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Histone deacetylase inhibitors: emerging mechanisms of resistance

Robert W. Robey[1,*], **Arup R. Chakraborty**[1,2], **Agnes Basseville**[1], **Victoria Luchenko**[1], **Julian Bahr**[1], **Zhirong Zhan**[1], and **Susan E. Bates**[1]

[1]Medical Oncology Branch, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD, 20892

[2]Department of Biological Sciences, Texas Tech University, Lubbock, TX, 79409

Abstract

The histone deacetylase inhibitors (HDIs) have shown promise in the treatment of a number of hematologic malignancies, leading to the approval of vorinostat and romidepsin for the treatment of cutaneous T-cell lymphoma and romidepsin for the treatment of peripheral T-cell lymphoma by the U. S. Food and Drug Administration. Despite these promising results, clinical trials with the HDIs in solid tumors have not met with success. Examining mechanisms of resistance to HDIs may lead to strategies that increase their therapeutic potential in solid tumors. However, relatively few examples of drug-selected cell lines exist, and mechanisms of resistance have not been studied in depth. Very few clinical translational studies have evaluated resistance mechanisms. In the current review, we summarize many of the purported mechanisms of action of the HDIs in clinical trials and examine some of the emerging resistance mechanisms.

Keywords

histone deacetylase inhibitor; resistance; romidepsin; vorinostat; panobinostat

Introduction

In the nucleus, DNA is wound around four core histone proteins (H2A, H2B, H3 and H4, see Figure 1) to form the nucleosomes, which, when compacted, form the condensed structure of chromatin. Each histone protein in the nucleosome has a lysine-rich tail that extends outside of the nucleosome and the accessibility of DNA within the nucleosome is, in part, controlled by modifications of the tail. Histones can be modified in a number of ways, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation and citrullination. As shown in Figure 1, the lysines of the histone tails can be modified by methylation and acetylation. Acetylation is controlled by two enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs transfer acetyl groups to the lysine residues of the histones, which neutralizes the positively charged lysines, decreasing attraction of the negatively charged DNA, thereby resulting in greater access by transcription factors and RNA polymerase. HDACs, on the other hand, remove acetyl groups, resulting in decreased access to DNA[1]. Changes in global histone acetylation were found to be associated with tumorigenesis in some disease models[2–4].

[*]corresponding author: Robert W. Robey, 9000 Rockville Pike, Bldg 10 Rm 12N226, Bethesda, MD 20892. Phone 301-496-0796 Fax 301-402-1608 robeyr@mail.nih.gov.

Most of the human HATs function as co-activators of transcription[2]. The most studied function of this group of proteins is the acetylation of the histone tails, although they can also acetylate various cellular transcription factors[2]. Similarly, while HDACs are primarily known for their ability to deacetylate histones, they have also been shown to deacetylate other proteins such as tubulin, p53, Hsp90, Bcl-2, and Ku70[5, 6]. Based on their similarity to yeast HDAC proteins, HDACs are divided into four classes (compiled from references[1, 7]):

Class I

HDACs 1-3, and 8, 40–55 kD proteins, belong to this class and are ubiquitously expressed in human tissues. HDACs 1, 2, and 3 are localized in the nucleus while HDAC 8 is located in both the cytoplasm and the nucleus. This class of HDACs shares a structural similarity with the yeast transcription factor Rpd-3.

Class IIA, IIB

HDACs 4, 5, 7, and 9, make up class IIA and HDACs 6 and 10 make up the class IIB HDACs, all of which are 70–130 kD proteins. They share a structural similarity with the yeast HDA1 deacetylase. HDAC6 is unique in that it is a cytoplasmic HDAC that does not deacetylate histones.

Class III

This group of HDACs, known as the sirtuins, is made up of HDACs structurally similar to the yeast SirT2, and requires NAD^+ as a cofactor for enzymatic activity.

Class IV

The only known HDAC of this class is HDAC 11. Relatively little is known about this HDAC, which is localized to the nucleus.

Histone deacetylase inhibitors

The histone deacetylase inhibitors (HDIs) are a promising class of chemotherapeutic agents that have been added to the anticancer armamentarium. HDIs prevent the deacetylase activity of HDACs, leading to unrestricted HAT activity and increased gene transcription. Several HDAC inhibitors are currently in clinical trials both in monotherapy and in combination therapy with other anti-tumor drugs. The HDIs currently in clinical trials fall primarily into the short-chain fatty acid class, the hydroxamate class, the cyclic peptide class and the benzamide class. A partial listing of these HDIs is provided in Table 1, and structures for several are provided in Figure 2. The HDACs that are targets for the HDIs discussed are all zinc-dependent enzymes. To date, most of the responses using HDAC inhibitors as single agents were observed in advanced hematological cancers and only very few were observed in solid tumors. In hematological malignancies, clinical efficacy has been observed in cutaneous T-cell lymphoma (CTCL), peripheral T cell-lymphoma (PTCL), Hodgkin and non-Hodgkin lymphoma, while only a few responses were observed in patients with myeloid malignancies[8].

