Invest New Drugs (2010) 28 (Suppl 1):S3–S20
DOI 10.1007/s10637-010-9596-y

SPECIAL ISSUE ARTICLE

Histone deacetylase inhibitors: potential targets responsible for their anti-cancer effect

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Received: 10 November 2010 / Accepted: 12 November 2010 / Published online: 14 December 2010

Summary The histone deacetylase inhibitors (HDACi) have demonstrated anticancer efficacy across a range of malignancies, most impressively in the hematological cancers. It is uncertain whether this clinical efficacy is attributable predominantly to their ability to induce apoptosis and differentiation in the cancer cell, or to their ability to prime the cell to other pro-death stimuli such as those from the immune system. HDACi-induced apoptosis occurs through altered expression of genes encoding proteins in both intrinsic and extrinsic apoptotic pathways; through effects on the proteasome/aggresome systems; through the production of reactive oxygen species, possibly by directly inducing DNA damage; and through alterations in the tumor microenvironment. In addition HDACi increase the immunogenicity of tumor cells and modulate cytokine signaling and potentially T-cell polarization in ways that may contribute the anti-cancer effect in vivo. Here, we provide an overview of current thinking on the mechanisms of HDACi activity, with attention given to the hematological malignancies as well as scientific observations arising from the clinical trials. We also focus on the immune effects of these agents.

Keywords Histone deacetylase inhibitor · Mechanism of action

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Introduction

Histone deacetylase inhibitors (HDACi) induce a plethora of molecular and extracellular effects that singly, or in combination, result in potent anti-cancer activities. The clinical development of HDACi has been rapid, but fundamental questions about the mechanisms of anticancer activity remain: which HDAC(s) must be targeted to mediate the observed anticancer effects? Which molecular processes (ie chromatin remodeling, regulation of transcription factors, acetylation of non-histone targets) are critical? Finally, is apoptosis, differentiation or another biological effect responsible for the clinical responses we see? While it is clear that HDACi induce apoptosis associated with altered transcription of proteins involved in the intrinsic and extrinsic pathways, other mechanisms are in play, such as those relating to the aggresome/proteasome system. Through hyperacetylation of histone and non-histone targets, HDACi can induce quite diverse cellular effects. These include: altering immune responses through effects on the host and/or target cells; inducing permanent (i.e. senescence) or temporary (quiescence) cell cycle arrest usually at the G1/S transition; inhibiting angiogenesis; inhibiting apoptosis; and autophagy [1–5]. HDACi not only induce injury to the cell, they also modulate its ability to respond to stressful stimuli. Moreover, the anti-tumor effect is due to targeting not only the tumor cell itself, but also the tumor microenvironment and the immune milieu.

Inhibition of histone deacetylases and classification of the HDACi

HDACs are classified by their homology to yeast HDACs. Eighteen are known, of which the 11 zinc-dependent

enzymes belonging to class I, II, and IV constitute the focus of research, and of this review. HDACs, usually in conjunction with other corepressors, deacetylate lysine moieties in amino-termini of histones [6]. The acetylation status of the histone depends on the balance between deacetylase activity and histone acetyl transferase (HAT) activity. Deacetylation results in a relatively closed chromatin conformation that often leads to repressed transcription [6]. Thus, HDAC inhibitors are generally considered to be transcriptional activators [7]. However, gene expression profiling shows that as many genes may be repressed as derepressed after exposure to an HDACi. This is likely to be a consequence of the direct and indirect effects of these drugs on other transcriptional regulators and cell signaling pathways and/or due to the dynamic and complex interrelations between chromatin remodeling and regulated gene transcription [8, 9].

HDACi are currently classified according to their chemical structure, and each agent varies in its ability to inhibit individual HDACs (Table 1). HDACi share a common pharmacophore containing a cap, connecting unit, linker and a zinc binding group that chelates the cation in the catalytic domain of the target HDAC [10]. The pan-deacetylase inhibitors include vorinostat (suberoylanlide hydroxamic acid, SAHA), panobinostat (LBH589) and trichostatin A which inhibit class I, II and IV HDACs, while valproate, entinostat (MS-275) and romidepsin (depsipeptide, FK228) are considered class-I—specific, and tubacin, HDAC6-specific. (Table 1)

HDAC6 warrants special attention as a HDAC predominantly, [11, 12] but not exclusively [9] localized to the cytoplasm. HDAC6-specific effects, particularly those on cell motility and the proteasome and aggresome pathways (discussed below) are considered by some investigators to be responsible for much of the cytotoxicity of the HDACi. This is one example of how HDACi vary in their targets—the pan-HDACi include HDAC6 amongst their targets, while the class1-selective HDACi (such as romidepsin) do not. Such differences provide the rationale for the development of novel, highly HDAC-specific agents. For now, it is easiest to group the HDACi in commercial development into the pan-HDACi versus those that are class 1-specific, and it is probably not unreasonable to make generalizations about HDACi targets on that basis.

Apoptosis

HDACi can induce high rates of apoptosis at sub-micromolar concentrations in many cell-line models of hematological malignancy. Precisely which of the effects discussed below is most important remains a matter of conjecture and may well be cell-type and agent-specific. The two major apoptotic pathways are the death receptor (direct) and mitochondrial (indirect) pathways. HDACi have been shown to induce apoptosis through effects on mediators within either pathway or by inducing other signals within other cellular pathways that activate apoptosis.

