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Commentary

HDAC inhibitors: Clinical update and mechanism-based potential

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ABSTRACT

Recently, the role of transcriptional repression through epigenetic modulation in carcinogenesis has been clinically validated with several inhibitors of histone deacetylases and DNA methyltransferases. It has long been recognized that epigenetic alterations of tumor suppressor genes was one of the contributing factors in carcinogenesis. Inhibitors of histone deacetylase (HDAC) de-repress genes that subsequently result in growth inhibition, differentiation and apoptosis of cancer cells. Vorinostat (SAHA), romidepsin (depsipeptide, FK-228), belinostat (PXD101) and LAQ824/LBH589 have demonstrated therapeutic benefit as monotherapy in cutaneous T-cell lymphoma (CTCL) and have also demonstrated some therapeutic benefit in other malignancies. The approval of the HDAC inhibitor vorinostat (Zolinza™) was based on the inherent sensitivity of this type of lymphoma to alterations in acetylation patterns that resulted in the induction of repressed apoptotic pathways. However, the full potential of these inhibitors (epigenetic modulators) is still on the horizon, as the true breadth of their utility as anti-cancer agents will be determined by the careful analysis of gene expression changes generated by these inhibitors and then combined with conventional chemotherapy to synergistically improve response and toxicity for an overall enhanced therapeutic benefit to the patient. The question that must be considered is whether the current HDACIs are being utilized to their fullest potential in clinical trials based on their mechanism-based alterations in disease processes.

1. Histone deacetylase inhibitors

The role of gene regulation by physical alterations of either DNA or the structural components of chromatin has recently been highlighted as a major process in neoplastic transformation and maintenance of the malignant phenotype. The discovery that chromatin contains a dynamic group of nuclear proteins that regulate transcription of many genes and especially some tumor suppressor genes came about with the discovery that the histone deacetylases (HDACs) were the target for a potent natural product that induced differentiation of neoplastic cells [1]. Several other compounds were initially discovered as inducers of differentiation and as mimetics of growth factor pathways (TGFβ) that were subsequently shown to have a mechanism of action that involved inhibition of histone deacetylase enzymes [2–7]. Recently, there have been several excellent reviews of the HDAC field both preclinical characterization of histone deacetylase inhibitors (HDACIs) and clinical development of HDACIs [8–11]. A brief overview of the mechanism of HDACIs and their road to the clinic is warranted here.

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Regulation of gene transcription occurs by various mechanisms including (1) DNA methylation, (2) post-translational histone modifications (primarily acetylation but also includes methylation, phosphorylation, poly-ADP-ribosylation, ubiquitinylation, sumoylation, carbonylation and glycosylation), and (3) RNA-associated silencing [8,10]. It has long been recognized that neoplastic cells exhibit aberrant gene expression; therefore, strategies have been investigated to correct these genetic perturbations through pharmacological manipulation of the epigenome, namely, modulation of DNA methylation and histone acetylation. As the most promising clinical data have emerged from modulation of histone acetylation the focus here will be on this unique balance that maintains nucleosomal DNA in either an active (open, acetylated) or inactive (closed, deacetylated) form. This balance is controlled by the reciprocal activities of the acetylating enzymes, histone acetyltransferases (HATs) and deacetylating enzymes, histone deacetylases (HDACs). Epigenetic alterations are crucial to the onset and progression of cancer, and HDACIs have been demonstrated to reverse some of the aberrant epigenetic states associated with cancer through induction of hyperacetylation of nucleosomal histones resulting in expression of repressed genes that produce growth arrest, terminal differentiation, and/or apoptosis in carcinoma cells.

HDACs were first identified as the target of a natural product, trapoxin, which caused differentiation of cancer cells and was used as an affinity ligand to pull out the target, HDAC1, from the cell lysate [1]. Other various differentiation assays or promoter/reporter construct assays for the TGFβ pathway identified other compounds whose mechanism of action was later elucidated to be inhibition of HDACs. The biological activity of compounds such as the spiruchostatins A and B [6], diheteropeptin [7,12], scriptaid, and A-161906 were all discovered using these assays and subsequently, the mechanism of action of all these compounds was determined to be inhibition of HDACs. These observations emphasize the prominent role of HDACs in the signaling pathways regulated by TGFβ and how modulation of chromatin structure can produce desired pharmacological effects.

1.1. *Deacetylase enzymes—the HDAC family*

The HDACs can be divided into two families, (1) the Zn^{+2}-dependent HDAC family composed of class I (HDACs 1, 2, 3 and 8), class II a/b (HDACs 4, 5, 6, 7, 9 and 10), and class IV (HDAC 11) and (2) Zn^{+2}-independent NAD-dependent class III SIRT enzymes (Table 1). The class I HDACs, apparently the ''true'' histone deacetylases, are localized to the nucleus of cells. The classes II a/b deacetylases have both histones and non-histone proteins as substrates and are primarily localized to the cytoplasm but are known to shuttle in and out of the nucleus through association with 14-3-3 proteins. The class II enzymes are characterized by either a large N-terminal domain or a second catalytic domain (e.g., HDAC 6 which contains both a histone and a tubulin deacetylase catalytic domain). The class III SIRTs are NAD^{+}-dependent deacetylases with non-histone proteins as substrates (in mammalian cells) and have been linked to regulation of caloric utilization of cells (only in yeast are the SIR proteins known to be histone deacetylases) [8]. HDACs do not function independently but rather in concert with multi-protein complexes (e.g., NCoR, SMRT, MEF, MeCP2, Sin3A, etc. [13]) that are recruited to specific regions of the genome that in turn generate the unique spectrum of expressed and silenced genes that are characteristic of the expression profile(s) responsible for the malignant phenotype of cancer cells.

Table 1 – HDACs

HDAC	~MW[a] human	~MW[a] murine	AAs[b] human	AAs[b] murine	%Similarity to human (nucleic acid/amino acid)
Zn^{+2}-dependent					
Class I					
1	55,103	55,076	482	482	90.8/99.4[c]
2	55,364	55,331	488	488	91.1/98.6[c]
3	48,848	48,821	428	428	92.5/99.6[c]
8	41,758	41,772	377	377	90.9/96.3[c]
Class IIA					
4	119,070	118,562	1084	1076	86.3/94.2
5	121,992	120,941	1122	1113	91.1/95.6
7	102,927	101,286	952	938	86.8/90.3
9	111,297	65,631	1011	588	90.394.8
Class IIb					
6	131,431	125,703	1215	1149	81.1/78.7[d]
10	71,445	72,111	669	666	78.1/76.4[d]
Class IV					
11	39,183	39,157	347	347	87.3/91.9

[a] Estimated molecular weight based on amino acid sequence, may be different than observed molecular weight on SDS-PAGE gels.
[b] AAs—number of amino acids in the open reading frame.
[c] Class of HDACs with the greatest similarity between murine and human species.
[d] Class of HDACs with the greatest difference between human and murine species.