Some of the HDAC inhibitors that are being studied in clinical trials have demonstrated therapeutic potential in CTCL and other malignancies and are detailed below:

Vorinostat (suberoylanilide hydroxamic acid, SAHA)

An orally-available, pan-HDAC inhibitor, vorinostat was found in *in vitro* studies to facilitate transcription of genes associated with growth arrest, differentiation, and apoptosis[9, 10]. Responses in patients with refractory CTCL led to the approval of vorinostat

in 2006 by the Food and Drug Administration (FDA) for the treatment of patients with relapsed or refractory CTCL[11]. In the registration trial, patients were treated with 400 mg daily of oral vorinostat, with an overall response rate of approximately 30% and a response duration of over 6 months[11]. Promising results have also been observed in follicular lymphoma and marginal zone lymphoma[12]. Combinations with the proteasome inhibitor bortezomib in the treatment of multiple myeloma have also seen clinical success[13]. In contrast, single agent trials with vorinostat for the treatment of most solid tumors have not met with success[14].

Romidepsin (FK228, FR901228, NSC630176, depsipeptide)

Romidepsin is unique among the HDIs in that it is actually a prodrug; the disulfide bond of romidepsin must be reduced to yield the active form[15]. Most studies seem to suggest that romidepsin is an inhibitor of class I HDACs[16], but some studies also find romidepsin treatment leads to Hsp90 acetylation, leading to speculation that it might somehow affect HDAC6[17]. In 2001, we first reported the efficacy of romidepsin in a phase I trial where partial responses (PRs)were observed in three patients with CTCL and a complete response(CR) was observed in one patient with PTCL[18]. These early successes in T-cell lymphoma led to two registration trials, culminating in the approval of romidepsin in November 2009 for the treatment of CTCL patients who had received at least one prior systemic therapy[8]. In the trial sponsored by the National Cancer Institute, among 71 patients with CTCL, the overall response rate was 34% with a median duration of response of 13.7 months[19]. In a second, independent, international trial of 96 patients with CTCL, the overall response rate was 38% and the median duration of response was 15 months[20]. In PTCL, an overall response rate of 38% was observed with a median duration of response of 8.9 months in a number of subtypes[21]. Romidepsin was recently approved by the FDA for the treatment of patients diagnosed with PTCL. As with vorinostat, results in solid tumors have been disappointing[22–24].

Panobinostat (LBH589)

The first clinical trials with this pan-HDAC inhibitor were conducted in patients with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and myelodysplastic syndrome (MDS)[25]. Panobinostat is currently in phase I and II clinical trials, with the most significant anti-tumor activity of this drug observed in patients with refractory CTCL and other hematologic malignancies[26, 27]. As with the other HDIs, panobinostat has not been successful in solid tumor clinical trials[26], so that the overall activity in T-cell lymphoma and inactivity in solid tumors appears to be a class effect.

Belinostat (PXD101)

Belinostat is another pan-HDAC inhibitor that has been studied in multiple clinical trials as a single agent or in combination with chemotherapeutic agents. A phase I trial of this drug reported disease stabilization in patients with hematological malignancies[28]. Like romidepsin, belinostat has produced responses in patients diagnosed with PTCL[29]. Preliminary results from a phase II trial in lymphoma have been promising[30].

Other HDIs

Less clinical data are available for some of the other HDIs. Some clinical activity was observed with valproic acid in pediatric patients with central nervous system tumors[31], but not in a phase I trial for prostate cancer[32]. In a phase I trial, entinostat, an HDAC1-specific inhibitor, produced a partial remission in a patient with melanoma[33]; however, a subsequent phase II trial did not yield any objective responses[34]. Mocetinostat is a class I selective HDI that has demonstrated clinical activity in non-Hodgkin lymphoma and in relapsed or

refractory Hodgkin lymphoma[35]. One patient with myelodysplastic syndrome and two patients with AML responded to single-agent mocetinostat in a phase I trial in leukemia[36], while a phase II trial of mocetinostat in patients with chronic lymphocytic leukemia did not yield any responses[37]. A phase I trial of dacinostat in solid tumors demonstrated degradation of CRAF, an Hsp90 client protein, and increased histone acetylation in circulating peripheral blood mononuclear cells, but no responses were observed[38].

Mechanisms of action

One global effect of HDI treatment is an increase in histone acetylation. In clinical trials of HDIs, increased histone acetylation has been monitored in circulating peripheral blood mononuclear cells as a surrogate marker for the inhibition of HDACs[39]. However, this effect alone is apparently inadequate to confer activity, as solid tumor trials have demonstrated increased histone acetylation in tumor samples despite little clinical effect[40, 41] In the search for the mechanism of action of HDIs, several have been suggested, including cell cycle arrest, activation of apoptotic pathways, induction of autophagy, reactive oxygen species generation, Hsp90 inhibition, and disruption of the aggresome pathway[1, 7, 14]. We elaborate on several of these potential mechanisms of action below:

Alteration of gene expression

Studies with cDNA arrays have shown that treatment with HDIs such as sodium butyrate, entinostat, vorinostat or romidepsin leads to a two-fold or greater change in the expression of approximately 7–10% of the genes examined[1]. HDI treatment was found to induce about as many genes as were repressed. The histone deacetylase inhibitors induce p21 expression[42], leading to G1 cell cycle arrest, and frequently downregulate cyclin D and c-myc. Whether or not these gene expression changes result in cell death probably depends upon cellular context. For example, decreased expression of c-myc will probably be more important for myc-dependent cancers, such as Burkitt's lymphoma, than for cancers that do not rely on c-myc for survival[43]. Vorinostat treatment has been shown to lead to decreased cyclin D1 expression and cell death in mantle cell lymphoma cell lines[44].