Death receptor pathway

The death receptor pathway is triggered by the ligation of death receptors (Fas, TNF-R1, TRAIL) on the cell surface by tumor necrosis factor (TNF)-super family receptor ligands (Fas-L, TNFα, TRAIL, TR1a). In the case of TRAIL-induced death though ligation DR4 /TRAIL-R1 or DR5/TRAIL-R2, the adaptor molecule FADD is recruited, leading to caspase 8 activation through formation of the multi-protein death inducing signaling complex (DISC) and activation of final common effector, caspase 3. C-FLIP may inhibit or potentiate the binding of FADD and caspase 8, but is generally seen as an inhibitor of apoptosis [13, 14]. Tumor cells are more sensitive to TRAIL-induced death than normal cells, [15] and HDACi may further sensitize malignant cells to death-receptor-mediated apoptosis.

HDACi can increase expression of death receptors DR5 expression can be induced by HDACi in a dose and time—dependent manner in AML (HL-60), CML (K-562) [16] and myeloma cell lines [17]. Similarly, in mouse models of acute promyelocytic leukemia (APL) and AML-ETO dependent leukemia, valproic acid increased expression of death receptors and their ligands by the leukemic cells and cell death was dependent on the death-receptor pathway. These observations were confirmed in primary APL and AML-ETO samples, and were not seen on CD34+ stem cells from the same patients [18].

HDACi can induce apoptosis by directly stimulating the death receptor pathway Increased susceptibility to TRAIL-mediated death may occur without altered receptor expression. HDACi were able to re-sensitize Jurkat T-cell leukemia cells with acquired resistance to TRAIL-mediated death without changes in surface expression of death receptors, suggesting that in these cells post-DISC changes to the apoptotic cascade was important [19]. CLL cells are typically resistant to TRAIL-mediated death, [20] yet romidepsin and sodium valproate can sensitize CLL cells to DR4-mediated death through increased recruitment of FADD to the DISC [21–23].

TRAIL/Fas sensitization may also occur through down-regulation of c-FLIP [24–29] or increased expression of APAF1 [30]. These observations have been replicated in a mouse model where vorinostat augmented the effects of a murine DR5 agonist through down-regulation of c-FLIP

Table 1 Classes of DAC inhibitors, their HDAC targets and HDAC cellular distribution

		HDAC specificity										
	HDAC Cellular distribution	Nuclear				Nuclear, Cytoplasmic				Cytoplasmic[a]		Nuclear
	HDAC Class	I				IIa				IIb		IV
HDACi Class	HDAC	1	2	3	8	4	5	7	9	6	10	11
Short chain fatty acids	Butyrate	■	■	■	■	■	■	■	■			
	Valproate	■	■	■	■	■	■	■	■			
Hydroxamic acid derivative	Trichostatin A	■	■	■	■	■	■	■	■	■	■	■
	Vorinostat (suberoylanilide	■	■	■	■	■	■	■	■	■	■	■
	hydroxamic acid, SAHA)											
	Panobinostat (LBH589)♣	■	■	■	■	■	■	■	■	■	■	■
	Belinostat (PXD101)	■	■	■	■	■	■	■	■	■	■	■
	Tubacin									■		
Benzamide	Entinostat (MS-275)	■	■	■								
	Mocetinostat (MGCD0103)	■	■	■								■
Cyclic tetrapeptide	Romidepsin (depsipeptide)	■	■									

[a] HDACs 6 & 10 are typically found in the cytoplasm [12] however both have also been found in the nucleus and are likely to affect transcription [9, 220].

and XIAP and without changes in the expression of the receptor or of TRAIL [31].

Moreover, synergy of HDACi with death receptor agonists, in vitro and in vivo, has been demonstrated with different putative and possibly cell-line-dependent mechanisms [15, 17, 24, 31].

Death receptor signaling may not be essential for HDACi induced apoptosis Recent evidence shows that death receptor signaling is not essential for HDACi-induced apoptosis in various experimental model systems. Panobinostat and the related pan-HDACi LAQ824 were able to induce apoptosis and have a therapeutic effect in a transgenic murine model of Burkitt lymphoma (Eμ-*myc*) [32, 33]. This effect was preserved in an Eμ-*myc*/TRAIL$^{-/-}$ murine model in which TRAIL was not expressed.

Intrinsic (mitochondrial, stress-activated) pathway

The intrinsic pathway is activated by cell stress stimuli, such as free-radical generation, the generation of misfolded proteins, chemotherapy, and radiation or DNA damage. The increased mitochondrial permeability that ensues through activation of Bax and Bak results in the release of pro-apoptotic proteins, which in turn activate caspase 9 and finally, the common effector caspase, caspase 3. This pathway is partly controlled by the interplay between pro-apoptotic multidomain BCL-2-family proteins which may initiate mitochondrial membrane permeability (Bax and Bak), the pro-apoptotic BH3-only proteins (Bad, Bik, Bid, Bim, Bmf, Hrk, Puma, Noxa) that act as "sensors" of cellular stress and activate the intrinsic apoptotic pathway and pro-survival BCL-2-family proteins (BCL-2, BCL-XL, BCL-W, MCL-1, A1) that serve to "protect" mitochondrial integrity. There is interconnection between the extrinsic and intrinsic pathways through activation of Bid following cleavage by caspase-8 [34]. HDACi modify the cell's ability to respond to stressors, favoring apoptosis, and in addition probably contribute directly to cellular stress.

Gene expression profile studies show that HDACi alter the expression of members of the intrinsic apoptosis cascade such that the overall profile is pro-apoptotic [15, 17, 35–40]. For example, BCL-XL and BCL2 are often down regulated, and Bim, Bax and Bak are consistently up regulated [35, 40–43].

