Rational Combinations Using HDAC Inhibitors

Michael Bots1 and Ricky W. Johnstone1, 2

Abstract
In addition to well-characterized genetic abnormalities that lead to cancer onset and progression, it is now recognized that alterations to the epigenome may also play a significant role in oncogenesis. As a result, epigenetic-modulating agents such as histone deacetylase inhibitors (HDACi) have attracted enormous attention as anticancer drugs. In numerous in vitro and preclinical settings, these compounds have shown their vast potential as single agent anticancer therapies, but unfortunately equivalent responses have not always been observed in patients. Given the pleiotropic effects HDACi have on malignant cells, their true therapeutic potential most likely lies in combination with other anticancer drugs. In this review we will focus on the anticancer effects of HDACi when combined with other cancer therapeutics with an emphasis on those combinations based on a strong molecular rationale.

Cancer is a disease caused by insult to the normal genome and it is apparent that both genetic and epigenetic alterations can play a critical role in tumor initiation and progression (1–3). Compounds targeting enzymes such as histone deacetylases (HDAC) that are actively involved in chromatin remodeling and have been shown to be aberrantly expressed and/or inappropriate activated or localized in human tumors have generated great interest as anticancer drugs (4, 5). Indeed, treatment of malignant cells with HDAC inhibitors (HDACi) can induce a range of anticancer effects including tumor cell apoptosis, cell cycle arrest, differentiation and senescence, modulation of immune responses, and altered angiogenesis (5). As a consequence of their therapeutic potential in laboratory studies, the single-agent activities of numerous HDACi have been tested in the clinic or are currently the subject of ongoing early-phase clinical trials (6, 7). As monotherapies, HDACi have thus far been shown to be effective against a defined subset of hematological tumors however there is less than convincing evidence that these agents will be effective against solid tumors as single agents (8, 9).

Considering the diverse anticancer effects mediated by HDACi and their moderate and largely manageable clinical side effects, the full therapeutic potential of HDACi will probably be best realized through combination with other anticancer agents. There have been numerous reports documenting the enhanced antitumor effects (predominantly enhanced apoptosis) of combining HDACi with a vast array of cancer therapeutics in studies done in vitro with human or mouse tumor cell lines (5, 7). The agents tested in combination with HDACi range from traditional anticancer agents such as cytotoxic drugs and γ-irradiation to small molecule inhibitors of defined molecular cancer targets (5). Most of the published reports do not provide a preconceived molecular rationale for combining an HDACi with a given agent(s). Moreover, the molecular and biological events that underpin any observed additive or synergistic combination effect are largely ill-defined. As a result, it is clear that HDACi can augment the antitumor activities of a plethora of pharmacological and biological anticancer agents, however little is known about how this occurs, and accordingly, there is little useful information to direct the future clinical use of HDACi in combination with other agents.

In this review, we will summarize current data on combination strategies that utilize HDACi. We will focus on those studies in which a rationale for combining a given HDACi with another agent is apparent and those studies that provide preclinical and/or clinical trial data.

Preclinical Combination Studies Using HDACi

**HDACi and modulators of transcription.** The promoter regions of genes that play important roles in regulating cell cycle, apoptosis, DNA repair, differentiation, and cell adhesion are often hypermethylated and therefore transcriptionally silenced in tumors, and this provides strong circumstantial evidence that aberrant epigenetic control may be an important oncogenic event (3, 10). Abnormal patterns of DNA methylation in tumors can occur either as a result of overexpression of DNA methyltransferases (DNMTs) or the inappropriate recruitment of these enzymes to promoters (11), and transcriptional silencing via DNA hypermethylation can often be associated with poor clinical outcome (3, 12). If we accept that tumor-selective silencing
of tumor suppressor genes is an oncogenic driver, then targeting the reexpression of these genes is a logical therapeutic approach. Although inhibition of DNMT activity using pyrimidine nucleoside analogs such as decitabine (Dacogen, SuperGen, Inc.) and azacitidine (Vidaza, Celgene) can result in the reexpression of hypermethylated genes, this response is greatly enhanced through the combined effects of HDACi and DNMTi inhibitors (DNMTi; refs. 13, 14) (Fig. 1A). Indeed this strategy has proven successful in vitro with the combination of HDACi and DNMTi demonstrating enhanced anticancer responses (15, 16). Among the reactivated genes are tumor suppressor genes such as CDKN1A, CDKN2A, and CDKN2B encoding the cyclin-dependent kinase inhibitors p21, p16, and p15, respectively (13, 17, 18).