1.2. *Which HDACs are responsible for aberrant transcription?*

One key question relates to which of the HDAC(s) are important to inhibit to obtain the desired pharmacological profile for the therapy of cancer. Genetic studies, knockout in yeast and siRNA in mammalian cells, have indicated that the class I HDACs are essential to cell proliferation and survival [14,15]. However, few studies have addressed the *in vitro* HDAC enzyme selectivity of low molecular weight HDAC inhibitors between classes I and II HDACs, most likely due to the difficulty in obtaining isolated isozymes free of other HDACs [16,17]. As described above, the HDACs function in complexes with other co-repressor proteins and in concert with DNA and histone methylation; therefore, one should exercise caution in the strict interpretation of results with siRNA knockdown or any other type of genetic knockdown/knockout that eliminates these proteins entirely from the complex and may therefore have effects other than those observed when using small molecule inhibitors. Minucci and Pelicci [11] state "... no conclusive experimental evidence that points to specific HDACs as being selectively involved in any form of disease, including cancer." However, experiments have demonstrated that HDAC1 knockout (recombinant) is embryonic lethal, HDAC2 knockdown by siRNA regulates cell survival [11], and that siRNA knockdown of HDACs 1 and 3 (class I) but not 4 and 7 (class II) results in an antiproliferative phenotype [18]. All these data suggest the role of class I HDACs in cancer cell proliferation and survival and that dysregulation of their normal function is potentially a driving force in neoplastic transformation and progression.

So exactly how do HDACIs cause cancer cell death? As with most pharmacological agents, the type of cell death induced by HDACIs can be cell type dependent and context dependent. Several recent reviews have addressed this issue and have detailed each possible mechanism [8,10]. One assumption is that cell death is caused by the re-expression of repressed genes upon HDACI treatment; however, HDACIs have the ability to induce cell death by other mechanisms independent of re-expression of genes. It has also been noted that combination of epigenetic inhibitors (DNA methylation inhibitors and HDACIs) results in synergistic cell death and it is yet unclear if this synergism reflects the re-expression of silenced genes or potentiation of cell death through acetylation of non-histone proteins [10]. HDACI mediated cell lethality can be generalized into several different mechanisms: (1) acetylation and disruption of the activity of client proteins for the heat shock proteins; (2) perturbation of the NFκB pathway; (3) up-regulation and activation of the extrinsic apoptotic pathway (death receptor pathways); (4) induction of oxidative injury (ROS); (5) generation of pro-apoptotic second messengers such as ceramide. Again the data clearly suggests that the mechanism actually causing cancer cell death is very cell type specific. Understanding which of these mechanisms is operative in a specific cancer type and which HDAC(s) are responsible may be critical to optimizing their rational incorporation into combination regimens [10].

2. HDAC inhibitors in clinical development

HDACIs currently in clinical development cover pan-HDACIs (vorinostat, belinostat, and LBH589) and somewhat isotype selective agents (romidepsin, MS-275 and MGCD0103) (Tables 2 and 3). With the approval of Zolinza (vorinostat, SAHA) by the FDA for the treatment of CTCL and with other histone deacetylase inhibitors awaiting approval for various cancers, this will hopefully prompt the investigation of histone deacetylase inhibitors into a broader range of disease states where altered chromatin function may play a role in their pathophysiology (see Section 3.7).

2.1. *Vorinostat (SAHA, Zolinza™): clinical update*

The sensitivity of CTCL cells to vorinostat was demonstrated in cell lines (Hut78, HH and MJ cells) and in primary peripheral blood lymphocytes from CTCL patients [19]. In normal PBLs vorinostat increased apoptosis from 6 to 13% whereas in CTCL patient PBLs vorinostat increased apoptosis from 15 to 32%, suggesting selectivity of vorinostat (HDACIs) for malignant versus normal cells. Vorinostat increased the acetylation of histones H2B, H3 and H4 and also increased the expression of p21 and Bax while decreasing the expression of STAT6 and decreasing levels of phospho-STAT6; all ultimately leading to the activation of caspase 3, cleavage of PARP and apoptosis [19]. Similar results have been seen for romidepsin (depsipeptide, FK228) in Hut78 cells [20].

Generally, vorinostat has been well tolerated in Phase I studies administered either i.v. or orally. The dose limiting toxicities (DLTs) observed in these studies included gastrointestinal (nausea, vomiting and/or diarrhea), anorexia, dehydration, fatigue and myelosuppression (thrombocytopenia,

Table 2 – HDAC inhibitors in clinical development

HDACI	Zn^{+2}-chelator[a]	Selectivity[b]	Clinical development
Zolinza™, vorinostat, SAHA	HA	Pan	Approved
LBH 589	HA	Pan	II/III
Belinostat, PXD101	HA	Pan	I/II
Romidepsin, depsipeptide, FK228	SH	Class I	I/II
MS-275	AN	Class I	I/II
MGCD0103	AN	Class I	I/II

[a] HA: hydroxamic acid; SH: sulfhydryl; AN: anilide.
[b] Pan: inhibitor of both classes I and II HDACs (enzyme and cellular); class I: inhibitor of primarily class I HDACs (enzyme) or compounds that lack inhibition of α-tubulin deacetylation in cells.

