaperone function through hyperacetylation of Hsp70, leading to increased association of Bcr-Abl with Hsp70 in an unstable Hsp70/Bcr-Abl complex, resulting in subsequent degradation of the oncogenic client protein. Interestingly, in our laboratory we found that Hsp90 hyperacetylation and disruption of the Hsp90/Bcr-Abl interaction could be achieved with both vorinostat and the HDAC1/2 selective inhibitor MRLB-223, further questioning the importance of HDAC6 in this effect [133].

HDAC inhibition can supress the oncogenic function and expression of c-Myc, which is a somewhat underappreciated effect of HDACis, particularly in tumors driven by aberrant Myc activity. Myc target gene expression is perturbed through direct downregulation of *Myc* and/or modulation of Myc-mediated transcription. Downregulation of *Myc* expression has been observed in a wide range of tumor types [137-143]. Furthermore, Myc interacts with a number of HDACs including HDAC1 [144], HDAC2 [145] and HDAC3 [146, 147]. Myc interacts with HDAC1 in a complex with Sin3b to regulate Myc-target genes including Myc itself [144]. Additionally, neuroblastoma and pancreatic cancer cells driven by N-Myc and c-Myc respectively, exhibit upregulated HDAC2 expression that contributes to Myc-driven proliferation, at least in part through silencing of cyclin G2 as a result of Myc recruiting HDAC2 to the cyclin G2 promoter [145]. Treatment of these cells with HDACis, or knockdown of HDAC2, reactivated cyclin G2 and inhibited tumor cell growth providing strong pharmaco-genetic evidence that HDAC2 can play a fundamentally important role in Myc-mediated oncogenesis. Moreover, a recent study demonstrated that in transformed cells Myc represses the miR-15 and let-7 families that target anti-apoptotic *Bcl-2* and *Bcl-xL* respectively. HDAC inhibition resulted in Myc-mediated upregulation of miR-15 and let-7, downregulation of Bcl-2 and Bcl-xL and subsequent apoptosis [148]. Taken together, these data indicate that under certain conditions, HDACis may indirectly target Myc expression and/or function in a similar fashion to the proposed activity of BET-family bromodomain inhibitors that have recently gained prominence as putative anti-cancer drugs for the treatment of Myc-driven tumors [149, 150].

CONCLUSION

HDACis are a group of natural and synthetic compounds that can mediate a diverse range of effects on tumor cells, including alterations in proliferation, survival and immunogenicity. Although these agents have been approved for clinical use, their anti-tumor effects as single agents have been limited to a subset of hematological malignancies, in particulat T cell lymphomas. In light of this, it is likely that in the future HDACi will be most effective as part as combination regimens. In terms of single agent HDACi activities, it is still unclear precisely how or why some patients experience extremely robust clinical responses. To date, there are no clearly defined biomarkers for response or genetic, biological or biochemical biomarkers of resistance to these agents. It is therefore likely that the true therapeutic potential of HDACis has yet to be realised. Further development of more selective agents that target individual HDACs, may be required to enhance the clinical benefit of HDAC inhibition. Studying the molecular and biological responses of tumor cells to HDAC-selective inhibitors will likely provide insight into the critical proteins and pathways regulating the anti-tumor effects of small molecules that broadly inhibit a range of different HDACs. Ultimately, this may lead to the development of even more potent and predictable therapeutic regimens and improved patient outcomes. The most recent studies indicating that HDACi can modulate tumor immunogenicity coupled with the compelling clinical data demonstrating the effectiveness of immune checkpoint inhibitors provides a strong rationale to combine these two modalities and the field awaits formal demonstration that these combination approaches will provide clinical benefit. Moreover, initial studies showing that HDACi can suppress the self-renewal properties of tumor initiating cells (CSCs and LSCs) provide another direction for further investigation. While HDACi have rapidly progressed through the drug-development pipeline to the point that four have been approved by the FDA for use in cancer patients, our view is that they have still not been used to their full potential as oncology agents. As stated above, their immunomodulatory effects and potential effects on tumor initiating cells provide the foundation for future combination approaches.