It is currently not known if the reactivation of epigenetically silenced genes is the molecular event that underpins the antitumor effects observed when DNMTi and HDACi are combined, and if so, if specific reactivation of only one or a number of epigenetically silenced genes is necessary.

There is a strong molecular rationale for combining HDACi and all-trans retinoid acid (ATRA) for the treatment of acute promyelocytic leukemia (APL) and acute myeloid leukemia (AML; refs. 19, 20). These tumors often harbor either the oncogenic fusion protein PML-RARα or PLZF-RARα that results in abnormal recruitment of HDAC-containing repressor complexes to retinoic acid response elements (RARα). Although APL can often be highly sensitive to treatment with ATRA concomitant with release of repressor complexes from retinoic acid response (RAR)α, leukemias driven by PLZF-RARα and certain PML-RARα variants can be relatively unresponsive to ATRA. Combination with HDACi however results in the release of the repressor complexes and restores the sensitivity to ATRA (Fig. 1A). The combination of HDACi and retinoids has been successfully shown in vitro and in vivo in experimental models (20–22). Consistent with these preclinical findings, treatment with ATRA and the HDACi sodium phenylbutyrate induced a clinical response in a retinoic acid-resistant APL patient (23). Although retinoids have some clinical benefit for the treatment of neuroblastoma, intrinsic and acquired resistance to this form of therapy has been observed (24). As such it was hypothesized that combination therapy using HDACi and retinoids may be effective for the treatment of neuroblastoma and encouraging results have been obtained in preclinical studies (25–27). Although HDACi treatment increases the expression of retinoic acid receptor beta (RARβ; ref. 28) and RAR genes are certainly activated following combination treatment, it remains unclear if
the activation of RAR genes is necessary for the observed combination effect.

**HDACi and Regulators of the Misfolded Protein Response**

In addition to histones, a range of cellular proteins are posttranslationally modified by HDACs resulting in their altered function, stability, or cellular localization (5, 29). As such, in addition to HDACi-induced anticancer effects caused by altered gene transcription, the biological activities of HDACi may be mediated by functional changes to nonhistone proteins such as transcription factors (e.g., p53, Bcl6, E2F1), DNA repair proteins (e.g., Ku70), cytoskeletal proteins (e.g., α-tubulin), and chaperone proteins (e.g., Hsp90; ref. 29).

The accumulation of misfolded and damaged proteins that can be toxic to cells is circumvented through their removal either through the activity of chaperones such as Hsp90 and/or through the transportation of protein aggregates from the cytoplasm by dynein motors via the microtubule network to a novel organelle termed the aggresome where they are processed (30). This processing of misfolded and/or damaged proteins may be particularly important for tumor cells that produce large amounts of these aberrant proteins. HDAC6 plays a key role in the misfolded protein response by deacetylating Hsp90, resulting in the stabilization of client proteins that can include overexpressed (i.e., ErbB2/Her2), fusion (i.e., Bcr-Abl), and mutated (i.e., c-kit, FLT3) oncoproteins, the expression of which tumor cells can often be reliant on (“addicted”) for their growth and survival (Fig. 1B; refs. 31, 32). Moreover, HDAC6 plays an important role in the aggresome pathway through its ability to bind both polyubiquitinated misfolded proteins and dynein motors, thereby acting to recruit misfolded protein cargo to dynein motors for transport to aggresomes (Fig. 1B; ref. 33). Accordingly, tumor cells are potentially vulnerable to HDAC6 inhibition and this has been exploited by combining HDACi with Hsp90 inhibitors such as 17-allylamino-demethoxy geldanamycin (17-AAG; refs. 34–37) and with proteosome inhibitors such as bortezomib (Velcade, Millennium Pharmaceuticals; refs. 38–41). In addition, the combined effect of directly targeting oncogenic client proteins such as ErbB2/Her2 and Bcr-Abl using Herceptin and Imatinib respectively and destabilizing these proteins through hyperacetylation of Hsp90 by HDACi has a potent antitumor effect in vitro (42–44). Although it is tempting to speculate that targeting the misfolded protein response pathway at two points (Hsp90 and the aggresome pathway) by inhibition of HDAC6 is the molecular event necessary for the observed effects of combining HDACi with agents such as 17-AAG and bortezomib, romidepsin has higher affinity for HDAC1 and 2 than HDAC6 (45), yet this HDACi can also function synergistically with proteosome inhibitors (46, 47) indicating that other HDACs may be involved in the combined antitumor responses.
Combining HDACi with Agents That Directly Target Apoptotic Proteins