Degradation of Hsp90 client proteins

Hsp90 is required to stabilize a number of "client" proteins that play a role in cancer cell proliferation, such as the human epidermal growth factor receptor 2 (ErbB-2, Her-2) and epidermal growth factor receptor (ErbB-1, EGFR); the fusion oncogene Bcr-Abl; and signaling transduction molecules such as Akt[45]. HDAC6 has been shown to be a deacetylase of both tubulin and Hsp90[46]. Treatment of cells with compounds that are known to inhibit HDAC6 leads to increased tubulin acetylation, increased Hsp90 acetylation and degradation of Hsp90 client proteins, having much the same mechanism of action as Hsp90 inhibitors.

Romidepsin was one of the first HDIs found to induce Hsp90 acetylation and cause degradation of the Hsp90 client proteins EGFR, Her-2 and Raf-1[17]. In breast cancer cell lines overexpressing Her-2, treatment with vorinostat or panobinostat has been shown to lead to increased Hsp90 acetylation, Her-2 degradation and cell death, and synergy has been demonstrated when the HDIs were combined with Hsp90 inhibitors or Her-2 inhibitors such as trastuzumab or lapatinib[47, 48]. Similarly, treatment of lung cancer cell lines expressing mutant EGFR with panobinostat resulted in decreased EGFR expression and synergized with the EGFR inhibitors erlotinib or lapatinib[48, 49]. Leukemia cells expressing the Bcr-Abl fusion protein are particularly sensitive to treatment with vorinostat, dacinostat, romidepsin or panobinostat and cotreatment with imatinib or nilotinib results in synergistic cell death[50–53]. Again, the ability of an HDI to inhibit HDAC6 would probably be only effective

in cancers where proliferation is driven by Hsp90 client proteins, although cell death could also occur as a result of deacetylation of other HDAC6 substrates[54].

It is not clear, however, whether HDAC6 is the sole deacetylase of Hsp90. Romidepsin is considered a rather weak inhibitor of HDAC6, as romidepsin treatment does not result in acetylation of tubulin[42], yet has still been shown to cause acetylation of Hsp90[17]. As seen in Figure 3, treating HEK293 (Flp-In-293) human embryonic kidney cells with varying concentrations of romidepsin, vorinostat, panobinostat or valproic acid results in increased histone H3 acetylation, but only results in tubulin acetylation in cells treated with vorinostat and panobinostat. Additionally, entinostat treatment has been shown to result in apoptosis due to degradation of mutant FLT-3, another Hsp90 client protein, in leukemia cells that express the mutant protein despite the fact that entinostat does not inhibit HDAC6[55]. It has been suggested the HDAC1 mediates this effect[56]. One study has suggested that acetylation of Hsp70 mediates degradation of the Bcr-Abl fusion protein[57]. Further study is needed to determine how HDIs that are weak inhibitors of HDAC6 mediate apoptosis in cell lines where proliferation is driven by Hsp90 client proteins.

Increased production of reactive oxygen species (ROS)

Several studies have suggested that the generation of reactive oxygen species (ROS) is a key event in HDI-induced cell death. ROS generated by HDIs leads to DNA damage and the addition of free radical scavengers such as N-acetyl cysteine during the time of HDI treatment has been shown to result in decreased ROS generation and decreased HDI-mediated cell death[58–61]. HDI treatment has also been shown to prevent repair of DNA damage[62, 63]. Another mechanism by which HDIs increase ROS production is through downregulation of thioredoxin and upregulation of thioredoxin-binding protein 2 (TBP-2). Thioredoxin is a thiol reductase that acts as a scavenger of ROS, and TBP-2 has been shown to be a negative regulator of thioredoxin, decreasing its reducing activity[64]. Vorinostat treatment has been shown to not only increase TBP-2 expression, but also to suppress thioredoxin expression[65].

Alterations in the apoptotic pathway

Apoptosis proceeds either via the extrinsic or cell death receptor-mediated pathway or the intrinsic or mitochondria-mediated pathway. Several HDIs have been shown to facilitate death by the extrinsic pathway by causing an increase in the expression of TRAIL, DR-4, DR-5, Fas and FasL as well as a decrease in c-FLIP, a protein associated with resistance to TRAIL-mediated apoptosis[66–68]. Activation of the apoptotic machinery associated with the intrinsic pathway, by decreasing expression of the anti-apoptotic proteins Bcl-2, Bcl-XL, Mcl-1 and survivin and increasing expression of the pro-apoptotic proteins Bax and Bim, seems to be a class effect of the HDIs[47, 49, 58, 69].

Mechanisms of resistance

As the clinical course of the HDIs is pursued, it becomes important to identify potential mechanisms of resistance so as to increase efficacy and identify potential drug combinations. Despite the fact that HDIs have been in development for several years, relatively few drug-selected cell lines have been developed. Most of these *in vitro* selections have been with romidepsin alone, where emergence of P-glycoprotein (Pgp) seems to be the dominant mechanism of resistance[70–72]. Non-Pgp mechanisms of resistance have only been observed when cells are selected with romidepsin in the presence of a Pgp inhibitor[71]. However, some mechanisms of resistance have been identified by transfection with purported resistance mechanisms. Summarized below are some of the major mechanisms of resistance to HDIs that have been characterized.

As will be noted below, the search for resistance mechanisms has primarily centered on laboratory models, and sparse clinical data have been gathered. Most studies have detected histone acetylation, whether in peripheral blood mononuclear cells or in biopsy samples, and some have concluded that the presence of histone acetylation does not correlate with response to therapy[73]. Our data with romidepsin suggested otherwise, when we compared histone acetylation analyzed by an immunodot blot assay with response on a clinical trial in cutaneous and peripheral T-cell lymphoma. It appeared that higher and more durable levels of histone acetylation in peripheral blood mononuclear cells were associated with better clinical response[39]. These findings may have been unique to romidepsin, which as a prodrug, requires reduction of the disulfide bond to an active form[15].