Table 3 – Ongoing clinical trials with HDACIs (www.ClinicalTrials.gov)

Clinical trial	Sponsor	Clinical phase	Description
MGCD0103			
NCT 00323934	Pharmion/MethylGene	I	3x/wk oral, advanced solid tumors or non-Hodgkins lymphoma
NCT 00324194		I	2x/wk oral, leukemia and MDS
NCT 00324129		I	3x/wk oral, leukemia and MDS
NCT 00359086		II	3x/wk oral, relapsed and refractory lymphoma
NCT 00358982		II	Relapsed and refractory Hodgkins lymphoma
NCT 00374296		II	AML/high risk MDS in the elderly and previously untreated or adult w/relapsed/ refractory disease
NCT 00372437		I/II	Combination with gemcitabine (Gemzar) in refractory solid tumors/ MG0103-Gemzar to Gemzar naïve patients w/locally advanced (non-resectable stage III) or metastatic (stage IV) pancreatic cancer
NCT 00324220		I/II	Combination with Vidaza (azacitidine) in high risk MDS or AML
Belinostat (PXD101)			
NCT 00413075	CuraGen Corp.	I	Oral PXD101 in advanced solid tumors
NCT 00336804	U. Chicago/NCI	I	Oral, combination w/azaditidine in AML, ALL, APL, CML and MDS
NCT 00274651	CuraGen Corp.	II	Oral, CTCL, PTCL, non-Hodgkins lymphoma
NCT 00411476	MD Anderson/CuraGen Corp.	I	Oral, QD or BID in advanced solid tumors
NCT 00413322	CuraGen/TopoTarget A/S	I	Combination w/5-FU in advanced solid tumors and advanced colorectal cancer
NCT 00348985	U. Colo./NCI	I	Combination w/bortezomib (PS-341) in advanced solid tumors and lymphoma, i.v. day 1–5 30 min infusion
NCT 00421889	CuraGen/TopoTarget A/S	I/II	Belinostat (PXD101) combination w/carboplatin or paclitaxel in advanced solid tumors—ovarian, epithelial ovarian and fallopian tube cancers
NCT 00351975	U. Chicago/NCI	I	Combination w/azacitidine (5-aza) in advanced hematological malignancies, CML, leukemia, MDS or MPD
NCT 00334789	CA Cancer Consortium/NCI	I	Combination w/isotretinoin in metastatic or unresectable solid tumors
NCT 00357162	Mayo Clinic/NCI	II	MDS (i.v. days 1–5 30 min infusion)
NCT 00357032	CA Cancer Consortium/NCI	II	Relapsed or refractory AML or older patients w/newly diagnosed AML (i.v. days 1–5 30 min infusion)
NCT 00321594	Cancer Therapeutics Research Group/NCI	I/II	Liver cancer, unresectable HCC
NCT 00303953	SWOG/NCI	II	Relapsed or refractory aggressive B-cell non-Hodgkins lymphoma
NCT 00365053	CA Cancer Consortium/NCI	II	Malignant mesothelioma
NCT 00301756		II	Platinum-resistant epithelial ovarian cancer and micropapillary/borderline (LMP) ovarian tumors
NCT 00354185	U. Wisconso/NCI	I	Combination w/17-AAG in metastatic or unresectable tumors or lymphoma
LBH 589			
NCT 00419536	Novartis	I	Single agent and combination w/docetaxel and prednisone (oral)
NCT 00412997	Novartis	I	Advanced solid tumors or CTCL (oral)
NCT 00425555	Novartis	II/III	CTCL (refractory)
Romidepsin (depsipeptide)			
NCT 00106431	Gloucester Pharm.	II	CTCL
NCT 00379639	Gloucester Pharm.	I/II	Combination w/gemcitabine in pancreatic cancer
NCT 00299351	Royal Marsden NHS Foundation Trust	II	PTCL patients who have completed a prior clinical study w/FK-228
NCT 00112463	Wake Forest Univ./NCI	II	Metastatic or unresectable soft tissue sarcoma

NCT 00084682	Albert Einstein Coll. Of Med./NCI	II	Unresectable, recurrent or metastatic squamous cell carcinoma of the head and neck
NCT 00079443	U. Maryland Greenebaum Cancer Center/NCI	I/II	Combination w/rituximab and fludarabine in relapsed or refractory low grade B-cell non-Hodgkins lymphoma
NCT 00098644	NCI	I	Combination w/flavopiridol in advanced lung, esophageal or pleural caner
NCT 00085540	North American Brain Tumor Consortium/NCI	I/II	Recurrent high-grade glioma
NCT 00052767	NCI	I	Refractory thyroid or other advanced solid tumor
NCT 00383565	MD Anderson/NCI	II	Relapsed or refractory non-Hodgkins lymphoma
NCT 00020436	NCI	II	CTCL and relapsed PTCL
NCT 00041158	NCI	I	Combination w/decitabine in unresectable advanced lung, esophageal, pleural mesothelioma, or lung metastases
MS-27-275			
NCT 00387465	Sidney Kimmel Comp. Cancer Ctr./NCI	I/II	Combination w/5-azacitidine in recurrent advanced non-small cell lung cancer
NCT 00101179	Sidney Kimmel Comp. Cancer Ctr./NCI	I	Combination w/5-azacitidine in MDS, CMMoL and AML
NCT 00313586	ECOG/NCI	II	Combination w/5-azacitidine in MDS, CMMoL and AML
Vorinostat (SAHA)			
Ongoing studies—41		I–III	Combinations w/tamoxifen, bortezomib, temozolomide, doxorubicin, idarubicin, azacitidine, isotretinoin, cytarabine/etoposide, decitabine, flavopiridol, trastuzumab, capecitibine, bevacizumab, 5-FU, leucovorin, oxaliplatin, gemzar, paclitaxel/bevacizumab, targretin, carboplatin/paclitaxel

neutropenia and/or leukopenia) [21]. HDAC inhibition was demonstrated in patients as increased accumulation of acetylated histones in tumors, bone marrow and peripheral blood cells. Phase I studies with vorinostat have resulted in complete and partial responses (CRs and PRs, respectively) in both refractory solid and hematological malignancies. The major adverse events (AEs) observed with vorinostat differ by route of administration, i.v. or oral, possibly due to differences in pharmacokinetics; oral vorinostat produced fatigue, diarrhea, anorexia and dehydration as major AEs, whereas i.v. vorinostat produced myelosuppression and thrombocytopenia as major AEs [22]. Clinical improvement was observed in Phase I studies with vorinostat in renal cell carcinoma, head and neck squamous carcinoma, mesothelioma, B- and T-cell lymphomas and Hodgkins disease [23].

