Histone deacetylases (HDAC) represent a new target for cancer therapeutics. Four classes of HDACs have been identified (1). Class I human HDACs are homologous to the yeast HDAC Rpd3 and include HDAC1, HDAC2, HDAC3, and HDAC8. Class II HDACs include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10. HDAC11 makes up class IV HDACs. The key catalytic residues have been conserved in both class I and class II and IV HDACs. The third class of human HDACs consists of homologues of yeast Sir2. Class III HDACs require nicotinamide-adenine dinucleotide NAD+ for activity and differ from classes I and II in their catalytic site. Class I and II HDACs have been shown to be overexpressed, aberrantly recruited to oncogenic transcription factors, and mutated in cancer, thus representing potential targets for small-molecule inhibitors. Several structural classes of HDAC inhibitors (HDI) are currently undergoing evaluation in the clinic and include the short-chain fatty acids (e.g., valproate and phenylbutyrate); hydroxamic acid derivatives [e.g., vorinostat (suberoylanilide hydroxamic acid), belinostat (PXD-101), and panobinostat (LBH-589)]; benzamides [e.g., SNX-275 (MS-275) and MGC0103]; and cyclic tetrapeptides [e.g., romidepsin (FK-228); refs. 2, 3]. These HDIs under clinical development have been shown to induce differentiation, cell cycle arrest, and apoptosis in a variety of transformed cell lines; inhibit tumor growth in animal models; and show antitumor activity in clinical trials. Vorinostat, which has shown clinical responses in ~30% of patients with advanced cutaneous T-cell lymphoma, is the first HDI approved for the treatment of cancer, and it is currently being evaluated in other indications. A better understanding of the molecular determinants of resistance to HDIs may provide the basis for therapeutic combinations with improved clinical efficacy. Poor response to treatment could be linked to systemic factors like pharmacokinetics or to tumor-specific factors both at the level of the malignant cells (tumor intrinsic) or the tumor microenvironment. This review focuses on the tumor intrinsic mechanisms of drug resistance (excluding mechanism of acquired resistance due to chronic exposure). In particular, attention is given to selected mechanisms that are relevant across chemical classes of HDIs and that can aid in the design of rational combination strategies.

Mechanisms of Resistance to HDAC Inhibitors

Inhibition of HDACs trigger a number of cellular responses that interfere with growth-promoting signals, increase cellular stress, and ultimately lead to tumor cell death. Cancer cells, however, are highly adaptable in their ability to respond and resist growth inhibitory and damaging factors. In fact, the process of tumorigenesis is characterized by constant selection of those malignant cells that adapt to cope with damaging agents and environmental constraints and to become more independent from external proliferative/survival stimuli. In addition to these general mechanisms of survival or resistance to death that arise as a consequence of natural selection, stable alterations that may hinder the mode of action of HDIs can also become determinants of response. For instance, DNA hypermethylation represents a mechanism of resistance uniquely relevant to HDIs, which interferes with their ability to induce transcriptional activation of silenced tumor suppressor genes. Furthermore, exposure of tumor cells to first-line therapeutic treatments such as chemotherapy and radiotherapy may select for those with increased capacity to deal with high levels of HDAC activity leads to accumulation of acetylated proteins including histones, transcription factors, tubulin, and heat shock protein 90 (4, 5). The increase in the acetylation state of these proteins alters their function, leading to alterations in transcription, mitosis, and protein stability. Collectively, these changes interfere with tumor cell proliferation, survival, and maintenance. In addition, HDIs have shown antiangiogenic and immunomodulatory activity that may play an important role in mediating their antitumor effects. Indeed, the mechanism of action of HDIs is complex, and the mechanisms of resistance to HDIs described to date are diverse because alterations at various cellular levels can potentially block their activity.
DNA damage and oxidative stress. Ultimately, those adaptations could also contribute to block cell death and interfere with the activity of HDIs.

The antitumor activity of small-molecule compounds can be affected by efflux mechanisms, drug deactivation, status of drug target, bypass/repair mechanisms for drug-induced damage, and alterations in the cell death pathway. Cellular factors that have been implicated as determinants of resistance in HDIs include (a) drug efflux, (b) target overexpression and desensitization, (c) chromatin/epigenetic alterations, (d) stress response mechanisms, and (e) antiapoptotic/prosurvival mechanisms (Fig. 1).