ATP-binding cassette transporters

Treatment with histone deacetylase inhibitors, such as sodium butyrate, was found several years ago to induce a more differentiated phenotype, accompanied by increased expression of the multidrug-resistance gene, *MDR1* (*ABCB1*), and its product, Pgp[74, 75]. Trichostatin A treatment was also found to increase Pgp expression [76]. Romidepsin was first brought to our attention after it was identified as a Pgp substrate based on rhodamine efflux patterns in the NCI Anticancer Drug Screen and was also found to induce expression of Pgp[77]. Valproic acid and apicidin have also been shown to increase *ABCB1* expression[78, 79]; thus upregulation of ABCB1 and increased expression is believed to be a class effect of histone deacetylase inhibitors.

Romidepsin is unique among the HDIs in that it is also a substrate of Pgp, while other HDIs, such as vorinostat and belinostat, are not [80, 81]. Cell lines expressing the multidrug resistance-associated protein-1 (*MRP1*/*ABCC1*) have also been found to be resistant to romidepsin, although less so than cells that express Pgp[82]. Cell lines selected for resistance to romidepsin express Pgp and are also resistant to other Pgp substrates such as vincristine or taxol; resistance to romidepsin can be overcome by Pgp inhibitors[70, 72, 83]. Despite the major role of Pgp in romidepsin resistance in cancer cell lines, *ABCB1* gene expression in tumor biopsy samples from patients with CTCL enrolled on the NCI phase II trial did not appear to correlate with resistance to romidepsin treatment[39], even though increased *ABCB1* expression has been observed in peripheral blood mononuclear cells and circulating tumor cells obtained from patients receiving depsipeptide[70]. Thus, other mechanisms of resistance are likely to play a role in the intrinsic resistance observed in clinical trials with HDIs.

In an attempt to identify non-Pgp mechanisms of resistance, we selected the HuT 78 cell line, derived from a patient with Sézary syndrome, with romidepsin in the presence of the Pgp inhibitor verapamil[71]. One of the resulting romidepsin-resistant cell lines, HuT DpVp50, is maintained in 50 ng/mL romidepsin in the presence of 5 μg/ml verapamil. As seen in Figure 4, although the DpVp50 cells express some Pgp, this is not the dominant mechanism of resistance; treatment of the resistant line with 10 ng/ml romidepsin for 48 h in the presence of the Pgp inhibitor tariquidar does not result in increased cell death compared to romidepsin treatment without tariquidar. HuT 78 parental cells, which do not express Pgp are exquisitely sensitive to romidepsin; accordingly, the addition of tariquidar does not have any effect on cytotoxicity. We are currently working to characterize the mechanism of resistance to romidepsin in these cell lines.

Cell cycle proteins

It has been postulated that the induction of p21, which is responsible for the G1 arrest caused by HDI treatment, might serve a protective role. We reported that, when p21-deficient HCT116 cells were treated with romidepsin, cells arrested only in G2 and were more sensitive to treatment compared to wild-type cells[84]. In accordance with that result,

U937 leukemia cells transfected with a p21 antisense construct were found to be more sensitive to vorinostat treatment when compared to untransfected cells[85]. Additionally, cotreatment with flavopiridol has been shown to potentiate the cytotoxicty of romidepsin, sodium butyrate and vorinostat, due in part to the prevention of p21 upregulation[86–88]. Temisirolimus treatment has also been shown to decrease p21 expression in mantle cell lymphoma cell lines and to synergize with sublethal concentrations of vorinostat[89]. Clinical trials with vorinostat and temisirolimus or sirolimus are currently ongoing.

Increased thioredoxin levels

As mentioned above, thioredoxin is a scavenger of reactive oxygen and expression of TBP-2 has been shown to decrease its reductive capacity. Ungerstedt and colleagues found that high levels of thioredoxin in normal cells served to protect cells from ROS induced by HDI treatment[90]. Additionally, when HDI-sensitive transformed cells were transfected with a small-interfering RNA (siRNA) against thioredoxin, the cells exhibited higher ROS levels and increased cell death compared to untransfected cells[90]. Similarly, Chen and colleagues reported in romidepsin-treated, human lung cancer cells, that thioredoxin expression negatively correlated with ROS generation and apoptosis[91], supporting the idea that HDIs in combination with compounds that decrease thioredoxin expression or function may lead to increased activity.

Apoptosis-related proteins

Enforced expression of anti-apoptotic proteins has been shown to prevent HDI-mediated cell death. High levels of Bcl-2 or Bcl-XL have been shown to confer resistance to treatment with vorinostat, dacinostat, panobinostat or oxamflatin, while only high levels of Bcl-2 were found to confer resistance to romidepsin treatment[85, 92, 93]. Bcl-2 expression has also been linked to sensitivity to panobinostat treatment in CTCL cell lines[94]. Increased apoptosis has been observed when HDIs are combined with Bcl-2 inhibitors such as ABT-737 or others in several model systems[94–98], suggesting that the combination should be tested in the clinical setting.

When Shao and colleagues knocked down expression of the pro-apopototic Bax in panobinostat-sensitive, T-cell lymphoma cell lines, toxicity was diminished[94]. Similarly, knockdown of anti-apoptotic Mcl-1 was found to potentiate HDI-mediated apoptosis in primary chronic lymphocytic leukemia cells and K562 cells[99]. Expression of Bim has also been shown to be required for romidepsin-mediated apoptosis in lung cancer cells[100]. Thus, in cells where these proapoptic proteins are silenced, HDI-mediated cell death might be blunted. Treatment with targeted therapeutics such as erlotinib or imatinib has been shown to increase levels of Bim in some model systems[101, 102], suggesting that combination with an HDI might be advantageous.