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CONFLICTS OF INTEREST

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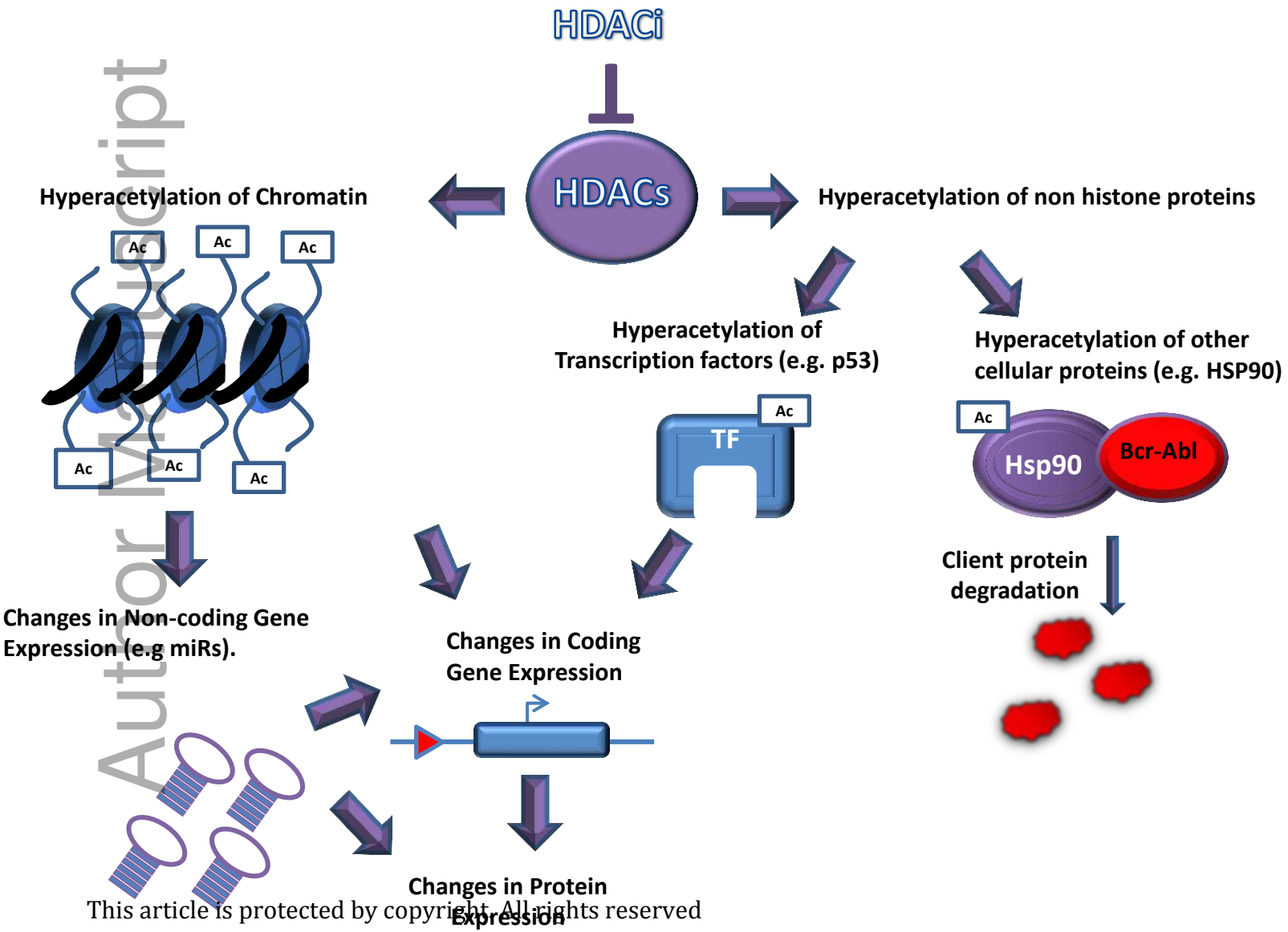
Figure Legends

Figure 1. Molecular responses to HDACis. HDACs primarily target histone proteins however these enzymes also deacetylate other cellular proteins. HDACi treatment results in histone hyperacetylation, chromatin remodeling and altered

expression of coding and non-coding genes. Non-coding genes such as microRNAs (miRs) that can subsequently regulate gene and protein expression. Transcription factors such as p53 are regulated by acetylation and HDACi can hyperacetylate and activate a wide variety of transcription factors that then alter target gene expression. Hyperacetylation of the protein chaperone Hsp90 following HDACi treatment results in release of client oncoproteins such as Bcr-Abl resulting in their proteasome-mediated degradation.

Figure 2. Biological responses to HDACis. HDACis induce a range of anti-tumor responses including: (i) cell death mediated by various pathways and processes including the intrinsic and extrinsic apoptotic pathways, enhanced ROS production and induction of DNA damage responses; (ii) Cell cycle arrest at the G1/s and G2/M checkpoints; (iii) Cellular senescence; (iv) terminal differentiation; (v) autophagy; (vi) enhanced immunogenicity through altered cell surface expression of immune regulatory molecules.



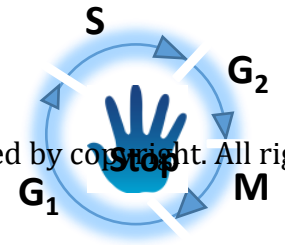
HDACi

- ↑ intrinsic apoptosis: ↑ Bid, Bim, Noxa, Puma; ↓ Bcl-2, Bcl-xL
- ↑ extrinsic apoptosis: ↑ DR4, DR5, Fas, TRAIL, FasL
- ↑ ROS production; ↓ free radical scavengers
- ↑ DNA damage: ↓ DNA repair protein expression, function, recruitment (Ku70, DNA-PK, RAD51); ↑ replication-mediated DNA damage
- ↑ immunogenic cell death
- ↓ oncoprotein expression and stability

cell death

- G1/S arrest: ↑ p21, p15, p19, p57 and ↓ Cyclin D1, Cyclin E1, CDK4, CDK6
- G2/M arrest: ↓ Cyclin A, Cyclin B1, Aurora A, PLK1

cell cycle arrest

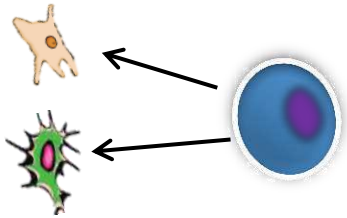


- morphological changes
- ↑ β-galactosidase staining, $p21^{CIP1/WAK1}$ expression
- ↓ Bmi1 and EZH2 expression

senescence

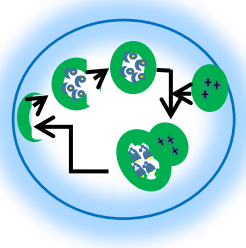
- de-repression of differentiation genes
- repression of stemness genes

differentiation



- controversial role in HDACi efficacy
- mechanisms unclear
- ↑ FOXO1 important for autophagy

autophagy



- ↑ antigen presenting capacity
- ↑ co-stimulatory and inhibitory molecules
- ↑ & ↓ NK cell ligand expression

tumor immunogenicity