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Prospects: Histone Deacetylase Inhibitors

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Abstract Histone deacetylase (HDAC), inhibitors represent a new class of targeted anti-cancer agents. Several of these compounds are in clinical trials with significant activity against a spectrum of both hematologic and solid tumors at doses that are well tolerated by the patients. The HDAC inhibitors are a structurally diverse group of molecules that can induce growth arrest, differentiation, apoptosis, and autophagocytic cell death of cancer cells. While the base sequence of DNA provides the genetic code for proteins, the expression of genes is regulated, in large part, by the structure of the chromatin proteins around which the DNA is wrapped (epigenetic gene regulation). The acetylation and deacetylation of the lysines in the tails of the core histones, among the most extensively studied aspects of chromatin structure, is controlled by the action of two families of enzymes, histone deacetylases (HDACs) and histone acetyltransferases (HATs). Protein components of transcription factor complexes and many other non-histone proteins are also substrates for HDACs and HATs. The structure and activity of these non-histone proteins may be altered by acetylation/deacetylation with consequent effects on various cell functions including gene expression, cell cycle progression, and cell death pathways. This review focuses on several key questions with respect to the mechanism of action of HDACi, including, what are the different cell phenotypes induced by HDACi, why are normal cells compared to transformed cells relatively resistant to HDACi induced cell death, why are certain tumors more responsive to HDACi than others, and what is the basis of the selectivity of HDACi in altering gene expression. The answers to these questions will have therapeutic importance since we will identify targets for enhancing the efficacy and safety of HDACi. J. Cell. Biochem. 96: 293–304, 2005.

Key words: apoptosis; thioredoxin; thioredoxin-binding protein; transcription factor complex

HISTONE DEACETYLASES (HDACs) AND HISTONE ACETYLTRANSFERASES (HATs)

The structure of chromatin is complex, made-up of DNA, histones, and non-histone proteins [Luger et al., 1997; Marks et al., 2001; Lehrmann et al., 2002]. The basic repeating unit of chromatin is the nucleosome, composed of approximately 146 bp of DNA wrapped around the histone octamer composed of two copies of each of four histones, H2A, H2B and H3 and H4. It was proposed almost four decades ago that structural modification of histones by acetylation plays a role in regulation of gene expression [Allfrey et al., 1964]. There is now abundant evidence that remodeling of the chromatin proteins around which the DNA is wrapped is a fundamental epigenetic mechanism for regulating gene expression, involving the reversible post-translational modification of amino acids in the histone tails by acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines, and ubiquination and sumoylation of lysines [Zhang and Reinberg, 2001; Spotswood and Turner, 2002; Fischle et al., 2003]. Two groups of enzymes, histone deacetylases (HDACs) and histone acetyltransferases (HATs), determine the pattern of histone acetylation. It has been hypothesized that histone modifications acting alone, sequentially or in combination represent a "code" that can be recognized by non-histone proteins forming complexes involved in the regulation of gene expression.

In humans, 18 HDAC enzymes have been identified and categorized in three classes based on homology to yeast HDACs ([De Ruijter et al.,

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2002; Blander and Guarente, 2004; Marks et al., 2004]). Class I includes HDAC 1, 2, 3, and 8 which are related to yeast RPD3 deacetylase with molecular weights of 22–55 kDa and share homology in their catalytic sites. Class II includes HDACs 4, 5, 6, and 9 which are larger molecules, with molecular weights between 120 and 135 kDa, and they are related to yeast HDA1 deacetylase. A subclass of HDACs is represented by HDAC 6 and 10, which contain two catalytic sites. HDAC 11 has conserved residues in the catalytic core region that are shared by both class I and class II enzymes. A third class of HDACs has been identified that have an absolute requirement for NAD, the so called Sir2 family of deacetylases, which are not inhibited by compounds that inhibit class I and II HDACs. The Sir2 class of histone deacetylases appear not to have histones as their primary substrates.