In a Phase II study of vorinostat in CTCL no CRs were observed and 8 out of 33 patients achieved PRs (1 early stage disease and 7 advanced stage disease). Pruritis is a major clinical symptom of CTCL and 14 out of 31 patients (45%) with baseline pruritis had symptomatic relief. Histologically in these CTCL patients a significant decrease in dermal vessel density occurred after 4 weeks of therapy (11 out of 18 or 61%) and correlated with an increase in thrombospondin-1 (TSP-1, a known antiangiogenic protein) expression in the dermis. Overall in this Phase II study there was a 24% response rate in a heavily pretreated and refractory patient population where 8 out of 33 patients achieved PRs and 11 out of 33 patients had significant relief of pruritis and/or SD, providing for an overall clinical benefit in 58% of these patients [21].

The Phase IIb of vorinostat in CTCL was an open label 400 mg once daily dose with an overall response rate of 27%. There was an 81% reduction in SWAT scores and a 32% decrease in overall pruritis with a 43% improvement in severe pruritis. Fifteen out of 74 patients have been treated for >1–2 years. Vorinostat has a medium time to response of 55 days and maximum duration of response has not been reached with patients now beyond 448 days of treatment. Fatigue and GI were the most common AEs and Grades 3–4 thrombotic events occurred in 5% of the patients (4 out of 74). Patient PBMCs were analyzed by gene array and 2 h after a dose there were decreases in genes associated with proliferation and an increase in genes associated with apoptosis. Proteinuria was only seen as a Grades 1–2 toxicity [24]. Zolinza was approved in 2006 for treatment of refractory CTCL and is currently in multiple clinical trials in combination with other chemotherapeutic agents (Table 3).

2.2. *Romidepsin (depsipeptide, FK228, FR901228): clinical update*

Romidepsin is a novel natural product bicyclic tetrapeptide HDACI [5]. Romidepsin was isolated from *Chromobacterium violaceum* and was found to reverse the transformed phenotype of Ha-Ras transformed cells and was antiproliferative in a wide variety of murine and human tumor cell lines both *in vitro* and *in vivo* [25]. Romidepsin is a pro-drug, the active moiety being a sulfhydryl group acting as the Zn^{+2}-chelator [26]. This pro-drug structure provides stability allowing both *in vivo* dosing and its use in humans [26]. As romidepsin is not a hydroxamic acid, and similar to other non-hydroxamates [27],

it is a more selective inhibitor of the class I HDACs (HDACs 1 and 2 versus HDACs 4 and 6) [26]. Romidepsin, possibly due to being a natural product tetrapeptide, is a substrate of MDR-1; however, cross-resistance has not been observed with other cytotoxic agents [28].

There have been multiple Phases I and II trials with romidepsin. Generally, romidepsin is well tolerated and has a similar toxicity profile as vorinostat. Two of the early Phase I trials demonstrated changes in the ECG of patients. DLTs observed with i.v. infusion on a 3- out of 4-week schedule consisted of fatigue, nausea, vomiting, and transient thrombocytopenia and neutropenia. The MTD was determined to be 17 mg/m^2 on days 1 and 5 every 21 days. In the Phase I studies cardiac arrythmias manifested by changes in the ECG were observed with a case of atrial fibrillation [29]. Acetylation of histones in patient's PBMCs was observed confirming inhibition of HDACs by romidepsin and there was one response out of 37 patients indicating limited activity in patients with solid tumors [29]. Another Phase I trial by the National Cancer Institute (NCI) demonstrated significant activity in CTCL patients with three PRs and one CR in a peripheral T-cell lymphoma (PTCL) [30]. The activity observed in this Phase I trial prompted the Phase II trial in CTCL.

Phase II clinical trials in T-cell lymphoma continued to demonstrate the efficacy of romidepsin in this disease. In patients with PTCL, 10 out of 27 patients demonstrated activity with a weekly infusion of romidepsin for 3 out of 4 weeks. In a recent Phase II study in CTCL and PTCL the overall response rate for CTCL was 31% with 3 CRs, 10 PRs and 9 SD, and for PTCL was 30% with 3 CRs and 8 PRs. An increase in histone acetylation was observed in normal and malignant blood cells and there was also an up-regulation of MDR-1 in these cells, consistent with *in vitro* findings [31]. In Phase II studies in solid tumors, romidepsin continued to demonstrate only marginal activity. These studies demonstrated the same DLTs as observed in the Phase I studies; fatigue, nausea and vomiting as well as myelosuppression and abnormalities in ECGs. A study of the cardiac risks in these patients determined that the ECG abnormalities were not indicative of myocardial dysfunction or myocardial damage. The clinical effect of the observed prolongation in the QTc interval on the safety profile of romidepsin is still under investigation [32]. Romidepsin is still undergoing multiple Phase II investigations to determine efficacy and the effect of QTc prolongation on the utility of this novel HDACI (Table 3).

2.3. MS-275: clinical update

MS-275 is a novel benzamide-based HDACI which like other non-hydroxamic acid inhibitors is somewhat selective for the class I HDACs [8,27,33]. MS-275 inhibits the proliferation of multiple carcinoma cell lines in the micromolar range and its mechanism of inducing cell death appears to involve generation of ROS [9,34]. MS-275 inhibits the growth of tumors implanted into mice in a dose-dependent manner and recently, through the use of a fluorescence-based gene expression reporter system based on the p21 promoter, it was demonstrated that a single dose of MS-275 can induce fluorescence in tumors in a time- and dose-dependent manner [35]. This reporter system demonstrated direct *in vivo* epigenetic regulation by this HDACI and can potentially be used to predict long-term anti-tumoral efficacy for MS-275 in animal models [35].

Phase I studies in advanced solid tumor and lymphoma patients demonstrated that the half-life of MS-275 was much longer than predicted based on preclinical models, 39–80 h in humans. Therefore, it is not surprising that a daily dose for 28 days on a 6-week schedule was not tolerated even at the initial dose of 2 mg/m^2 [36]. The MTD on a q14 day schedule was 10 mg/m^2 with DLTs of nausea, vomiting, anorexia and fatigue after oral dosing. In all these studies and all dose levels of MS-275, an increased acetylation of histone H3 in PBMCs was observed indicating that MS-275 was biologically active at the doses being evaluated [36]. In another Phase I study in adult refractory and relapsed acute leukemias, MS-275 had an MTD of 8 mg/m^2 on a weekly $\times$ 4 every 6-week schedule. In this study, DLTs were primarily infections and neurologic toxicity that included unsteady gait and somnolence, but also included fatigue, anorexia, nausea, vomiting, hypoalbuminemia and hypocalcemia [37]. In all dose groups as before, increased accumulation of acetylated histones H3 and H4, increased p21 expression and activation of caspases was observed in bone marrow mononuclear cells. However, in this study no responses based on classical criteria were observed [37]. MS-275 is currently undergoing Phase II studies in combination with 5-azacitidine in non-small cell lung cancer, MDS, CMMoL and AML (Table 3).