Alterations in HDAC protein levels

Another cell line selected for resistance to HDIs was generated by selection of the HL-60 leukemia cell line with dacinostat, resulting in the HL-60/LR cell line maintained in 200 nM dacinostat[103]. In addition to dacinostat, the resistant line was also highly cross-resistant to vorinostat, panobinostat and sodium butyrate[103]. While levels of BCL-XL and XIAP were attenuated, levels of Bim and Bax remained unchanged[103]. Interestingly, HL-60/LR cells expressed higher levels of HDAC1, 2, and 4, but lacked expression of HDAC6 and had higher levels of Hsp90 acetylation compared to the parental line[103]. The resistant line also demonstrated collateral sensitivity to Hsp90 inhibitors[103]. A separate study demonstrated that increased HDAC1 expression prevented sodium butyrate-mediated toxicity in a melanoma cell line[104]. It is not clear whether this mechanism of resistance has clinical relevance; no studies have yet linked altered HDAC protein expression levels with clinical

response to HDIs. However, HDAC2 expression levels were found to correlate with histone acetylation in a phase I trial of doxorubicin and vorinostat[105] in solid tumors and combined tamoxifen and vorinostat treatment in a phase II trial in breast cancer[106].

Signaling proteins

Activation of the mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K) pathways has increasingly been associated with resistance to HDIs. Combination of romidepsin with mitogen-activated protein kinase kinase (MEK) inhibitors has been shown to increase cell death, suggesting that activation of the MAPK pathway is an important mechanism of resistance to romidepsin[107, 108]. In agreement with this hypothesis, Yu and colleagues found that enforced expression of constitutively active MEK1, but not Akt, in lung cancer cell lines reduced romidepsin-mediated cytotoxicity[109]. Other studies, however, seem to suggest that phosphorylated Akt is, in fact, an important resistance mechanism to romidepsin, as combination of romidepsin with inhibitors of Akt results in synergistic cytotoxicity[110]. Combination of panobinostat with compounds that abrogate MAPK and PI3K signaling has also been shown to result in synergistic cytotoxicity, possibly due to increased ROS[111]. Similarly, Jane and colleagues found that treatment of glioma cells with vandetanib inhibited both the MAPK and PI3K pathways and was synergistic with vorinsotat treatment[112]. Vorinostat in combination with PI3K inhibitors has shown promise in T-cell lymphoma cell line models[113]. These combination studies, while targeting different signaling molecules, do suggest that activation of one or more of these pathways may confer clinical resistance.

Activation of the signal transducer and activator of transcription (STAT) pathway has also been linked to vorinostat resistance. In a panel of almost 40 lymphoma cell lines, expression of STAT1, 3 and 5 was higher in cell lines that were more resistant to vorinostat compared to sensitive lines; phosphorylation levels of the STAT proteins were also higher in the resistant lines[114]. In a series of skin biopsy samples, patients with higher nuclear staining of phosphorylated STAT3 were more likely to be resistant to vorinostat treatment[114], again implicating activation of signaling pathways in resistance to HDIs. Combined treatment with vorinostat and compounds shown to inhibit STAT3 phosphorylation, such as lestaurtinib[115], may therefore increase efficacy in T-cell lymphoma.

NFκB activation

Activation of the NFκB pathway is a hallmark of a number of cancers and leads to deactivation of the apoptotic pathway and increased cell survival[116]. Acetylation of the p65, or RelA, subunit of NFκB has been shown to increase the acitivity of NFκB[117], and HDI treatment has been shown to result in increased p65 acetylation[118, 119]. Constitutive activation of NFκB has been linked to resistance to HDIs in cell line models[94], as has p65 acetylation caused by HDI treatment; combination of an HDI with an inhibitor of NFκB activation leads to synergistic cytotoxocity[120]. The increased cytotoxicity observed when HDIs are combined with proteasome inhibitors is believed to be due, at least in part, to decreased NFκB activity mediated by proteasome inhibitors[121, 122]. However, while a phase II trial with vorinostat and bortezomib in multiple myeloma had a 42% response rate and elicited some responses from patients whose disease was refractory to bortezomib, response did not correlate with levels of NFκB or IκB[13] in CD138+ bone marrow cells, casting some doubt on the relevance of NFκB status.

Conclusion

The histone deacetylase inhibitors have shown promise in the treatment of peripheral and cutaneous T-cell lymphomas. Their lack of success in clinical trials for solid tumors has

been disappointing. As noted above, a long list of mechanisms of action has been compiled through *in vitro* studies. It can be concluded from this list that we still do not really understand how the HDIs work when they are effective, as in T-cell lymphomas. We have yet to validate a marker that predicts clinical response to HDI treatment. We also do not understand why they work in T-cell lymphomas and not in solid tumors. Perhaps in the context of the overall sensitivity of lymphomas to anticancer therapy, a decades-old observation, this is not so surprising. But the activity in T-cell lymphoma seems beyond that in other hematologic malignancies and is a class effect. It is likely that a dominant mechanism of action, not necessarily on the list of mechanisms outlined above, is responsible for the efficacy.