Over expression of BCL-2 or BCL-XL appears to be an important mechanism of resistance to HDACi, which can be overcome with small molecule inhibitors of BCL-2 such as ABT-737 [27, 30, 32, 33, 44–49]. Exploratory gene expression profile studies on clinical samples from patients with cutaneous T cell lymphoma (CTCL) treated with the HDACi panobinostat showed altered expression of Bcl2 family genes [50]. However, whether these are direct effects of the altered histone structure or associated with alterations in other mediators of transcription remains unclear.

The absence of a functional apoptosome does not preclude a efficacy from the HDACi. In an Eμ-*myc* mouse model in which either apaf-1 or caspase 9 were deleted, apoptosis was reduced, however HDACi were still able to kill the tumour cells and doubled the survival of the mice affected by this aggressive model of lymphoma [33].

Accumulation of reactive oxygen species (ROS) occurs after exposure to HDACi, and may trigger apoptosis selectively in cancer cells [51–56]. Normal cells appear to be spared of this effect, possibly through up regulation of the Trx-binding protein -2, which protects cells from the effects of ROS in normal but not tumor cells [51, 57]. The relative importance of ROS to HDACi induced apoptosis is suggested by the ability of the PEITC (a glutathione depleting compound) to enhance the cytotoxicity of vorinostat in leukemia cell lines and primary samples [56].

The ubiquitin/proteasome system and the misfolded protein response

Another potential trigger of HDACi-induced cell death arises through the potential effect of these agents on the *misfolded protein response* (MPR). The MPR is comprised of a number of cellular processes which protect the cell from toxicity arising from the accumulation of misfolded proteins. Misfolded proteins may arise as a consequence of defective protein synthesis, or due to other cellular derangements that result in a change in conformation of pre-formed protein [58]. Folding of proteins occurs in the endoplasmic reticulum (ER) and is reliant on the chaperone function of HSP90 [59, 60]. In this way, HSP90 prevents degradation of client proteins.

The ER responds to increased transcriptional activity in the cell by activation of the ER stress response. Through signaling from the ER three responses to increased ER stress can be initiated: 1. Decreased protein transcription, 2. Increased transcription of genes of the ER to increased long term processing capacity, or 3. apoptosis [60]. Apoptosis may be initiated by a number of trans-membrane receptors in the ER that then activate the intrinsic apoptotic pathway via c-Jun terminal kinase (JNK) [61]. Misfolded proteins may also be targeted for destruction through the proteasome. Targeting to the proteasome occurs through a number of protein modifications most importantly, ubiquitinylation.

Aggregates of misfolded protein are relatively resistant to destruction by the proteasome, and form in the context of proteasome inhibition, insufficiency or dysfunction [62]. Misfolded proteins accumulate focally in into an aggresome via a microtubule—an HDAC-6-dependent mechanism. The aggresome is then targeted for destruction by the autophagosome [12, 62–64]. Overall, the aggresome pathway is a homeostatic and cytoprotective mechanism which may rescue the cell in the context of proteosomal overload, inhibition or dysfunction. The ubiquitin-proteasome-aggresome pathways are thought to be particularly relevant targets for anti-cancer therapy of myeloma, where production of immunoglobulin requires a properly functioning endoplasmic reticulum and proteasome.

HDAC inhibitors affect functioning of the proteasome / aggresome pathways in three key ways (which make their combination with proteasome inhibitors particularly appealing) [65]. Firstly, inhibition HDAC6 results in hyperacetylation of HSP90 and HSP70 [66] which subsequently promotes misfolding and depletion of client proteins, including c-RAF, AKT and CDK4 and induces ER stress [67–70]. Recent evidence suggests that in a model of mantle cell lymphoma, induction of the ER stress-response gene CHOP is critical to panobinostat-induced cytotoxcity [68]. Secondly, HDAC hyperacetylation of tubulin leads to defective function of the dynein motor complex required for aggresome formation [7, 62, 63, 65, 68]. Inability to compensate for additional ER stress through a functioning aggresome pathway primes the cell for, and potentially initiates, apoptosis. Finally, a loss of function screen, discovered that proteasome deregulation through a pathway involving HR23B (RAD23B) was in part responsible for HDACi-induced apoptosis [72]. HR23B possesses ubiquitin-like domains and shuttles proteins to the proteasome. It also has a role in nucleotide excision repair, which was not shown to be critical in the effects of the HDACi. In this study, CTCL cells possessing higher levels of HR23B were more sensitive to HDACi induced death, and HDACi were shown to decrease proteasome function in treated cells in an indirect manner. Experimental depletion of the HR23B restored proteasomal function and reduced HDACi sensitivity. These observations were expanded in a subsequent study [73] in which an association between reduced HR23B expression in CTCL tissue biopsies and clinical response was observed. The authors concluded that HR23B expression may prove to be a useful biomarker to predict responsiveness to HDACi.