2.4. LAQ824, LBH589, belinostat, MGCD0103: clinical update

LAQ824 [38] and the more potent analog LBH589 are pan-HDAC inhibitors developed by Novartis. LBH589 and LAQ824 are unique in that they have been extensively studied for their role in the regulation of Hsp90 and degradation of Hsp90 client proteins [39]. Enhanced efficacy or synergy *in vitro* can clearly be demonstrated when LBH589 is used in combination with direct inhibitors of the client proteins of Hsp90, e.g., bcr-abl in CML [40,41] and FLT3 in AML [42]. HDAC inhibition results in the accumulation of acetylated Hsp90 and inhibition of its chaperone function with its client protein(s) results in ubiquitination and degradation of the client protein(s); therefore, the combination of a direct inhibitor of the enzyme (client protein) and decreasing client protein levels results in synergistic inhibition of the pathway and enhanced killing of those cells. Recently, LBH589 has also been demonstrated to deplete members of the polycomb repressive complex 2 (EZH2, SUZ12 and EED proteins) and DNMT1 in CML cells [43]. Currently, LBH589 is in Phase II/III clinical development in patients with CTCL (Table 3). At an MTD dose of 20 mg M, W, F, two patients achieved CRs, four attained PRs and one achieved SD for an overall response rate of 60% in patients with advanced-stage CTCL [44]. Multiple Phase I trials are ongoing with LBH589 and it is entering Phase II/III clinical studies (Table 3).

Belinostat (PXD101) is a novel hydroxamic acid HDACI (Table 2) and is cytotoxic to numerous cancer cell lines with IC_{50} values in the range of 0.2–3.4 μM [45,46], consistent with the activity of most other hydroxamate-based HDACIs. Belinostat has demonstrated *in vivo* activity against ovarian

and colon cancer xenograft models without significant toxicity in these murine tumor models. In ovarian cancer models, Belinostat demonstrated additive to synergistic activity when combined with standard cytotoxic agents such as carboplatin and paclitaxel [47]. Gene expression studies with belinostat and have identified regulation of target genes that should guide the selection of therapeutically effective combinations (e.g., 5-FU and thymidylate synthase as discussed below) [48]. Down-regulation of Aurora A and B kinases at the mRNA and protein levels was also observed which may contribute to the G2/M delay observed with belinostat [49]. The initial Phase I trial of belinostat was conducted in patients with advanced solid tumors. The most common adverse events were fatigue, nausea, vomiting and phlebitis (no grade 4 toxicities were observed in this initial Phase I trial). An MTD of 1000 mg/m^2/day was determined for progression into Phase II trials [50]. Interestingly, similar to vorinostat, belinostat did have 33% bioavailability when administered orally in these clinical trials. A Phase Ib study in colorectal carcinoma in combination with 5-FU is ongoing, no grade 3 or 4 toxicities have been observed at 1000 mg/m^2/day and the common adverse events include fatigue, nausea, vomiting, dysgeusia, dehydration and anorexia [51]. Currently multiple Phase I trials are ongoing in combination with agents such as Velcade and Vidaza (5-azacytidine) in multiple myeloma and hematological malignancies, respectively (Table 3). A Phase II study was recently reported in patients with advanced multiple myeloma who received monotherapy belinostat for >2 cycles (12 pts), there were six SD and six PD demonstrating that belinostat treatment resulted in stabilization of advanced and progressive disease. The combination of belinostat with dexamethasone (standard of care for MM patients) led to one MR as well as long duration of stable disease even in patients who have received multiple dexamethasone regimens [52]. Belinostat is currently in multiple Phase I/II clinical trials (Table 3).

MGCD0103 is a novel, orally bioavailable anilide-based HDACI developed by MethylGene, Inc. (http://www.MethylGene.com). This molecule is selective for the class I HDACs. This profile of HDAC inhibition has been determined by siRNA and antisense oligonucleotides to be optimal for inhibition of cell proliferation and survival. MGCD0103 is antiproliferative in a wide variety of liquid and solid tumor cell lines, causes the accumulation of acetylated histones (not α-tubulin), induces gene changes characteristic of other HDACIs and has been shown to enhance the activity of several different chemotherapeutics. MGCD0103 is in multiple Phases I and II clinical trials as a single agent or in combination with various chemotherapeutics including gemzar and Vidaza (azacitidine). Cancers being targeted by MGCD0103 include pancreatic (combination with gemzar), MDS and AML in combination with Vidaza, diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, and relapsed or refractory Hodgkin's lymphoma. In a Phase I study in AML and MDS, as monotherapy, MGCD0103 was dosed twice weekly in a 3-week cycle, no MTD has been reached up to 66 mg/m^2/day. Non-dose-limiting toxicities included fatigue, nausea and vomiting. Inhibition of HDAC activity in PBMCs from the majority of treated patients was observed in this Phase I trial [53]. MGCD0103 is currently in multiple Phase I/II clinical trials (Table 3).

3. Mechanism-based potential of HDACIs: are HDACIs being utilized in combinations that make mechanistic sense to achieve optimal therapeutic potential?

Several factors enter into paradigms of therapeutic combinations with epigenetic modulators: first, and the first demonstrated utility of HDACIs [54], the presence of oncogenic fusion proteins that incorporate HDACs or make high affinity complexes with HDACs; second, what are the genes regulated by these agents and how are they regulated; third, are these direct effects on proteins involved in apoptosis or client protein stability; fourth, are these effects due to direct induction of oxidative injury in cells (Fig. 1). Several of these aspects are discussed below.

3.1. *Oncogenic fusion proteins that incorporate HDACs*

Hematological malignancies containing an oncogenic fusion protein were the first demonstration of the mechanism-based utility of HDACIs. Acute promyelocytic leukemia normally responds to retinoic acid (RA), inducing differentiation of the neoplastic cells and growth arrest. However, when the retinoic acid receptor (RARα) is expressed as a fusion protein with promyelocytic leukemia (PML) or promyelocytic leukemia zinc finger (PLZF), these cells are resistant to physiological levels of RA. In fact the PML fusion protein requires pharmacological doses of RA and the PLZF fusion protein with RARα does not respond at all to RA as a result of physical association of HDACs with these fusion proteins and repression of genes induced by RA that normally result in differentiation. In APL patients, treatment with HDACIs and RA is extremely effective in de-repressing RA target genes and inducing cellular responses to RA both *in vitro* and *in vivo* [11]. In at least one patient with the PLZF-RAR fusion protein, combination therapy of RA with the HDACI sodium phenylbutyrate generated disease remission [54]. This patient achieved a fourth CR that was sustained for several months [55]. When these types of fusion proteins are present, combinations with HDACIs has clear therapeutic potential for those patients.