The insensitivity of solid tumors also appears to be a class effect. Unfortunately, *in vitro* models do not typically reflect the insensitivity of solid tumors in the clinic. This may be due in part to the rapid doubling time of cell lines in culture, and the epigenetic alterations that accompany that cell growth rate. Combined with the duration of exposure typically used in cell culture sensitivity assays, responses to HDIs in the laboratory are homogenous across different tumor types. The negative impact of this ubiquitous responsiveness is to divert those working in the field from focusing on a few candidate solid tumor types for targeted drug development.

One approach to overcoming resistance in solid tumors is to exploit the diverse effects of HDIs in solid tumors in combination therapies. Thus, if altered gene expression does not itself prompt cytotoxicity, it may allow promising combination therapies to be developed. Induction of the sodium iodide symporter and increased sensitivity of thyroid cancer cells to radioiodine uptake is one such example[123]. If altered handling of reactive oxygen species or reduced DNA repair are insufficient to induce cell death following HDI exposure, it may still allow for effective combination therapies with DNA damaging agents, whether chemotherapy or radiotherapy[124, 125]. It is critical, however, in developing the rationale for combination studies that attention be paid to sequence of administration. For example, in small cell lung cancer cells, simultaneous exposure to an HDI and cisplatin or etoposide was more effective than sequential exposure[126]. Among the plethora of HDI effects on cells are likely to be some that are detrimental to cytotoxicity (e.g. p21 induction).

Further, the *in vitro* paradox also has the potential to yield misleading clinical directions from combination assays that require durations of exposure for efficacy that are not achievable in the clinic by any HDI developed to date. The length of the list of mechanisms of resistance generated from laboratory models confirms that we do not understand resistance, either. We can only hope that somewhere on those lists are the answers and that translational studies will help us separate the important mechanisms from those that are trivial. What we do know is that development of the HDIs is a major step in bringing epigenetic therapy to the anticancer armamentarium. We need to figure out how to exploit them more fully and in many more tumor types.

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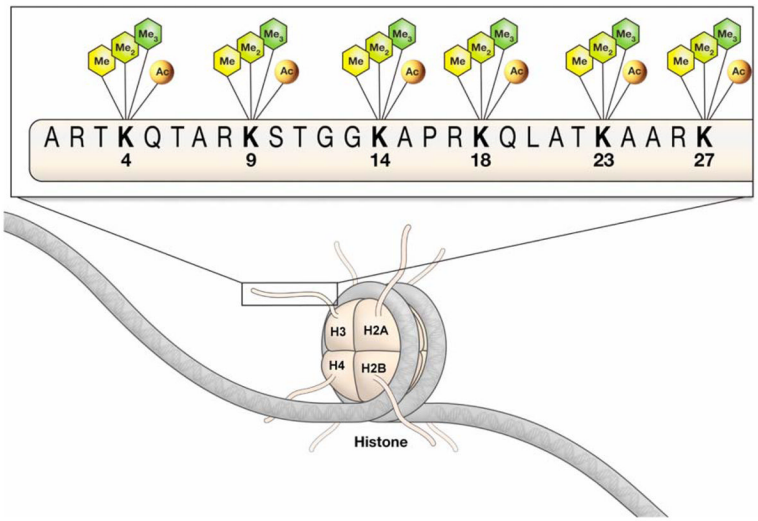

Figure 1.

Modification of lysines in histone tails. DNA is wound around four core histone proteins: H2A, H2B, H3 and H4. Each of the histones possess lysine-rich tails and accessibility of the DNA is controlled by modifications to the tail. Lysines can either be multiply methylated or acetylated. Methylation and deacetylation of lysines both contribute to a more condensed chromatin structure, preventing transcription of genes. Demethylation and acetylation promote a more open chromatin structure allowing for increased gene transcription.