HDACi are potent inducers of tumor cell apoptosis and recent preclinical studies have defined a direct correlation between the ability of HDACi to kill tumor cells, and therapeutic efficacy (48–50). It was recently shown that constitutive Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling may be useful as a biomarker for nonresponsiveness in patients with cutaneous T-cell lymphoma (CTCL) receiving the HDACi vorinostat as part of a phase IIb clinical trial. Decreased expression of anti-apoptotic proteins following inhibition of JAK activity sensitized previously resistant CTCL cells to vorinostat-mediated apoptosis (51). This observation provides circumstantial evidence that although HDACi can elicit a range of biological responses that may affect tumor growth and/or survival, direct tumor cell apoptosis induced by HDACi is likely to play a very important role in mediating the therapeutic responses observed. Therefore there exists a strong impetus to design combination strategies aimed at augmenting HDACi-mediated death of tumor cells as detailed below (Fig. 1C).

**HDACi and death receptor agonists.** HDACi can increase the expression and/or activity of proteins that directly transmit an apoptotic signal through death receptor pathways such as death receptors [i.e., Fas, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-receptors], death receptor ligands (i.e., Fas ligand, TRAIL), and downstream caspases (caspase-8 and -3), and can downregulate proteins that negatively regulate death-receptor signaling (i.e., c-FLIP, XIAP, survivin, c-IAP1/2; refs. 5, 50, 52–57). These results provides a strong rationale to combine HDACi with death receptor stimuli and given the tumor-selective activity attributed to TRAIL and agonistic anti-TRAIL receptor antibodies and their clinical development as anticancer agents (see ref. 58 and references therein), this combination seems most attractive. In vitro studies have shown that sublethal doses of HDACi can sensitize tumor cells to TRAIL-mediated apoptosis (58) and importantly, HDACi do not sensitize normal cells to TRAIL-mediated apoptosis (59). We have recently extended the synergistic antitumor activity of HDACi and agonistic anti-TRAIL receptor antibodies (MD5-1) observed in vitro to significant therapeutic responses in vivo. Complete regression of established syngeneic breast cancers was observed using a vorinostat/MD5-1 combination, whereas either agent used alone caused only a minimal delay in tumor growth (60). Interestingly, synergistic tumor cell apoptosis mediated by vorinostat and MD5-1 was concomitant with proteosome-mediated downregulation of c-FLIP with no change in TRAIL, TRAIL-receptor, or death receptor regulatory proteins observed (60).