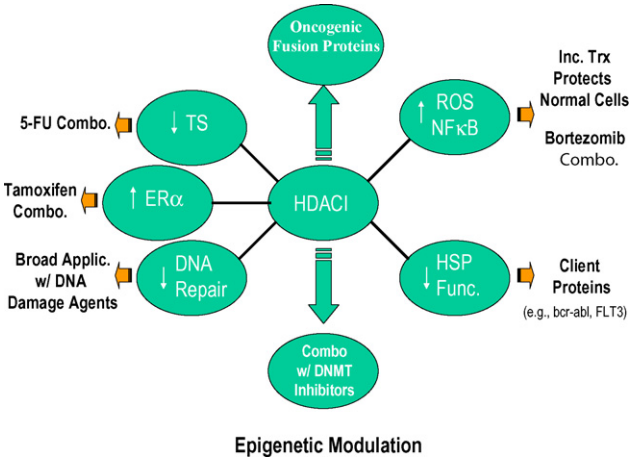

Fig. 1 – Avenues of epigenetic modulation with HDACIs based on known modulation of genes and pathways in cancer cells.

Several mutations (G289R and P407S) in the DNA binding domain of the PML-RARα also confers resistance to RA, which can be overcome by addition of a known HDACI [55]. These types of mutations occur frequently after multiple RA treatment relapses. The responses seen with RA plus sodium phenylbutyrate in these patients may be related to the potential of sodium phenylbutyrate to circumvent the blocked RA-regulated gene response pathway [55]. Therefore, alleviation of HDAC-induced repression restores normal function and differentiation capabilities ultimately leading to growth arrest and apoptosis in malignant cells.

So why with so much evidence of HDAC association with these oncogenic fusion proteins, is it that HDAC inhibitors have not performed as well as expected? Tabe et al. [28] demonstrated the critical importance of mechanism-based sequential therapy in future clinical trials that combine HDACI, ATRA and the anthracyclines; this apparently needs to be exploited clinically now that HDACIs are approved. Understanding the correct combination from a mechanistic viewpoint can lead to optimal tumor response, something not observed in the clinic in some forms of APL. Combining HDACIs with other types of agents that target co-repressor complexes therefore warrants further investigation to ultimately provide an optimal therapeutic response in these APL patients with RA non-responsive disease [10].

3.2. *Re-expression of the estrogen receptor-α (ERα)*

Yang et al. [56] described re-expression of the silenced ERα induced by TSA in MDA-231 ER-negative cells and implicated histone deacetylation as a critical component of ER gene silencing in human breast cancer cells. This observation suggested that activation of the silenced ER by various HDACIs could open a new avenue for therapy of a subset of advanced breast cancer with hormonal resistance [56]. Subsequently, the use of HDACIs to re-activate the silenced *ERα* gene demonstrated increased sensitivity of ER-negative cells to tamoxifen. It was also recognized that the *ERα* gene has a methylated promoter region in hormone resistant disease and the re-expression of the *ER* gene is enhanced by treatment with a HDACI and a methyltransferase inhibitor, 5-aza-2′-deoxycytidine [57]. Studies with LBH589 in MDA-231 and MDA-435, ER-negative breast cancer lines, demonstrated that ERα could be reactivated without demethylation of the ER promoter [58]. It was also demonstrated that LBH589 released DNMT1, HDAC1 and SUV39H1 (a histone demethylase) from the ER promoter; this was associated with an increased acetylation of histones H3 and H4, a decrease in methylation of histone H3-K9, and impaired binding of heterochromatin protein 1 (HP1 alpha). The re-activation of ERα by LBH589 was sustained for 96 h after withdraw of the HDACI. LBH589, as with other HDACIs, enhanced 4-hydroxy-tamoxifen sensitivity in MDA-231 cells [58]. Re-expression of ERα in MDA-231 cells with the combination of TSA and 5-aza-2′-deoxycytidine (5-aza-dC) restored tamoxifen sensitivity and also recruited distinct co-repressor complexes to the tamoxifen bound ERα to form a repressive ER complex at ER target genes, turning off these ER responsive genes. The complex was characterized by the presence of recruited HDAC3/NCoR and TBL1 [59]. Interestingly, in ER-positive breast cancer cells (MCF-7), ERα is actually repressed by HDACIs and results in an antiestrogenic effect. This effect on ERα, which also includes pS2 and cyclin D1, is mediated by the recruitment of the methylated DNA binding protein, MeCP2, and the exclusion of DNMT1 from the promoter regions by agents such as valproic acid [60].

Here is an example where use of an HDACI as monotherapy would probably not produce a clinical benefit to the patient; however, when combined appropriately based on the response to HDAC inhibition (with or without DNMT inhibition), re-expression of ERα may lead to significant clinical responses in a subset of refractory advanced cancer patients. Clinical trials combining vorinostat with tamoxifen are on going to investigate the clinical potential of this combination (Table 3).

3.3. *Regulation of DNA repair by HDACIs*

It has been demonstrated that all HDACIs to date synergize with ionizing radiation (γ-irradiation) to kill tumor cells *in vitro* and several have shown this synergy *in vivo* [8]. In the absence of double strand DNA breaks caused by radiation, the HDACIs can independently induce or mimic the DNA damage response by activation of ATM which phosphorylates and activates downstream effectors such as BRCA1, 53BP1, CHK2 and γ-H2AX, ultimately leading to apoptosis. One mechanism where HDACIs can synergize with radiation is through down-regulation of the genes/proteins involved in the DNA damage response such as Ku70, Ku80, Rad50 and DNA ligase IV [8,61]. By reducing the proteins responsible for repairing the DNA damage due to ionizing radiation, the greater the overall DNA damage and the greater the apoptotic response. It has also been demonstrated that HDAC4 is recruited to DNA-damage-induced foci and co-localizes with 53BP1 in irradiated cells. Knockdown of HDAC4 by siRNA decreased levels of 53BP1, abrogated the DNA damage-induced G2 delay, and sensitized HeLa cells to radiation-induced apoptosis [62]. In addition to reducing the expression of some of the DNA damage-induced response genes, HDACIs (e.g., vorinostat) in combination with radiation increase the relative number of γ-H2AX foci in cells, which can potentially be used as a predictive marker of radiotherapy response to vorinostat [61].