The evidence pointing to aggresome and proteasome dysfunction after HDACi therapy and the importance of HDAC6 in the maintenance of ubiquitin-proteasome-aggresome function [74] has been the basis for combinations of HDACi with proteasome inhibitors [71, 75]. However HDAC6 inhibition and tubulin acetylation may not be required, either for HDACi efficacy as a mono therapy or for synergy from the combination of HDACi with the proteasome inhibitors [76]. Buglio et al, hypothesized that the HDAC-selective mocetinostat (which has no effect on HDAC6) would make a more attractive agent for combination with bortezomib due to a perceived lower risk of thrombocytopenia compared to the pan-HDACi. Their preclinical experiments showed that the combination of mocetinostat and bortezomib was synergistic in Hodgkin lymphoma cell lines through reduction of the NfKb levels typically associated with HDAC inhibition. This synergy was HDAC6-independent, and brings into question the necessity for HDAC6 inhibition for combination therapies with the proteasome inhibitors. In a recent clinical study,

the combination of bortezomib with the HDAC 1 and 2-specific romidepsin was able to rescue patients with bortezomib-refractory myeloma, adding weight to the concept that inhibition of HDAC6 is not required for this type of drug combination to be of benefit to patients [77]. Indeed, although not compared directly in a trial, romidepsin appears to be at least as effective as the pan-HDACi, vorinostat for cutaneous T-cell lymphoma, the only indication for which HDACi have earned FDA approval [78].

Changes to p53 and the cell cycle

p53 is one of the most commonly altered transcription factors in cancer, and is found to be inactivated or mutated in various acute leukemias, CLL, myeloma and lymphoma. It is a promiscuous transcription factor with interactions with many key cellular pathways, including, but not limited to, those of Rb-E2F, MAP-kinase, IGF-1/AKT, Wnt-beta-catenin and cyclin-CDK via p21 [79]. Wild-type p53 is activated and accumulates in the nucleus in response to stress signals such as DNA damage, hypoxia, spindle damage and heat shock, amongst others. This response is modified by kinases (ATM, AT, CHK2, CHK1), acetyl-transferases (CBP/ p300, pCAF, TRAF), PML, SUMO-1 and HMG1 and also deacetylases including the HDAC1/ mSin3 complex [79–85]. Ubiquitin-mediated proteasomal degradation contributes to the control of p53 levels. The overall effect of p53 activation is cell cycle arrest (predominantly by activation of $p21^{Waf1/Cip1}$) and apoptosis (through increased expression of pro-apoptotic genes of the intrinsic pathway).

Many of the pathways discussed in this review influence p53, and thus the HDACi have several means of modulating p53. The importance of the acetylation status of p53 (and thus the role of direct HDAC-p53 interactions) is controversial [86], however there is evidence that acetylation of p53 is enhanced in the setting of cellular stress [81], is required to interrupt Mdm-2 mediated repression of p53, [84, 87] increases the affinity of p53 for DNA [88], reduces ubiquitin-mediated degradation of the transcription factor [89], and can enhance expression of $p21^{Waf1/Cip1}$ [89]. A number of studies demonstrate activation of p53 after HDAC inhibition [89, 90]. However in most reports the apoptosis and p21 induction following HDAC inhibition can be induced in a p53 *independent* manner—an observation that may be clinically relevant for the treatment of tumors harboring mutated p53 [32, 48, 91–93].

It is postulated that the HDACi-mediated effects on the cell cycle may be a key reason for the differential toxicity and responses between normal and transformed cells. Cell cycle arrest at G1 associated with induction of CDKN1A/ $p21^{WAF1/CIP1}$ is a key response to almost all of the currently available HDACi [1, 7, 94, 95]. Down-regulation of CCND1/cyclin D may also contribute [96, 97]. However, induction of cell cycle arrest may protect cells against cytotoxic agents that require cell cycling for efficacy. Cell cycle arrest may also partly explain the tumor selectivity of HDACi [98, 99]. HDACi can also induce cell cycle arrest at G2/M. Tumor cells lacking a functional G2 checkpoint and that proceed into mitosis after HDACi therapy, undergo apoptosis. By contrast, normal cells (with an intact G2 checkpoint) are able to maintain arrest G2/M following withdrawal of HDACi treatment [98, 99]. This difference may go some way towards explaining the tumor selectivity of the HDACi.

Cytokine signaling

Hematological malignancies are frequently associated with altered cytokine dependency, with perturbation of cytokine expression, receptor abnormalities, or with dysfunction of the post-receptor signaling cascades. Generally, binding of a cytokine to its receptor results in receptor dimerization. The cytoplasmic domains of cytokine receptors bind JAKs (Janus kinases) which phosphorylate the receptor and activate each other. In turn, the signal transducer and activators of transcription (STATs) are activated, also by phosphorylation, and STAT dimers enter the nucleus to initiate transcription at specific promoter regions. Chromatin remodeling is required for maximal transcriptional effect, and this is achieved through recruitment of HATs, [100, 101] as well as HDACs [102–105].

Activation of the STAT3 signaling pathway is associated with multiple cellular effects including increased proliferation and cell survival (through induction of pro-survival Bcl-2 family members), induction of angiogenesis, inhibition of p53, and with activation of Rel/NFκβ [106]. STAT3 hyper activation is described in multiple myeloma (where IL-6 dependence is of particular significance), [107–109] Hodgkin lymphoma, [110–114] c-myc dependent lymphoma, [115] diffuse large B cell lymphoma, [116] and the T-cell lymphomas [42, 106]. Mycosis fungoides and Sézary syndrome are associated with constitutive activation of STAT3 and probably induced over activity of STAT5 [100, 117, 118]. STAT5 hyper activation is also described in Hodgkin lymphoma, [119, 120] as is IL-4/STAT6 activation (with production of the cytokine thymus and activation-regulated chemokine- TARC) [121–124]. For a review of the role STATs in cancer, the reader is referred to a review by Yu and Jove [125]. The aberrancy of STAT activation in many hematological malignancies makes the STATs a rational target for anticancer agents.