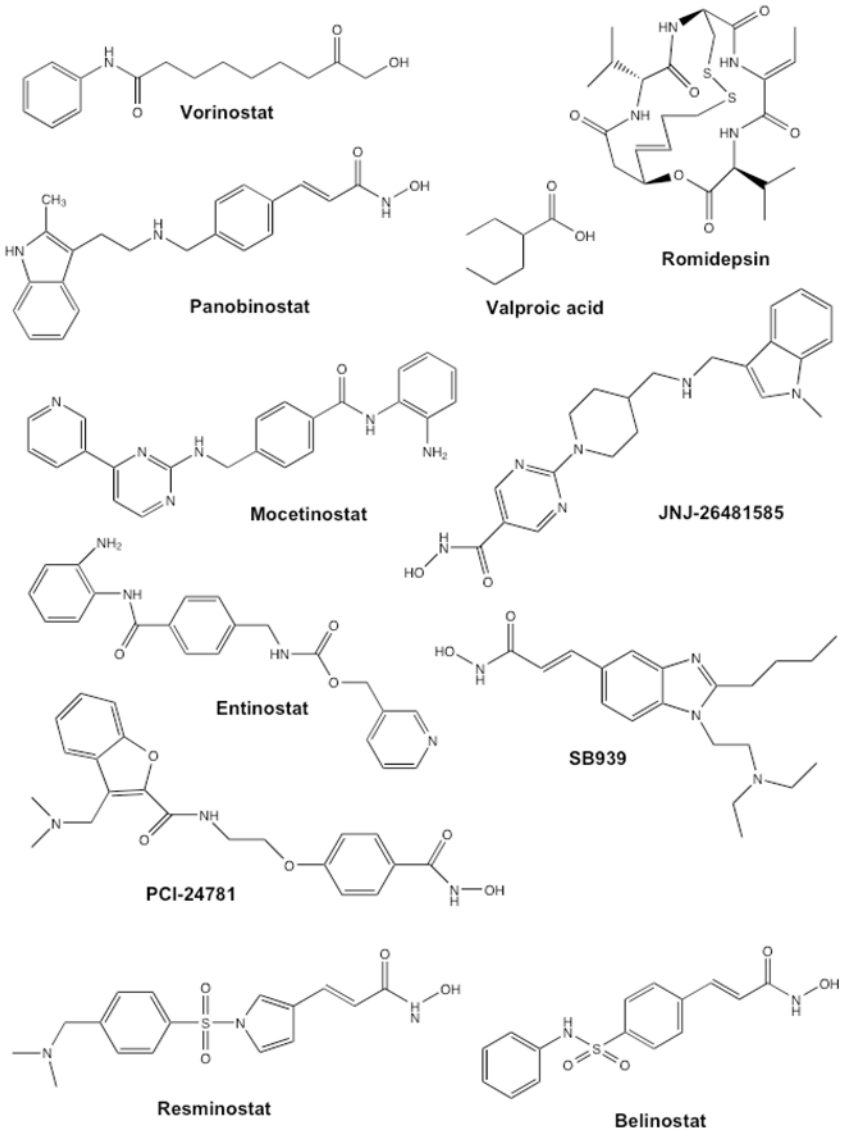

Figure 2.
Structures of some of the histone deacetylase inhibitors currently in clinical trials.

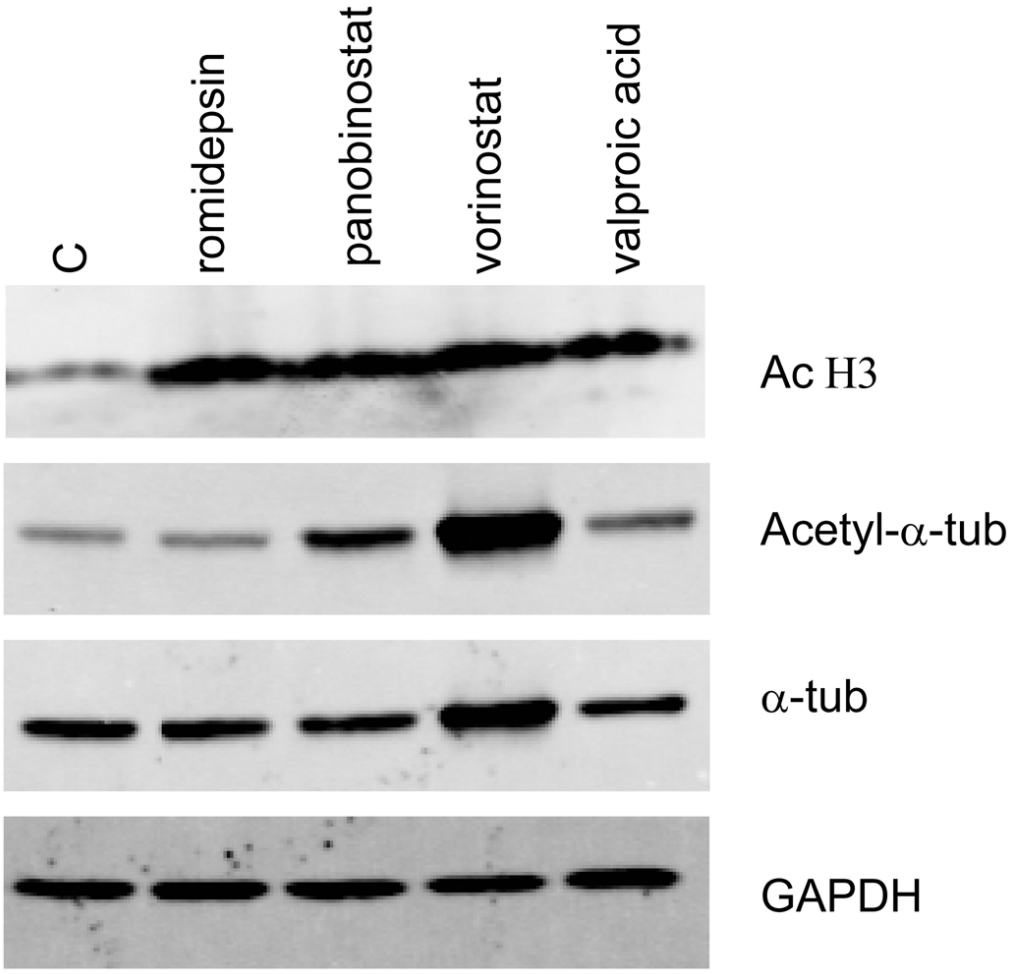

Figure 3.

Histone deacetylase inhibitors all cause increased histone acetylation but differentially cause tubulin acetylation. HEK293 (Flp-In-239) cells were treated with 46 nM romidepsin, 100 nM panobinostat, 10 μM vorinostat or 1 mM valproic acid for 24 h after which protein was extracted, subjected to electrophoresis, and transferred to a PVDF membrane. The membrane was subsequently probed for acetylated histone H3 (AcH3), acetylated α-tubulin (acetyl-α-tub), total α-tubulin (α-tub) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). While all of the HDIs were able to induce histone acetylation, only panobinostat and vorinostat were able to cause increased tubulin acetylation, suggesting that these HDIs also target HDAC6