Recent phylogenetic analysis of bacterial HDACs suggest that all three HDAC classes preceded the evolution of histone proteins. This raises the possibility that the primary activity of some histone deacetylation enzymes is directed against non-histone substrates [Gregoretti et al., 2004]. A recurring theme that emerged from these phylogenetic studies was the common nature of association between HDAC molecules. It is well established that class I and class II HDACs are often found as components of larger transcription factor protein complexes [De Ruijter et al., 2002; Lehrmann et al., 2002; Marks et al., 2004; Sengupta and Seto, 2004; Drummond et al., 2005]. The possible functional significance of association among HDAC molecules is further considered below in the context of studies on the effect of suberoylanilide hydroxamic acid, (SAHA) on proteins associated with the proximal promotor region of the p21 gene [Gui et al., 2004]. Contrary to what might be expected from the widespread distribution of HDAC within the chromatin, HDACi induce alterations in transcription of relatively few genes [Butler et al., 2002; De Ruijter et al., 2002; Johnstone and Licht, 2003; Marks et al., 2004]. We hypothesize that it is the structure of the complex of protein components of transcription factors, including HDACs and HATS, that accounts for the selectivity of HDAC inhibitors in altering gene transcriptions.

There is abundant evidence that HDACs are not redundant in their biological function [De Ruijter et al., 2002; Lehrmann et al., 2002; Glaser et al., 2003; Verdin et al., 2003; Marks et al., 2004]. HDACs targets include histones and nonhistone proteins which regulate gene expression and proteins involved in regulation of cell cycle progression, and cell death [Lehrmann et al., 2002; Johnstone and Licht, 2003; Warrener et al., 2003; Di Gennaro et al., 2004; Marks et al., 2004; Rosato and Grant, 2004; Drummond et al., 2005]. In addition, acetylation/deacetylation of histone and nonhistone proteins plays an important role in DNA replication and mitosis which does not involve directly altering gene expression. Class I HDACs are found almost exclusively in the cell nucleus, while class II shuttle between the nucleus and cytoplasm on certain cellular signals. Class I HDACs, such as HDAC1 and HDAC2, appear to be important in the regulation of proliferation of cancer cells. Different HDACs associate with different co-repressors and activators.

Some examples of the different functions of the various HDACs include (see reviews [Marks et al., 2004; Sengupta and Seto, 2004; Drummond et al., 2005]); HDAC1 complexed with myoD serves as a repressor of proliferating myoblasts. The expression of different HDACs through embryonic development changes with different stages of embryogenesis. Targeted disruption of HDAC1 results in embryonic lethality and reduced proliferation despite increased expression for HDAC 2 and 3. HDAC3 modulates the functions of transcription factors such as TFII-1, and is critical for repression of multiple nuclear receptors. Class 2 HDACs block myocyte enhancer factor 2 (MEF2) activation of cardiac hypertrophy. HDAC4 knockout mice display premature ossification due to the excessive proliferation of chondrocytes [Vega et al., 2004]. HDAC5 has been shown to interact with Ca(2+) calmodulin to inhibit MEF2a. HDAC5 knockout mice also develop cardiac hypertrophy phenotype. HDAC9 knockout mice are sensitive to hypertrophic signals and develop cardiac hypertrophy with advanced age. HDAC6 plays a critical role in the missfolded protein stress response. HDACs 7 has a role in regulating T-cell differentiation in the thymus which is not shared by other HDACs.

HATs, like HDACs, do not bind to DNA directly, but are recruited to multi-protein complexes associated with DNA which differ in their subunit composition [De Ruijter et al., 2002; Lehrmann et al., 2002; Marks et al., 2004;

Sengupta and Seto, 2004]. HDACs 1 and 2 are frequently found in complex with Sir3, NURD (nucleosome remodeling and deacetylation), N-Cor (nuclear receptor co-repressor), mSirN3A, Ni-2/NRD, and/or CoREST. Several major groups of proteins have been identified that have HAT activity including GCN; cyclic AMP response element binding protein (CREB); CBP/p300 and p300/CBP associated factor (P/CAF); TAFII p250, a component of the basic transcription complex TAFII; SRC-1 and ACTR, the co-activators for ligand-dependent nuclear receptors.