The STATs are among the non-histone proteins hyper acetylated in response to HDACi. In addition to phosphor-

ylation, STAT1 activity is partially regulated by CBP-induced acetylation or HDAC1-influenced deacetylation [101, 105, 126]. Acetylated STAT1 binds to the RelA subunit of NFκβ and prevents its nuclear translocation and anti-apoptotic effects [127]. Furthermore, STAT1- and STAT2 - mediated transcription of genes is reduced after HDAC inhibition [86, 102, 103, 128, 129]. HDAC inhibition prevents the transcription of the targets of STAT5, by preventing the recruitment of SMRT, rather than by alterations of histone acetylation [104].

In a murine xenograft model of CTCL, panobinostat reduced levels of (activated) pSTAT3 and pSTAT5 in biopsies, but not the overall quantity of either STAT protein [49]. An early study suggested that STAT6 expression was reduced after treatment with vorinostat in skin lymphoma biopsies without changes to expression to STAT3 [130]. Subsequently, an important study by Fantin and colleagues demonstrated that clinical response to vorinostat was associated with a change in localization of pSTAT3 from predominantly nuclear to predominantly cytoplasmic, presumably reflecting functional inactivation of pSTAT3 by vorinostat in these responding patients. A lack of in vitro and clinical response of CTCL to vorinostat appears to be associated with persistent accumulation of *nuclear* pSTAT3 [42, 118].

In Hodgkin lymphoma, mocetinostat downregulates the expression of STAT6 and its target cytokines TARC and IL-5, with paradoxical increases in IL-13 [121]. It is postulated that altered cytokine profile results in a shift to a T_H1-type cellular response to the Reed-Sternberg cell.

Together, these observations suggest that effects on the JAK/STAT pathways and altered cytokine signaling are putatively important therapeutic targets of the HDAC inhibitors that warrant further clarification. Indeed, STAT-dependency may explain why it is the hematological malignancies that show the most promising responses to these agents.

Impact on the NFkB system NFκβ is a key transcription factor, sometimes termed the "master regulator", with anti-apoptotic effects and control over a number of inflammatory cytokines. When activated, it increases transcription of a number of pro-survival genes in the indirect apoptosis pathway. Constitutive activation of the NFκβ pathway is a feature of CTCL and myeloma, ALL, NHL (particularly activated B cell subtype of diffuse large cell lymphoma and mantle cell lymphoma) and CLL [131]. The inhibitory protein IκB prevents transcription of NfKb target genes by preventing entry of NFkB into the nucleus of the cell [132]. During inflammation there is phosphorylation and ubquitinylation of IκB, which targets IκB for destruction by the proteasome. This results in increased translocation of NfKb to the nucleus with increased gene transcription. Although recently brought into question, [133, 134] one important effect of the proteasome inhibitor bortezomib in myeloma is to reduce NfKb translocation to the nucleus by reducing proteasomal degradation of IkB.

NfKb is acetylated by p300/CBP, the biological effect of which varies according to the acetylation site. HDACi block HDAC3-mediated deacetylation of the p65/RelA NfKb subunit, leading to impairment of the IkB/cNfKb binding, increased NfKB nuclear translocation and increased DNA gene transcription [135]. Histone deacetylase inhibitors also activate NFkB via induction of reactive oxygen species (ROS) and the ATM/ NEMO/ SUMOylation pathway as well as the DNA damage response [54]. Activation of NfKB following HDAC inhibition may well be cytoprotective (pro-survival) and an important mediator of HDACi-resistance. As already discussed, this activation of NfKb may be meaningfully addressed by combining HDACi with proteasome inhibitors [77].

Immuno-modulatory effects of HDACi

Cellular immunogenicity

In addition to altering cellular responses to cytokine receptor activation through the pathways discussed above, HDACi appear to modulate multiple arms of the immune system, and are able to also act in a pro- or anti-inflammatory manner. Presently it is uncertain if the net effect potentially improves or hinders anti-cancer immune surveillance.

Up regulation of surface molecules Romidepsin, trichostatin A and sodium butyrate were able to up regulate co-stimulatory (CD80, CD86) and adhesion (ICAM-1) molecules as well as HLA-DR on HL-60 (promyelocytic leukemia) cells, which was associated with an increased mixed leukocyte response when compared to untreated cells [136]. This up regulation, also observed in solid tumors models, [137–140] may reduce the ability of a tumor to escape immune surveillance [141, 142]. Tumor immunogenicity may also be increased through *increased expression of tumor-associated antigens*. The carcinoma/testis antigens (CTA) are an attractive target for immunotherapy because they are sparingly expressed in normal, non-testicular tissue [143, 144]. CTA-specific cytotoxic T-lymphocytes (CTL) are detectable in patients with CTA-expressing tumors, and CTA have become a attractive target for adoptive cellular immunotherapeutic strategies. Previous studies show that expression the MAGE proteins is under epigenetic control and may be altered by the HDACi [145] and DNA demethylating agents [146]. Conceivably, CTA-specific CTL-response can therefore be

promoted through the use of epigenetic modifiers, which may act to up-regulate the target antigen [147, 148]. CTA are expressed on the Reed-Sternberg cell in approximately a third of untreated cases of Hodgkin Lymphoma [143]. The class-1 isoform-selective HDACi entinostat increased the expression of testicular associated-associated antigens SSX2 and MAGE-A on Hodgkin lymphoma cell lines [149]. Similar observations have been made in myeloma [148, 150] and AML [151]. There is now rationale to assess whether the epigenetic modifiers can be used to modulate graft-versus-host/graft-versus-tumor effects [151] or improve adoptive cellular immunotherapeutic strategies.