**HDACi and inhibitors of prosurvival Bcl-2 proteins.** Overexpression of prosurvival Bcl-2 proteins (Bcl-2, Bcl-XL, Mcl-1, Bcl-w, and A1) is commonly observed in tumor cells and is associated with resistance to apoptosis mediated by chemotherapeutic drugs (61). These proteins play a major role in the regulation of the intrinsic apoptosis pathway by preventing mitochondrial outer membrane permeabilization and the release of mitochondrial proteins such as cytochrome c and...
smac/DIABLO (62). Although the finding that overexpression of Bcl-2 family proteins can potently inhibit HDACi-mediated apoptosis in vitro has been well-established (see ref. 5 and references therein), we have shown that these proteins can also inhibit the therapeutic activities of HDACi in preclinical cancer models (48, 49). Most recently, we have shown that ABT-737, a small molecule inhibitor of Bcl-2 can resestize Bcl-2-overexpressing, HDACi-resistant tumors to HDACi-mediated apoptosis in vitro and in vivo (63). Our demonstration that a significant decrease in tumor burden was achievable in mice following only a single treatment with ABT-737 and vorinostat raises the exciting possibility that this therapeutic regimen may be developed for clinical application. ABT-263 is an orally bioavailable homolog of ABT-737 that as detailed below is currently under investigation in early phase clinical trials as an anticancer agent (64–67) indicating that combination studies using HDACi and ABT-263 in human patients may be achievable in the near future.

**Clinical Studies Using HDACi in Combination**

As outlined in the previous section, many different combination strategies using HDACi have been successfully tested in laboratory studies. Based on these findings, it is not surprising that many trials have been started to test their safety and efficacy in clinical settings. Initial studies concentrated on combining HDACi with Food and Drug Administration (FDA)-approved, clinically validated chemo- and radio-therapeutics to augment their anticancer activities (8, 9). In addition, clinical studies using HDACi in combination with targeted therapeutics including those outlined above have been initiated (Table 1). Among these therapeutics are bortezomib, azacitidine, and decitabine, which all have already been approved by the FDA for treatment of multiple myeloma and myelodysplastic syndromes respectively. Although most of the clinical trials are still ongoing and concentrate predominantly on dosing schedule and safety of the combination therapies, so far some studies have shown encouraging results. In patients with advanced hematological or solid malignancies the combination of DNMTi and HDACi was well tolerated and showed biological effects such as DNA hypomethylation and histone acetylation (68–74). Importantly, this combination strategy showed clinical activity and in at least one preliminary study vorinostat in combination with azacitidine seems to be more effective compared with azacitidine alone (74). Although these data support further investigation, especially to evaluate