The activities of HDACs appear to be regulated, in part, by protein–protein interaction. In addition, HDACs are regulated by gene expression, subcellular localization, and post-translational modifications such as phosphorylation, sumoylation, proteolysis, and availability of metabolic cofactors [Lehrmann et al., 2002; Sengupta and Seto, 2004].

Alteration in both HATs and HDACs are found in many human cancers. Structural alterations in HDACs associated with cancers appear to be rare. HDACs are involved in the function of oncogenic translocation products in specific forms of leukemia and lymphoma [Rosato and Grant, 2003; Marks et al., 2004; Drummond et al., 2005]. The oncoprotein that is encoded by one of the translocation-generated fusion genes in acute promyelocytic leukemia, PML-RARα, represses transcription by associating with a corepressor complex that contains HDAC activity. In non-Hodgkin's lymphoma, the transcriptional repressor LAZ3/BCL6 (lymphoma-associated zinc finger-3/B cell lymphoma) is overexpressed and associated with aberrant transcriptional repression through recruitment of HDAC, leading to lymphoid oncogenic transformation. Acute myeloid leukemia m2 subtype is associated with the t(8;21) chromosomal translation, which produces an AML1–ETO fusion protein—a potent dominant transcription repressor—though its recruitment of HDAC activity. Increased expression of HDAC1 has been detected in gastric cancers, oesophageal squamous cell carcinoma, and hormone refractory prostate cancer [Choi et al., 2001; Halkidou et al., 2004]. Increased expression of HDAC2 has been detected in colon cancer. Increased expression of some of the Class II HDAC enzymes (HDAC6) has been linked to better survival in breast cancer, but reduced expression of Class II HDAC enzymes HDAC 5 and HDAC10 have been associated with poor prognosis in lung cancer patients [Osada et al., 2004].

HDAC 1 affects breast cancer progression by promoting cell proliferation by interacting with estrogen receptor alpha causing a loss in its expression [Drummond et al., 2005; Kelly and Marks, 2005]. The retinoblastoma tumor suppressor protein recruits HDAC1. The BRCA1 mutation which increases the risk for breast and ovarian cancers, codes for a protein which associates with HDAC 1 and 2. HDAC2 appears to be essential for survival of colon cancer cells. There are several examples of transcriptional repression and altered activity of proteins regulating cell cycle progression which are mediated by the recruitment of HDACs and provide a rational for the treatment of these neoplasm with inhibitors of HDAC activity.

Unlike the alterations in HDACs associated with neoplasms, structural alterations in HATs, including translocations, amplifications, deletions and point mutations have been found in various human cancers—both hematological and epithelial [Lehrmann et al., 2002; Rosato and Grant, 2003; Lindemann et al., 2004; Marks et al., 2004; Drummond et al., 2005]. For example, the HATs, CBP and p300, are altered in some tumors by mutation or translocation. Missense mutations in p300 and mutations associated with truncated p300, have been identified in colorectal and gastric primary tumors and other epithelial cancers. Individuals with the Rubinstein-Taybi syndrome—a developmental disorder—carry a mutation in CBP that inactivates its HAT activity. These individuals have an increased risk of cancer. Loss of heterozygosity of p300 has been described in 80% of glioblastomas and loss of heterozygosity around the CBP locus has been observed in hepatocellular carcinomas and in a subset of lung cancers.