Effect on NK cells The cytotoxic activity of NK cells is influenced by their engagement with stimulatory or inhibitory signals provided by the tumor target cells. NKG2D is an activating receptor expressed on NK cells, which also has co-stimulatory functions on CD4+ and CD8+T cells and macrophages. MICA, MICB and ULBP are among the stimulatory ligands for this receptor, which promote NK-cell mediated killing of tumor cells [152]. These ligands are expressed in response to cellular stress [153] Up regulation of NGK2D ligands solid tumor and AML cells with increased NK-mediated cell killing has been demonstrated after treatment by HDAC inhibitors [154–157]. In a CML-cell line, this affect was accentuated by treatment with hydroxyurea, presumably by accentuation of the DNA damage response [152, 158, 159]. These observations are tantalizing given the role of other NK-stimulatory agents in the management of hematological malignancies such as myeloma and MDS, and the potential for combination strategies [160, 161].

Effect on antigen-presenting cells HDACi appear to reduce differentiation and maturation of monocyte-derived human dendritic cells (DC), as well as reduce antigen uptake and antigen-specific immune responses after stimulation with Toll-like receptor (TLR) ligands [162, 163]. This effect was also seen in DCs in a murine model of graft versus host disease (GVHD), and in both contexts the effects were associated with reduced DC production of IL-12, IL-6 and TNF-a, and a reduced mixed leukocyte response (MLR) [164]. The mixed leukocyte response (MLR) to human and mouse-derived DCs treated with HDACi was consistently reduced, and in mice treated with HDACi, GVHD was ameliorated. These observations suggest a role for HDACi as anti-inflammatory agents, but also suggest that they may interfere with vaccine-based anti-cancer interventions (including dendritic cell vaccine therapy).

HDACi affect T cell polarization The changes to STAT signaling and cytokines described above and which are strikingly demonstrated in the setting of Hodgkin lymphoma, would be expected to shift the cellular immune response from a T_H-2 (IL-5/ IL-4 / IL-13 driven) to a T_H1 response [123]. Given the significant contribution of deranged cytokine signaling in HL, [165] and of the non-malignant cellular milieu responsible for much of the bulk of Hodgkin Lymphoma tumors, there is a real possibility that this hypothesized shift in T-cell polarization contributes to the observed clinical response [166].

T regulatory cells Generally, an increase in the number of T_{regs} is considered to be immune-suppressive and to impair anti-cancer immune surveillance [167–171]. The significance of increased T_{reg} numbers in the marrow of patients with marrow involvement is unknown, but in solid tumors T_{reg} assist in tumor immune-escape [172]. T_{reg} numbers are high in lymph nodes containing follicular lymphoma, but surprisingly portend an improved prognosis and chemotherapy sensitivity. By contrast elevated T_{regs} confer a poorer prognosis in AML. Reduced T_{reg} numbers are associated with clinical responses to the immune-modifying agents thalidomide and lenalidomide in myeloma and CLL. Notably, the transcription factor Foxp3 (which is uniformly expressed in T_{reg}) is under epigenetic control, [173] is stabilized by acetylation and is up-regulated after HDACi therapy [174]. While HDACi appear to increase T_{reg} numbers and function in mice, [167, 174] whether that occurs in humans in the setting of cancer is unknown.

Manipulation of this immune response provides some rationale for the use of HDACi to establish immune tolerance in GVHD but conversely provide a reason for caution for the use of these agents to augment anti-cancer immune responses [167]. These observations are curious given the FoxP3+/T_{reg} phenotype of the HDACi-responsive CTCL, and warrant further exploration in this disease [175].

When considered together, it is clear that there is sufficient evidence to consider HDACi immune modulating agents. Whether the overall effect is important for the anti-cancer effect, or limited to particular tumor types, remains to be seen.

Tumor microenvironment

Levels of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hypoxia-induced factor 1-α (HIF1α) are increased in the in a number of hematological malignancies, especially in the bone marrow microenvironment [176]. Targeting tumor angiogenesis has proven to be a valuable strategy in the therapy of solid tumors using VEGF/EGFR

inhibitors, as well as myeloma and myelodysplasia through the use of immunomodulatory agents (lenalidomide and thalidomide) and for the former, the proteasome inhibitors [176] Hypoxia-inducible factor 1-α (HIF1α) is considered a master regulator of the cell's response to normoxic and hypoxic conditions. Levels of HIF1α are modulated through ubiquitination and proteasomal degradation, with complex interactions between p300/CBP, pVHL and HDACs 1,3, 4, 6, and 7 influencing this process (see review by Ellis et al.) [177]. Increased levels of these HDACs appear promote angiogenesis, conversely HDAC5 appears to be an inhibitor of angiogenesis [178].

HDACi have been shown to suppress angiogenesis in a number of cell types across a range of experimental conditions [177, 179–186]. Down regulation of genes associated with angiogenesis (GUCY1A3, ANGPT1, COUP-TFII) has been documented in clinical CTCL samples after treatment with panobinostat [50] and TSP1 after treatment with vorinostat [118]. Similarly, reduction in VEGF, sVEGFR1, and bFGF were seen in samples from patients with myeloma who had been treated with panobinostat [187] and skin biopsies of cutaneous lymphoma from patients treated with vorinostat show reduced microvessel density [118].