This is a common mechanism exploited by combinations with HDACIs, reduction of repair or survival proteins, resulting in synergistic killing of tumor cells in combination with many of the classical chemotherapeutic compounds now used to treat cancer. Methylation and repression of the DNA mismatch repair gene hMLH1 results in resistance to cisplatin treatment. In this case, HDACIs in combination with DAC (2-deoxy-5-azacytidine) synergize to re-express hMLH1 and sensitize cells to cisplatin treatment. These results suggest that the combination of DNMT inhibitors and HDACIs could act synergistically to increase the efficacy of chemotherapy in patients that lack MLH1 expression due to MLH1 promoter hypermethylation [46]. Depending on the tumor type and the combination therapy being employed, HDACIs can either be used to induce pro-apoptotic proteins or reduce the enzymes that commonly repair damage induced by chemotherapeutic agents, again resulting in a more pronounced apoptotic response. Therefore, with the exception of CTCL where HDACIs have a profound direct effect on pro-apoptotic genes,

why would investigators be considering monotherapy for HDACIs when their greater promise lies in combinations based on the mechanism of HDAC inhibition in those particular tumor cells?

3.4. *Reduction of chemotherapy target proteins: thymidylate synthase*

Thymidylate synthase (TS) is the target of the chemotherapeutic agent 5-fluorouracil (5-FU). Resistance to 5-FU is a common reason for treatment failure, especially in the treatment of colorectal cancers [63]. Resistance to a chemotherapeutic can occur by several mechanisms in cancer cells: up-regulation of metabolizing enzymes or drug pumps such as MDR-1, up-regulation of the downstream effectors of the target protein requiring less of a initial signal to evoke a response and/or the up-regulation of the target protein itself therefore require increasing concentrations of the chemotherapeutic to obtain the desired pharmacological effect. It has been recognized that gene amplification of TS with consequent increases in TS mRNA and protein results in an acquired resistance to 5-FU and fluorodeoxyuridine (FUDR) [63]. In patients treated with 5-FU an improved response was observed in those patients with low tumoral TS expression, and high TS expression predicted a poor response to 5-FU-based chemotherapy.

Therefore, one potential to sensitize cells to 5-FU would be to lower the concentration of its target protein, TS, both in non-resistant cancer cells and those with acquired resistance to 5-FU treatment. Initial gene expression studies with HDACIs recognized that TS was one of the HDACI gene targets [64]. Addition of an HDACI to 5-FU treatment of cells results in the synergistic killing of those cancer cells [48,65,66]. It has also been demonstrated that knockdown of TS by antisense oligonucleotides decreases tumor cell growth and reduces drug resistance [67], and this has been demonstrated *in vivo* using cells containing a full-length antisense construct for TS [68]. Connection of the two observations, knockdown of TS by antisense [67] or by HDACI [64], could be used to enhance the chemosensitivity of resistant and non-resistant cancer cells to 5-FU and was only recently investigated with HDACIs [69]. The enhanced sensitivity of cells to 5-FU after TSA treatment was related to the repression of TS mRNA and protein synthesis [49,69]. However, these investigators demonstrated that like several other targets of HDACI, the decrease in protein could also be accomplished by regulation of Hsp90 function, and TS is a client protein of Hsp90. Therefore, there are multiple mechanisms by which HDACIs can regulate the expression of a protein, at the level of mRNA production and stability of the protein by altering chaperone protein function. The conclusion of these studies like the others done with HDACIs and 5-FU is that this combinatorial approach may be useful to overcome 5-FU resistance [69]. Vorinostat and belinostat are currently in clinical trials in combination with 5-FU recognizing that HDAC inhibition results in repression of TS in solid tumors [51].

3.5. *Acetylation of non-histone proteins: regulation of Hsp90 function*

It was demonstrated with romidepsin that treatment of cells results in the reduction of p53, Raf-1 and ErbB, all client proteins of Hsp90; therefore, it was investigated whether romidepsin would affect Hsp90 function similar to 17-AAG which also causes a reduction of these proteins [39]. It was also demonstrated that the binding of mutant p53 and Raf-1 to Hsp90 was inhibited by treatment with romidepsin and that this inhibition was associated with acetylation of Hsp90. Similarly, the hydroxamic acid HDACI LAQ824 reduced the levels of bcr-abl (also a client protein of Hsp90) in CML cells. This reduction in bcr-abl protein sensitized these cells to imatinib (Gleevec). The repression of bcr-abl was protein synthesis inhibitor sensitive suggesting that LAQ824 augments the level and activity of a transcriptional repressor for bcr-abl. Interestingly, the promoter region for bcr-abl was not acetylated on histones H3 or H4 after treatment with LAQ824, this is quite unlike the promoter region for p21 that is hyperacetylated upon HDACI treatment corresponding to its up-regulation. Treatment of CML cells with the HDACI LAQ824 was associated with the induced acetylation of Hsp90, suggesting that LAQ824 through Hsp90 acetylation disrupts the stable chaperone association of bcr-abl with Hsp90 therefore promoting proteosomal degradation of bcr-abl [40]. This phenomenon was also observed in AML cells with the FLT3 kinase inhibitor PKC412 where LAQ824 induced Hsp90 acetylation, resulted in proteosomal degradation of FLT3 and decreased P-FLT3, P-STAT5, P-AKT and P-ERK1/2 levels, and synergistic apoptosis in MV4-11 cells (AML cell line containing the FLT3-ITD mutant) and primary AML cells expressing mutant FLT3 [42]. Co-treatment of CML cells with either LBH589 or vorinostat and either AMN 107 [41] or dasatinib [70], respectively, led to decreased levels of bcr-abl and the gleevec-resistant mutants (bcr-abl T315I and E255K) with concomitant decreases in P-STAT5, P-ERK 1/2, Bcl-xL, P-CrkL and increases in p27 and Bim, a pro-apoptotic protein [41,70]. HDACIs regulate many of the client proteins of Hsp90 by inducing the acetylation of Hsp90; other epigenetic regulators such as DMNT1 can be regulated both at this post-translational step or at the transcriptional step as stated above [43].