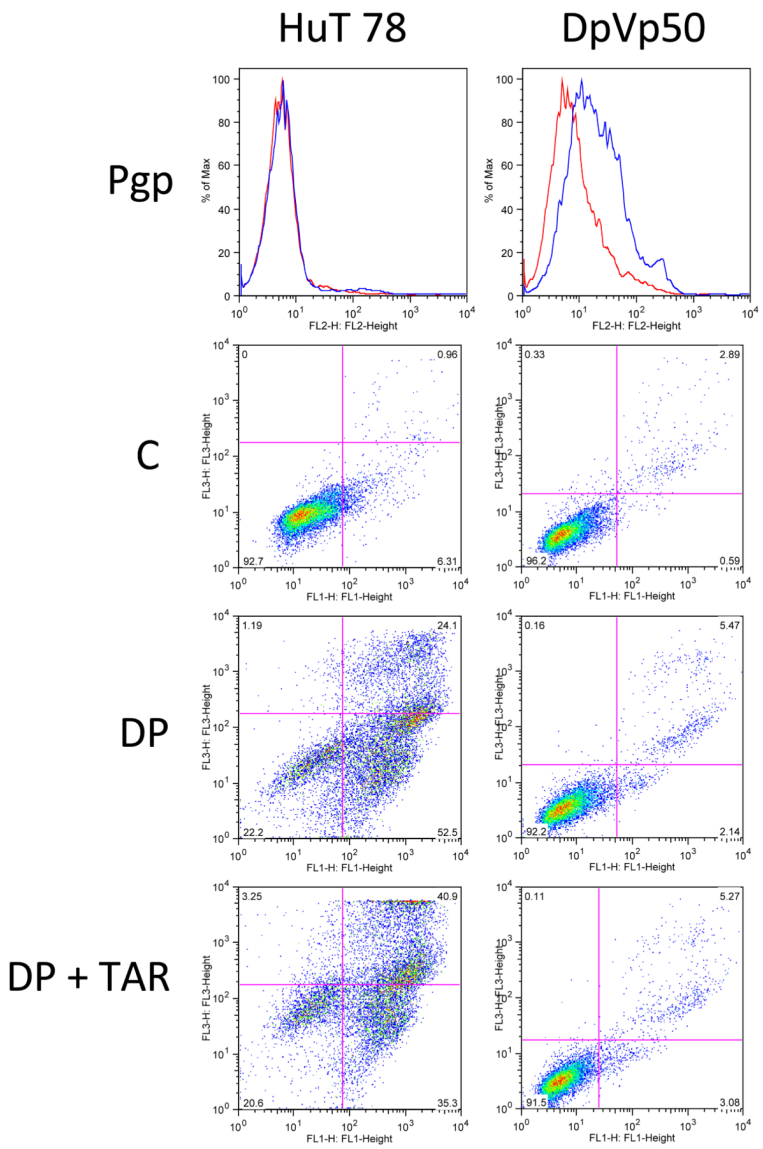

Figure 4.

Resistance to romidepsin is not mediated by Pgp expression in HuT78 DpVp50 cells. HuT78 parental and DpVp50 cells were incubated with the Pgp-specific antibody, MRK-16 (blue histogram), or IgG negative control antibody (red histogram) for 30 min after which cells were washed and incubated with phycoerythrin-labeled secondary antibody (top row, Pgp). While HuT78 parental cells are Pgp negative, the DpVp50 cells express low but detectable levels. Cells were also left untreated (second row, C) or were incubated with 50 ng/mL romidepsin for 48 h in the presence (third row, DP) or absence (bottom row DP + TAR) of 250 nM of the Pgp inhibitor tariquidar, after which cells were incubated with annexin V antibody and propidium iodide. Cells in the lower left quadrant are viable cells, while cells in the lower right quadrant are early apoptotic cells, and cells in the upper right quadrant are late apoptotic or necrotic cells. HuT parental cells readily undergo apoptosis after incubation with romidepsin either in the presence or absence of tariqudiar, so shown by the increase of cells in the upper and lower right quadrants. DpVp50 cells are resistant to romidepsin whether the inhibitor is added or not, suggesting a resistance mechanism that does not involve Pgp.

Table 1

Partial list of histone deacetylase inhibitors currently in clinical trials for the treatment of cancer.

Class	Compound
Aliphatic acids	Valproic acid
	AR-42 (OSU-HDAC42)
Hydroxamic Acids	*Vorinostat (suberoylanilide hydroxamic acid, SAHA)
	Belinostat (PXD101)
	Dacinosat (LAQ824)
	Panobinostat (LBH589)
	Resminostat (4SC-201)
	PCI-24781
	SB939
	CHR2845
	CHR3996
	JNJ-26481585
Benzamides	Entinostat (MS-275)
	Mocetinostat (MGCD0103)
	4SC-202
Cyclic peptides	*Romidepsin (depsipeptide, FK228, FR901228)

Compounds marked with an * are currently FDA approved. Belinostat is currently in registration trials.

Table 2

Summary of resistance mechanisms to HDIs

- ABC transporter expression
  - Increased levels of Pgp/ABCB1 or MRP1/ABCC1 (only true for romidepsin)
- Cell cycle proteins
  - Increased p21 expression
- Thioredoxin expression
  - Increased thioredoxin levels resulting in decreased ROS-mediated DNA damage
- Apoptosis-related proteins
  - Increased levels of antiapoptotic proteins such as Bcl-2 and Bcl-XL
  - Inability to upregulate proapoptotic proteins such as Bim
- Alterations in HDAC protein levels
- Signaling proteins
  - Increased signaling via MAPK, PI3K or STAT3
- NFkB activation
  - Acetylation of p65