Combination strategies with specific small-molecules inhibitors of angiogenesis are being explored in the solid tumor setting, and should be considered for haematalogical malignancies thought to depend on angiogenesis [181, 182, 188].

Histone deacetylase inhibitors and drug-resistant clones—targeting the cancer stem cell

Given that tumor-regrowth occurs after clinical remission implies the presence of subpopulation of cancer cells that are relatively resistant to primary treatment [189]. Such resistance may be present in a subset of cells prior to treatment or may develop as a consequence of exposure to drugs, through a process of natural selection. The *cancer stem cell* hypothesis somewhat controversially proposes that within a cancer there is a phenotypically distinct subpopulation of cells responsible for the clonogenic potential of the tumor [190–195]. The putative cancer stem cells are said to form the minority of overall cancer cell population, have the capacity for self-renewal, and importantly are potentially more resistant to anti-cancer agents. A host of mechanisms of resistance to a variety of anti-cancer treatments have been demonstrated in various putative cancer stem cell models, including hedgehog signaling in multiple myeloma [191, 194, 196], increased drug efflux, and changes in Notch and Wnt signaling in AML, CML and T-ALL. (See reviews by Lin *et al*. and references therein) [190, 193].

Sharma et al. recently demonstrated the ability to detect a subpopulation of PC9 lung cancer cells that were resistant to erlotonib, termed "drug-tolerant persisters" (DTPs) [197]. These DTPs all possessed the putative cancer stem cell marker CD133 that was present on only 2% of the original, untreated PC9 population. When grown in drug-free media, the cells re-acquired a drug-sensitive phenotype, this 'elasticity' implying an epigenetic mechanism of drug resistance. Supporting this was data from gene expression profiling of the two cell lines (drug-sensitive and drug-resistant) which was consistent with a global epigenetic modification. The authors identified that the retinoblastoma protein and HDAC-demethylating protein KDM5A was unregulated in the DTPs and found that histone H3 was consistently hypoacetylated in the DTPs. Trichostatin A was lethal to DTPs but not to the drug-sensitive cells, supporting the theory that the drug-resistance state was dependent on global chromatin changes and HDAC-dependence. Application of four different HDAC inhibitors to PC9 cells prior to exposure to erlotonib and a number of other anti-cancer drugs including cisplatin, prevented the development or expansion of DTPs without effect on the proliferation or survival of the PC9 cells. These observations offer the tantalizing possibility that HDACi can target the putative cancer stem cell or circumvent acquired drug resistance, and clearly offer a direction for further research.

Potential effect on leukemias with recurrent cytogenetic abnormalities

Fusion proteins associated with the acute leukemias interact with HDACs and offer appealing targets for the HDAC inhibitors. Fusion of the retinoic acid receptor-α (RARA) with PML or the PLZF loci results in acute promyelocytic leukaemia. The retinoic acid receptors (RAR) repress transcription through recruitment of corepressors that in turn recruit HDAC1 [198]. Ligation of RAR leads to dissociation of the HDACs and recruitment of HATs, and transcriptional activation [198]. Both fusion proteins require higher concentrations of retinoic acid to achieve the same level of HDAC dissociation. The result, phenotypically, is maturation arrest and proliferation at the promyelocyte stage [198–200]. This effect can be overcome by high concentrations of trichostatin A [199] an observation reciprocated in a mouse model, [201] as well as in patients with all-trans-retinoic-acid resistance [198, 202, 203].

An analogous situation arises with AML1/ETO, the commonest recurrent fusion protein in AML. AML1 is a transcriptional activator and achieves this effect through the recruitment of HATs [198, 204, 205]. The ETO portion of the AML1/ETO fusion instead appears to recruit a corepressor complex containing HDAC1, histone methyltransferase, DNA methyltransferase as well as meythl-CPG binding properties.

Transcription is repressed through dysfunction of RARA [206]. HDACi induce apoptosis in AML1/ETO-bearing cells [207, 208] and romidepsin has antileukemic activity (albeit limited) in patients with AML/ETO leukaemia [209].

The MLL locus on chromosome 11 is a subject to frequent translocation and participation in fusion proteins associated with myeloid or lymphoid leukaemia. The most common fusion partners in AML (of at least 54) are AF4 and AF9. MLL encodes a transcriptional regulator with two subunits: one is a transcriptional activator with histone methyltransferase activity, and an ability to recruit HATs, while the other has DNA methyltransferase homology, interacts with HDACs and is transcriptionally repressive [210–213]. The fusion proteins exert their proleukaemic effect through a gain of function effect on the MLL component of the fusion protein, and up regulation of Hox genes otherwise repressed by a normally functioning MLL complex [212–214]. The effect of HDACi in this context is unpredictable given the multiple potential epigenetic effects of the MLL complex [215]. P21-dependent cell cycle arrest and apoptosis has been observed in MLL/AF9 AML cells after treatment with valproate, [216] and we have observed a complete cytogenetic response to panobinostat in a patient with a MLL/CBP fusion protein-associated AML [217]. Further studies using in-vivo models are needed.