**Effect of drug efflux mechanisms on sensitivity to HDIs.** One of the molecular alterations most commonly associated with the multidrug resistance phenotype in cancer cells is that mediated by overexpression of efflux pumps in the ATP-binding cassette transporter family. The antiproliferative activities of vorinostat, belinostat, AN9, and romidepsin have been examined in multidrug-resistant human cancer cell lines with high levels of P-glycoprotein, the product of the multidrug resistance-1 gene (MDR1) as well as the multidrug resistance protein 1 (6–10). To date, romidepsin is the only HDI that has been established as a substrate for P-glycoprotein and multidrug resistance–associated protein 1. Overall, multidrug resistance mediated by efflux mechanisms does not seem to constitute a major driver of tumor cell resistance to HDIs that belong to either the hydroxamate or carboxylic acid class. Of note, the combination of romidepsin and all-trans retinoic acid was shown to up-regulate P-glycoprotein, inducing resistance to doxorubicin in promyelocytic cells (11). This effect was due, in part, to an increase in histone acetylation and chromatin remodeling at the MDR1 promoter. In addition to romidepsin, other HDIs including sodium butyrate have been shown to activate MDR1 transcription (12). Further studies will help to clarify the effect of other HDIs on P-glycoprotein expression to inform combination studies involving chemotoxins that are substrates of drug efflux pumps.

**Alterations in HDACs and their effect on response to HDIs.** The first indication that resistance to HDIs could be
traced to alterations at the level of their molecular target came from studies done with the hydroxamate derivative trichostatin A (13). Subsequent studies in melanoma cells established that overexpression of HDAC1 was sufficient to confer resistance to sodium butyrate (14). More recently, an inactivating mutation in HDAC2 was identified in various human colon and endometrial cancer cell lines with microsatellite instability (15). Treatment of HDAC2-deficient RKO and Co115 cells with trichostatin A failed to induce histone acetylation and inhibit proliferation. Interestingly, these cells remain sensitive to valproate and butyrate. The selective resistance to trichostatin A is intriguing, and additional studies are necessary to gain further insight into the molecular basis of this phenomenon. Noteworthy, the same frameshift mutation found in cell lines was also present in 48 of 228 (21%) tumor samples analyzed, suggesting that alterations in HDAC2 expression may play a role in clinical response to selective HDIs.

Epigenetic and chromatin alterations as determinants of response. In addition to genetic alterations, epigenetic mechanisms contribute to malignant transformation (16, 17). DNA methylation and histone acetylation alter the nucleosome structure to activate or repress gene transcription (18). Silencing of tumor suppressor genes is frequently mediated by DNA methylation at the promoter regions. Acetylation of histones, linked to methylation sites through the recruitment of HDACs by methylcytosine binding proteins (e.g., MeCP-2), constitutes a second level of regulation of epigenetic silencing (19–21). In addition, HDAC1 and HDAC2 have been shown to interact with DNA methyltransferases. The evidence thus far indicates that these two biochemical processes cooperate to induce gene silencing (22). Indeed, DNA methylation and hypoacetylation of histones H3 and H4 are frequently associated with silent genes. There are a number of cases of restoration of the expression of tumor suppressor genes after HDI treatment. Some studies, however, have shown examples of dominance of DNA methylation over histone repressive marks in cancer cells (23, 24). From that stand point, methylation of DNA hinders the ability of HDIs to fully restore expression of epigenetically silenced genes and represents a mechanism of resistance to HDI treatment. For instance, HDAC inhibition was not sufficient to restore expression of the tumor suppressor genes hMHL1 and TIPM3. Minimal methylation after a low dose of 5-aza-2'-deoxycytidine was required for robust reexpression of these genes in response to trichostatin A. Furthermore, cases in which either treatment alone was insufficient to induce significant reexpression of epigenetically silenced genes have been reported. Among those, synergistic reexpression of the MAGE family in testicular cancers and TMSC1 in hepatocellular carcinoma occurs only in response to the combination of 5-aza-2'-deoxycytidine and HDIs (25, 26). Although emerging new data suggest that in some cases HDIs can affect global and gene-specific DNA methylation (27, 28), most studies indicate that this repressive mark cannot be overcome by the single action of HDIs. These observations provided the rationale for the clinical evaluation of HDIs in combination with hypomethylating agents.

Cellular polyamines like spermidine and spermine are cationic molecules that regulate gene expression by affecting the chromatin microenvironment (29). The relative abundance of polyamines is controlled by ornithine decarboxylase, the rate-limiting enzyme in the process of polyamine biosynthesis. In vitro, polyamines have been shown to condense chromatin fibers and facilitate oligomerization of nucleosomal arrays (30, 31). A relationship between high polyamine levels and altered acetylation of core histones seems to be linked with proliferation. The basis for this correlation is not fully understood. Enhanced histone acetyltransferase activity, however, was detected in skin samples and skin fibroblasts from K6-ODC transgenic mice (29). Recently, the contribution of polyamines to the response of human cancer cell lines to inhibition of HDACs was evaluated (32). These studies showed that polyamines modulate the response to structurally diverse HDIs including trichostatin A, trapoxin A, and sodium butyrate, and that their depletion increases resistance to apoptosis in response to treatment. Examination of the correlation between polyamine levels and clinical response to HDI merits testing. Furthermore, the investigators proposed that elevated ornithine decarboxylase activity could represent a biomarker of response to HDIs.