HISTONE DEACETYLASE INHIBITORS

The structural details of the HDAC inhibitor enzyme interactions have been elucidated in studies of a homolog of HDAC (HDLP) with the HDAC inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) [Finnin et al., 1999]. More recently, the crystal structure of HDAC8/hydroxamate complex has been solved [Somoza et al., 2004]. These studies provide an understanding of the three dimensional structure of the catalytic site of HDACs

and insight into the mechanism for the deacetylation of acetylated lysine substrates. There is a direct interaction of the inhibitor with the active zinc site at the base of the catalytic pocket. The evidence that the different HDAC enzymes have different biological activities and that class I HDAC 1 and HDAC 2 maybe important in transformed cell proliferation, has stimulated efforts to develop selective HDAC inhibitors. A small molecule, tubacin, has been developed which selectively inhibits HDAC6 activity and causes an accumulation of acetylated alpha-tublin, but does not affect acetylation of histones and does not inhibit cell cycle progression [Haggarty et al., 2003].

HDAC inhibitors reported to date can be divided into several structural class including hydroximates, cyclic peptides, aliphatic acids, and benzamides (Table I) [Curtin and Glaser, 2003; Miller et al., 2003; Yoshida et al., 2003; Marks et al., 2004]. Trichostatin A (TSA) was the first natural product hydroximate discovered to inhibit HDACs. SAHA is structurally similar to TSA and a nanomolar inhibitor of partially purified HDAC class I and II [Richon et al., 1996; Richon et al., 1998]. Neither TSA or SAHA inhibit class III HDACs. *M*-carboxycinnamic acid bishydroxamide (CBHA) is another potent HDAC inhibitor which has been the structural basis for several derivatives including LAQ824 and a sulfonamide derivative, PXD-101, both of which inhibit class I and II HDACs in nanomolar concentrations. The cyclic peptide class is a structurally complex group of HDAC inhibitors, which includes the natural product depsipeptide (FK228), apicidin, and the Chaps group of molecules, all active in nanomolar concentrations. Depsipeptide is a prodrug of an active agent, red FK. Cyclic tetrapeptides containing trifluoroethyl and pentafluoroethyl ketone and zinc binding functional groups have been synthesized and are potent HDAC inhibitors [Jose et al., 2004].

The group of aliphatic acids, such as, phenylbutyrates and its derivatives and valproic acid, tend to be relatively weak inhibitors of HDACs being active at micromolar concentrations. Recently, a structural hybrid of 4-phenylbutyrate and TSA (BL1521) was reported to be an inhibitor at low micromolar concentrations. Both valproic acid and phenylbutyrate are relatively old drugs that have been on the market for non oncological uses and recently shown to have activity as HDAC inhibitors. The benzamide class of HDAC inhibitors includes MS-275 and CI994 which are active at micromolar concentrations. Newer benzamides are being developed which have activity both in vitro and in vivo in tumor bearing animal models [Miller et al., 2003; Marks et al., 2004].

Evidence has been developed to indicate that certain HDAC inhibitors may selectively inhibit different HDACs. For example, TSA was found to be a potent inhibitor of HDACs 1, 3, and 8 while MS-275 (2-Aminophenyl) 4-[(N-pyrydin-3-Metyloxycarbonyl)-(Aminomethyl)-(benzamide)] preferentially inhibited HDAC1 with IC50 at 0.3μM compared to HDAC3 with a IC50 of about 8 μM and no inhibitory effect against HDAC8 [Hu et al., 2003]. Two novel synthetic compounds, have been identified as HDAC inhibitors: SK 7041 and SK-7046 which preferentially target HDAC1 and 2 and exhibit growth inhibitory effects in human cancer cell lines and in tumor xenograft models [Park et al., 2004].