The ability of HDACIs to modulate Hsp90 function should be investigated fully to determine the breadth of client proteins affected by this mechanism and used in combination with inhibitors of these target proteins to obtain optimal synergistic killing of cancer cells. It is also of interest that in many of these cell types, the HDACIs also have a direct effect on the transcription of the client protein itself, thus adding a potential benefit to the use of HDACIs in cancers expressing these targeted client proteins. Another question posed by this data is which mechanism is dominant or responsible for the observed pharmacological effects of these HDACIs? Further investigations are clearly necessary to understand this phenomenon and determine the most beneficial utility of these HDACIs in relation to the client proteins being targeted.

3.6. *Methylation: DNA, histones, and other proteins*

Many of the strategies targeting epigenetic changes that drive malignant progression or tumorgenesis are combinations of an HDACI with a DNA methyltransferase (DNMT) inhibitor. Hypermethylation of specific promoter regions is known to silence genes and some cells use both hypermethylation and

repression by the incorporation of HDACs into co-repressor complexes to re-enforce gene silencing. Therefore, either an HDACI or DNMT inhibitor alone does not produce profound efficacy, but the combination of these two mechanisms sometimes generates a synergistic reactivation of specific genes and the desired pharmacological effect. It has become clearly evident that not only DNA methylation controls gene expression but also methylation of specific histone residues and other proteins/transcription factors. The histone code was described as a signaling mechanism through post-translation modification of histones (primarily acetylation, methylation and phosphorylation) required for the recruitment of the necessary components to repress or activate gene transcription [71–74]. DNA methylation is carried out by the DNA methyltransferases DNMT 1, 2 and 3a and 3b; histone methylation is carried out by the histone methyltransferases (HMTs) such as Suv4-20 and SET proteins [75]; methylation of transcription factors such as p53 is carried out by the Smyd family of proteins [76]. Many of the epigenetic genes regulating methylation or responses to methylated proteins are either over-expressed or severely dysregulated in many types of cancer [75].

The original dogma that implied histones were only static, structural proteins is now clearly not the case, this may now apply to methylation pathways. That is, methylation of DNA, histones and non-histone proteins has turned out to be a very dynamic process that modulates how cells respond to different stimuli (positive or negative). Methylation, like histone acetylation, involves the balance of methyltransferases and demethylation enzymes (of which only a few have been described to date and include the LSD-1 and JmjC protein families [77]). The approval of Vidaza (5-azacytidine) has validated the use of DNMT inhibitors for the treatment of myelodysplastic syndromes including chronic myelomonocytic leukemia and it is now in clinical trials combined with various HDACIs such as sodium phenylbutyrate, valproic acid and Zolinza (vorinostat). Again, once thought a rather static post-translational modification, DNA methylation is a dynamic process that not only controls such cellular functions as imprinting but also regulates active gene transcription and is a major contributor to tumorgenesis and malignant progression.

3.7. *Other diseases that may be treated with HDACIs*

Beyond cancer there may be several novel therapeutic arenas where epigenetic modulators may provide therapeutic benefit. Early in the investigations on HDAC inhibitors it was recognized that one of the effects was the activation of latent viruses (e.g., HIV). As this may seem problematic in the development of HDACIs, this mechanism can be utilized to reactivate a latent virus thus making it susceptible to subsequent treatment with targeted anti-viral therapy. This concept has been validated in humans with valproic acid, the HDACI, and highly active antiretroviral therapy (HAART) where resting cell infection declined significantly in three out of four patients treated with this combination [78,79]. HDACIs have effects on the acetylation of key factors that regulate immune cell function, such as STAT1, STAT3 and NFκB, and acetylation is known to regulate the function of these inflammatory transcription factors. HDACIs can reduce graft-versus-host disease following bone marrow transplantation by suppressing pro-inflammatory cytokines such as TNFα and IL-1 [8]. Similarly, with concentrations of HDACIs lower than those expected to have an antiproliferative effect, one can demonstrate alteration of genes during the inflammatory response. These gene changes effectively block the Th1 type but not the Th2 type inflammatory response. The genes modulated in monocytic and dendritic cells were fairly specific, not global, and in doing so provide abundant new targets for pharmacological intervention in inflammatory diseases [80]. HDACIs can also suppress the expression of key adhesion molecules such as VCAM-1, thus leading to a reduction in the number of activated monocytes binding to inflamed endothelium. This is yet another mechanism, selective inhibition of adhesion molecules, whereby HDACIs can have an anti-inflammatory effect [81]. In neurodegenerative states, HDACIs increase the expression of neuroprotective proteins such as Hsp70 and Bcl-2 in the ischemic brain and point to the therapeutic potential of HDACIs in stroke [82]. In other neurodegenerative diseases, dysregulation of transcription occurs through the inappropriate utilization of HATs and HDACs. HDACIs have shown activity in these diseases *in vitro* and in animal models of disease such as Huntington's disease, where expression of the mutant huntington protein displaces co-activators or directly binds and inhibits HATs resulting in the transcriptional repression observed early in the Huntington's disease process in animal models [83,84]. HDACIs therefore have the potential to reverse this disease process by re-expression of these silenced genes.

4. Summary

With the approval of vorinostat for the treatment of CTCL and PTCL, the application of epigenetic regulation as an avenue in treatment has expanded, not only for hematological malignancies, but also to a much broader range of cancers. The response rates in CTCL are impressive and the side effects are manageable. The greatest utility of these epigenetic modulators will be in combination with other therapeutics that synergize with the regulation being controlled by the epigenetic modulator. Only in this manner of combination will there be a sufficient response rate in solid tumors. These experiments are now ongoing in clinical trials of vorinostat, romidepsin, belinostat and LBH589.

As discussed herein, these are a few of the different disease that may benefit therapeutically with the use of HDACIs. One of the major issues still remaining is which of the HDACs are primarily responsible for the manifestation of the disease and can selective inhibitors be developed to address these specific diseases. It is clear that somewhat selective agents can be developed that distinguish at least the histone and non-histone protein deacetylation process [27,85]. As these more selective agents advance into clinical trials their utility will become apparent, but as discussed, their approval (efficacy in a disease state) may depend on effective combinations with other therapeutics to maximize the desired pharmacological benefit.

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