Mechanism of protection against oxidative stress as a determinant of resistance. Treatment of cancer cells with HDIs including vorinostat, SNDX-275, sodium butyrate, panobinostat, and romidepsin results in oxidative stress characterized by increased production of reactive oxygen species and decreased ratio of reduced to oxidized glutathione (33–37). Furthermore, cell death in response to several HDIs can be partially rescued by preincubation of cells with the glutathione precursor and free radical scavenging N-acetylcysteine (38). These data implicate oxidative damage as a significant component of the antiproliferative effect triggered by HDIs. Cellular redox homeostasis is maintained through a complex set of reactions, and the thioredoxin family participates in this process. Thioredoxins are proteins with disulfide reducing activity that provide reducing equivalents to ribonucleotide reductase for DNA synthesis and to peroxiredoxins for scavenging reactive oxygen species. Through these activities, thioredoxins play a role in proliferation, protection against oxidative damage, and inhibition of stress-induced cell death (39). The activity of thioredoxin-1 is modulated by thioredoxin-binding protein 2, which binds to the reduced form of the enzyme and interferes with its function. Response to vorinostat has also been associated with its ability to induce the expression of thioredoxin-binding protein 2 and down-regulate the expression of thioredoxin in cell lines that are sensitive to the compound (40). In addition, up-regulation of thioredoxin has been proposed to constitute a defense mechanism against HDI-induced increase in reactive oxygen species levels in nontransformed cells (41). In addition, thioredoxin was found to be differentially expressed across prostate cancer cell lines with diverse vorinostat sensitivity (42). In tumors, high levels of thioredoxin-1 have been associated with grade and resistance to standard therapy (39). Based on the existing in vitro data, it is tempting to speculate that mechanisms that contribute to alleviate cellular oxidative stress may antagonize the activity of HDIs. Thus, tumors with inherent high levels of antioxidants, including enzymes involved in the maintenance of cysteine thiol homeostasis, may be able to overcome HDI-mediated damage. In this scenario, proteins that participate in the stress response to oxidative damage could constitute determinants of clinical resistance to HDIs. This is an intriguing hypothesis that warrants rigorous clinical evaluation.
Molecular Pathways

Role of mediators of cell death pathway and of autophagic response in treatment outcome. In vitro, HDIs elicit apoptosis of a variety of malignant cells (43–47). Cell death proceeds mainly via the intrinsic apoptotic pathway and is characterized by mitochondrial damage, cytochrome c release, and production of reactive oxygen species (33, 48). Consistently, overexpression of antiapoptotic Bcl-2 or Bcl-X<sub>L</sub> is sufficient to render transformed cells resistant to HDIs (48–51). Therapeutic efficacy of HDIs was correlated with induction of apoptosis in mice bearing Eμ-Myc lymphoma cells (52). Extensive analysis of the role of the tumor suppressor p53 and intermediates involved in the mitochondrial apoptotic cascade on the response of the B-cell lymphomas indicates that the HDI-induced death is independent of p53 activity, requires the proapoptoticBH3-onlyproteinsBimandBid,andcanbeblockedbyBcl-2. Because Bcl-2 overexpression occurs in leukemias and lymphomas, and loss of Bim and Bid has been reported in human cancers, it is possible that alterations in these proteins may play a role in clinical response to HDIs. Future studies will provide clarity.

The antiapoptotic transcription nuclear factor κB (NF-κB) has been identified as a mediator of resistance to HDI treatment. Inhibition of HDACs stimulates transcriptional activation of NF-κB through acetylation of the RelA/p65 subunit and induction of an array of genes that mediate protection from cell death (53). The activation of NF-κB by HDIs interferes with their ability to trigger cell death in non–small cell lung cancer lines and leukemia cell lines (54, 55). In fact, inhibition of NF-κB activation by the compound BAY-11-7085 sensitizes the malignant cells to death in response to inhibition of HDACs. Because several HDIs are at various stages of clinical evaluation in these indications, it is important to understand the relevance of the pathway as a determinant of response to treatment.