**Table 1. Active clinical trials using HDACi in combination with other agents**

<table>
<thead>
<tr>
<th>HDACi</th>
<th>Synonym</th>
<th>Phase</th>
<th>Combination drug(s)</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vorinostat</td>
<td>SAHA</td>
<td>I</td>
<td>Azacitidine</td>
<td>Nasopharyngeal carcinoma, lymphoma</td>
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<td></td>
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<td>I</td>
<td>Decitabine</td>
<td>Advanced solid tumors, lymphoma, leukemia</td>
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<td></td>
<td></td>
<td>I</td>
<td>Decitabine</td>
<td>Hematologic cancer or other diseases</td>
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<tr>
<td></td>
<td></td>
<td>I</td>
<td>Decitabine</td>
<td>Myelodysplastic syndromes, AML</td>
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<td></td>
<td>I</td>
<td>Bortezomib</td>
<td>Lung cancer</td>
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<tr>
<td></td>
<td></td>
<td>I</td>
<td>Bortezomib</td>
<td>Metastatic solid tumors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>Bortezomib</td>
<td>Advanced lung cancer</td>
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<tr>
<td></td>
<td></td>
<td>I</td>
<td>Bortezomib</td>
<td>Advanced multiple myeloma</td>
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<tr>
<td></td>
<td></td>
<td>I</td>
<td>NPI-0052</td>
<td>Lung cancer, pancreatic cancer, melanoma, lymphoma</td>
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<tr>
<td></td>
<td></td>
<td>I/II</td>
<td>Azacitidine</td>
<td>Myelodysplastic syndromes, AML</td>
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<tr>
<td></td>
<td></td>
<td>II</td>
<td>Bortezomib</td>
<td>Recurrent glioblastoma multiforme</td>
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<td></td>
<td>II</td>
<td>Bortezomib</td>
<td>Recurrent lymphoma</td>
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<td></td>
<td></td>
<td>II</td>
<td>Bortezomib</td>
<td>Refractory multiple myeloma</td>
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<td>III</td>
<td>Bortezomib</td>
<td>Multiple myeloma</td>
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<td>Entinostat</td>
<td>MS-275</td>
<td>I</td>
<td>Azacitidine</td>
<td>Leukemia; myelodysplastic syndromes</td>
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<tr>
<td></td>
<td></td>
<td>II</td>
<td>Azacitidine</td>
<td>Lung cancer</td>
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<td></td>
<td>I</td>
<td>Decitabine</td>
<td>Leukemia; myelodysplastic syndromes</td>
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<tr>
<td>Panobinostat</td>
<td>LBH589</td>
<td>I</td>
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<td>I/II</td>
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<td>Myelodysplastic syndromes, AML</td>
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<td>I/II</td>
<td>Azacitidine; ATRA</td>
<td>Myelodysplastic syndromes; leukemia</td>
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<td>Bortezomib</td>
<td>Multiple myeloma</td>
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<td>Multiple myeloma</td>
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<td>PXD101</td>
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<td>Azacitidine</td>
<td>Leukemia; myelodysplastic syndromes</td>
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<td></td>
<td>I</td>
<td>Bortezomib</td>
<td>Advanced solid tumors, lymphomas</td>
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<tr>
<td></td>
<td></td>
<td>II</td>
<td>Azacitidine</td>
<td>Myelodysplastic syndrome; AML</td>
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the clinical efficacy of these combinations in randomized trials, it has to be noted that this strategy also resulted in some unfavorable outcomes and HDACi combined with DNMTi was dose-limited by encephalopathy (75). Such neurologic effects seem to be common adverse events following treatment with structurally diverse HDACi although the cause of these side effects is unknown (8). Compared with DNMTi, only limited data are available combining proteosome inhibitors such as bortezomib and NPI-0052 with HDACi. Results to date indicate that these combinations are well tolerated and seem to be clinically effective in both solid tumors and multiple myeloma (76, 77).

Clinical trials using HDACi in combination with recombinant TRAIL or agonistic anti-TRAIL receptor antibodies such as mapatumumab (anti-TRAIL receptor 1) or lecutumumab, AMG655 and Apomab (anti-TRAIL receptor 2), or pro-survival Bcl-2 family inhibitors have not yet been reported. However, a multicenter, phase 1b study of AMG 655 in combination with vorinostat in patients with relapsed or refractory low grade lymphoma, mantle cell lymphoma, diffuse large cell lymphoma, and Hodgkin’s disease has been initiated (78). Clinical trials using agents that activate the TRAIL pathway as mono-therapies have been initiated with encouraging safety profiles obtained (58). Clinical trials using ABT-263 as a monotherapy have been initiated and thus far it seems that predicted side effects are manageable. Although not the aim of these initial trials, antitumor responses in some of the patients have been achieved (66, 67).

**Future Directions**

HDACi hold enormous promise for the treatment of hematological and solid tumors, especially when used in combination with other anticancer agents. Although there are numerous reports demonstrating that HDACi can augment the antitumor effects of a diverse range of cancer therapies in vitro, in many instances the molecular events that underpin the combined effects remain ill-defined. However, as our knowledge of the mechanisms of action of HDACi continues to expand, and as we begin to understand how tumors may be intrinsically resistant to HDACi or acquire resistance, rational combination strategies can be implemented. Evidence already exists that this is achievable, either by combining HDACi with agents that augment the molecular (i.e., epigenetic/transcriptional) activities of HDACi, or enhance the biological antitumor responses (i.e., apoptosis) that HDACi elicit. Integrating functional cell-based assays with preclinical testing usingtractable mouse models of cancer in which mechanisms of action, therapeutic efficacy, and toxicity of any combination regimen using HDACi can be analyzed could provide important information about future clinical studies that could and should be initiated.

**Disclosure of Potential Conflicts of Interest**

R. Johnstone, commercial research grant, Merck, Novartis, Pfizer; consultant, Progen.

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**References**

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