EFFECTS OF HDAC INHIBITORS

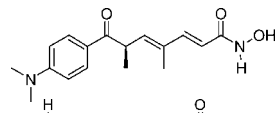
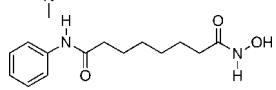
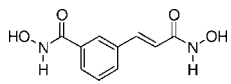
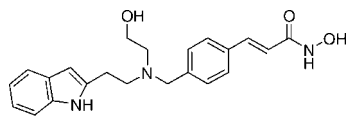
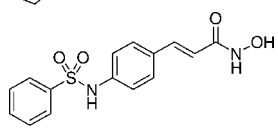
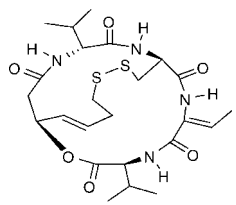
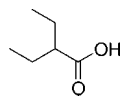
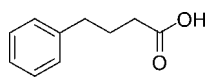
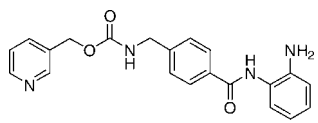
The mechanism of the anti-proliferative effects of HDAC inhibitors is complex, involving the accumulation of acetylated forms of histones and non-histone protein substrates which are involved in regulation of gene expression, cell proliferation, and cell death. Understanding the role of these different substrates is important in understanding the activity of HDAC inhibitors against a broad variety of hematologic and solid tumors.

Gene Expression and Non-Histones Proteins

HDAC inhibitors cause both increased and decreased expression of a finite number of genes. Microarray analysis of the effects of HDAC inhibitors on gene expression in different cancer cell lines has shown that the patterns of alterations of gene expression are quite similar for different HDAC inhibitors, as well as, showing definite differences induced by different agents in various transformed cells [Gray et al., 2004; Mitsiades et al., 2004; Peart et al., 2005].

For example, SAHA is a potent inducer of apoptosis of human multiple myeloma cells. Microarray analysis of gene expression in these cells revealed that a constellation of antiproliferative and/or pro-apoptotic genes was altered within 6 hrs of culture with SAHA, including down regulation of transcripts of a member of

TABLE I. Histone Deacetylase Inhibitors

Class	Compound	Structure	HDAC inhibitor activity[b]				
			HDAC[a]	CELLS	Animal bearing tumor	PhI	PhII
Hydroxamate	Trichostatin A (TSA)		nM	μM	×	NA	NA
	Suberoyl anilide hydroxamic acid (SAHA)		nM	μM	×	×	×
	CBHA		μM	μM	×	NA	NA
	LAQ-824		nM	μM	×	×	NA
	PXD-101		nM	μM	×	×	NA
Cyclic peptide	Depsipeptide (FK-228)		nM	μM	×	×	×
Aliphatic Acid	Valproic acid		μM	mM	×	×	×
	Phenyl Butyrate		μM	mM	×	×	×
Benzamide	MS-275		μM	μM	×	×	NA

[a]Concentration s indicated for HDAC and cells are range of HDACi activity. The × indicates the HDACi has been tested in tumor bearing animals (in vivo), in clinical trials, PhI and PhII. NA-HDACi has not been tested in this stage of development.
[b]See reviews [De Ruijter et al., 2002; Henderson et al., 2002; Lehrmann et al., 2002; Johnstone and Licht, 2003; Peart et al., 2003; Warrener et al., 2003; Di Gennaro et al., 2004; Marks et al., 2004; Rosato and Grant, 2004; Drummond et al., 2005].

the insulin like growth factor IGF/IGF-1 receptor and IL6 receptor signaling cascades and anti-apoptotic genes, such as, caspase inhibitors, oncogenic kinases, DNA synthesis repair enzymes and transcription factors such as E2F-1. SAHA also suppressed the activity of the proteasome and expression of its subunits and enhanced multiple myeloma cell sensitivity to proteasome inhibition and other pro-apoptotic agents. Among other genes commonly repressed by HDAC inhibitors are cyclin D1, erb-B_2, and thymidylate synthase.

HDAC inhibitor induced transcriptional repression may result from either effects on histone acetylation or alternately, from the increase in acetylation of transcription factors or components of the transcriptional machinery which alter the activity of these factors. For example, HDAC activity is required for transcriptional activation mediated by STAT5 (signal transducer and activator or transcription 5) [Rascle et al., 2003]. Inhibiting HDAC activity can prevent expression of genes for which STAT5 is required and result in repression of their expression.