Recently, we have uncovered a correlation between constitutive activation of various members of the signal transducer and activator of transcription family and lack of response to vorinostat in patients with cutaneous T-cell lymphoma. In vitro studies suggest that the protective role of these proteins is mediated, at least in part, by transcriptional control of several antiapoptotic genes.

In addition to its well-established role as a negative regulator of the cell cycle, the cyclin-dependent kinase inhibitor p21(CIP1/WAF1) has been implicated in the regulation of programmed cell death (56). Several studies have shown that HDIs induce the expression of p21(CIP1/WAF1) in a p53-dependent and p53-independent way, and that p21(CIP1/WAF1) up-regulation in response to HDI treatment mediates cell cycle arrest and differentiation or apoptosis (57–59). Thus, the role of p21(CIP1/WAF1) as a determinant of response is not totally understood. Early studies in U937 leukemia cells showed that HDI-mediated p21(CIP1/WAF1) induction protects cells from apoptosis, and that blocking it resulted in an increase in cell death (58). Subsequent studies with SNX-275, however, suggest that the p21(CIP1/WAF1)-mediated protection is dose dependent. In this case, low concentrations of SNX-275 induced cell cycle arrest in leukemia cells, and that effect was overcome at high concentrations of HDI (34).

The role of autophagy in the response of cancer cells to therapy is a matter of intense investigation. Autophagy is a process in which intracellular membranes sequester proteins and organelles (autophagosome) that are subsequently delivered to the lysosome for degradation (60). This cellular program enables elimination and/or recycling of proteins and intracellular components in response to starvation and stress. The contribution of autophagy to the response to anticancer therapy seems to depend on the cellular context. Whereas in some cancer cells activation of autophagy results in cell death and correlates with therapeutic efficacy, in others autophagy plays a protective role by which cells cope with therapy-induced bioenergetic stress and cellular damage (61, 62). In this second scenario, therapeutic strategies that incorporate inhibitors of autophagy may enhance the efficacy of anticancer therapy (63). Treatment of imatinib-resistant chronic myeloid leukemia with chloroquine, which blocks fusion of the autophagosome and lysosome, results in enhanced antineoplastic activity of the HDI vorinostat (64). This study underscores the role of protective autophagy as a determinant of response to HDI treatment in a defined subpopulation of chronic myeloid leukemia cells. Additional work will help to understand the relevance of this cellular process for other cancer types.

Clinical-Translational Implications

Undoubtedly, characterization of the molecular determinants of resistance to HDIs will help to stratify patients according to their likelihood of responding to treatment and to develop combination strategies to circumvent them. It is important to emphasize that most of the hypotheses drawn from preclinical studies on the mechanism of resistance to HDIs await clinical evaluation. One practical consideration is that a number of pharmacologic agents that can counteract the in vitro mechanisms previously described are in the discovery phase and are not available for clinical use. Inhibitors of efflux pumps (tariquidar and laniquidar), antiapoptotic Bcl-2 (oblimersen and ABT-263), and nuclear factor κB (oligonucleotide decoys), however, are at advanced stages of clinical development and could be evaluated in combination with HDIs in the near future.

As discussed earlier, certain tumor cells may have a high antioxidant protective barrier that undermines the antineoplastic activity of HDIs. Thus, one possible strategy to maximize the therapeutic efficacy of HDIs is to combine them with agents that also elicit oxidative damage as part of their mechanism of response and to potentiate this effect. This approach may suffer from high toxicity. Tolerability profiles for combinations of this nature may vary with the complementary compound selected. Whenever tolerated, however, pro-oxidant combinations may stress cells above their resistance threshold and push them down the apoptotic pathway. One of the combinations that has been explored preclinically involves the typical pro-oxidant agent 2-methoxyestradiol, which directly affects the mitochondria, the major source of cellular reactive oxygen species (65). A number of clinically approved compounds induce oxidative damage as part of their mode of action. Among them, the activity of bortezomib and doxorubicin may synergize with that of HDIs at various levels, including the increase in reactive oxygen species and depletion of reduced glutathione (66, 67). Clinical trials...

1 V.R. Fantin and V.M. Richon, unpublished data.
the potential of epigenetic therapy and warrants development of future trials to explore additional HDIs in combination with hypomethylating agents.

Conclusion

The molecular heterogeneity of tumor cells, together with the pleiotropic nature of the cellular response triggered by inhibition of HDACs, poses a major challenge in the quest for determinants of resistance. It is clear from preclinical studies that resistance to inhibitors of HDACs is multifactorial, and that the dominant pathway of resistance in tumors may be dependent on tissue of origin and genetic context. In the future, availability of large enough sets of clinical samples will enable us to further evaluate the clinical significance of these preclinical hypotheses of resistance.

References


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