Conclusion

The last decade of the research into HDACi has been one where dogma around their effects and targets are continually being challenged and refined. No longer can the HDACi be regarded as simple activators of transcription, or agents that achieve their activity predominantly through direct effects on the pathways of apoptosis. Apart from induction of cell death,

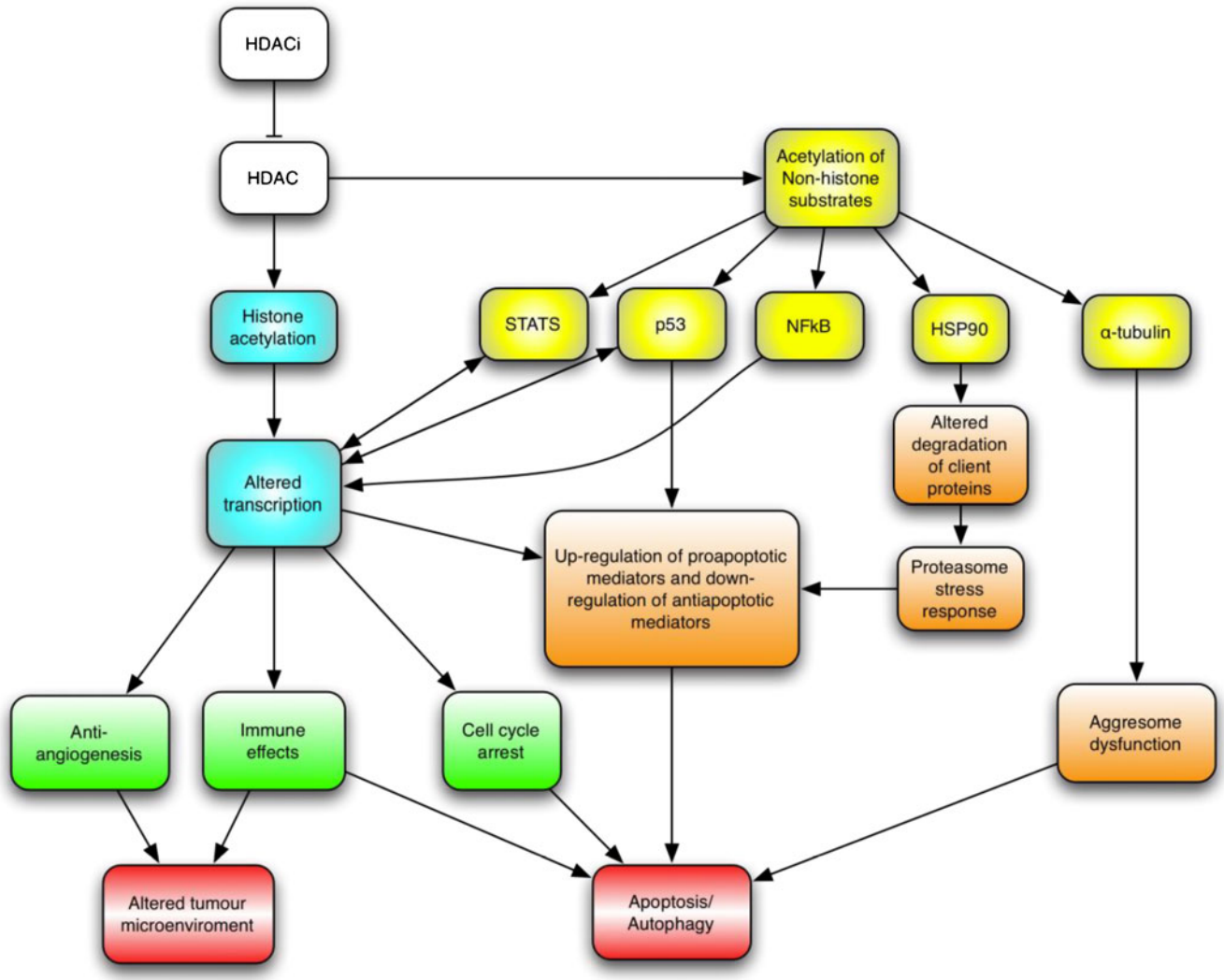

Fig. 1 Simplified schema of a selection of HDACi targets and downstream effects

these agents have complex effects on p53 and on cytokine signaling pathways, and must now be considered immune-modifiers as well as anti-angiogenic agents. Alteration of transcription is only one mechanism but non-histone targets are clearly critically important and we need more information on the effects on the host environment (Fig. 1).

One example of the challenges we face in developing these compounds is the developing story of HDAC6; tantalizing evidence that specific HDACs (such as HDAC6) make rational targets for drug development must be tempered by evidence that the targets of HDAC6 may not actually be necessary for clinical synergy with the drugs such as the proteasome inhibitors. Another challenge is to determine if HDACi can contribute to the therapy of AML with recurrent cytogenetic abnormalities.

The work now is to better dissect which on-target effects are most critical for HDACi efficacy, in which specific clinical situations, and how we may overcome the relatively modest single agent response rates using rational combination therapies [218, 219].

In vitro models do not replicate the tumor microenvironment or the immune milieu and therefore probably do not, alone, provide sufficient basis for clinical studies. A greater emphasis on immune-competent in vivo models and biomarker studies are essential to establish which targets are critical in patients, and which combinations will be the most promising.

Acknowledgements Michael Dickinson is supported by a Victorian Cancer Agency Translational Research Grant and this work is presented on behalf of the Victorian Epigenetics Group.

Conflict of interest Michael Dickinson: No conflict of interests

H. Miles Prince: Medical Advisory to Novartis, Merck. Gloucester Pharmaceuticals and Celgene.

Ricky W. Johnstone: The Johnstone Lab receives grant support from Novartis to study the anti-cancer effects of panobinostat.

Logistical support during submission of this article was provided by Springer Healthcare LLC. This support was funded by Novartis.

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