In addition to the effects on gene expression, HDAC inhibitor-induced accumulation of acetylated histones may effect cell cycle progression by altering the ability of tumor cells to undergo mitosis [Warrener et al., 2003]. The acetylation state is important for their proper deposition of histones during DNA synthesis and chromosome segregation. An increase in acetylated histones during the S phase (DNA synthesis) and G2 (pre-mitosis) phases of the cell cycle can activate a G2 checkpoint which leads to arrest of cells in the G2 phase. Loss of the G2 checkpoint is a frequent event in cancer cells and may account, in part, for the increased sensitivity of cancer cells compared to normal cells to the pro-apoptotic effects of HDAC inhibitors.

HDACs and HATs act on many proteins which are subject to reversible acetylation on lysine residues and consequent changes in activity [Johnstone and Licht, 2003; Marks et al., 2004; Rosato and Grant, 2004]. HDACs, such as SAHA, may be effective as anti-cancer agents by virtue of the fact that they target multiple protein defects that are present in most transformed cells. HDACi may block the activity of the proteins that regulate cell signal transduction pathways or cell death pathways by increasing non-histone protein acetylation. For example HDACi can cause accumulation of acetylated Hsp90, the chaperone protein for AKT, resulting in degradation of this anti-apoptotic client protein. Another example is the acetylation of the retinoblastoma tumor suppressor protein which results in its inactivation [Di Gennaro et al., 2004].

Effects on Cells in Culture

HDACi cause growth arrest, differentiation or cell death of a variety of hematologic and solid tumor cells in culture (Table II) [Drummond et al., 2005; Kelly and Marks, 2005] HDACi have also been reported to be synergistic or additive with radiation therapy, anthrocyclins, fludarabine, flavopiridol, imatinib, proteasome inhibitors, bortezomib and anti-angiogenic agents and nuclear receptors ligands, such as, all-trans retinoic acid, APOZi/Trail [Marks et al., 2004; Pei et al., 2004; Rosato and Grant, 2004; Yoshida and Melo, 2004; Drummond et al., 2005; Kelly and Marks, 2005]. The elucidation of the down stream pathways of HDAC inhibition should provide further mechanistic rationale for therapies to be administered in combination with HDAC inhibitors.

Tumor Bearing Animal Models

A number of HDAC inhibitors, including TSA, CHAP1, and CHAP31, SAHA, pyroxamide, CBHA, oxamflatin, MS-275, PCK101, and FK-228 have been shown to inhibit tumor growth in animal models bearing both solid tumors and hematological malignancies, with little toxicity [De Ruijter et al., 2002; Marks et al., 2004; Piekarz and Bates, 2004; Drummond et al., 2005]. The tumors models include human breast, prostate, lung and stomach cancers, neuroblastoma, medullablastoma, multiple myeloma and leukemias. HDAC inhibitors cause an accumulation of acetylated histones in tumor and normal tissue [spleen, liver, and peripheral mononuclear (PMN) cells], which is a useful marker of HDAC inhibitor biological activity and has been used to monitor dosing in clinical trials with cancer patient.

TSA, SAHA, valproic acid, and depsipeptide are reported to block angiogenesis in vivo [Marks et al., 2004; Piekarz and Bates, 2004]. Thus, HDAC inhibitors may inhibit tumor growth both directly by causing growth arrest, terminal differentiation and/or death of cancer cells, and indirectly, by inhibiting neovascularization of tumors.

TABLE II. HDACi Induced Phenotypic Changes in Normal and Transformed Cells

Cell line	Cell type[a]	HDAC inhibitor	Concentration	Phenotypic outcome	Reference
Normal					
WI-38	Lung fibroblast	SAHA; MS-275	1.25–10 μM	G1/G2 arrest	Ungerstedt et al. [2005]
Hs 578 Bst	Breast fibroblast	SAHA	1.25–10 μM	G1/G2 arrest	Ungerstedt et al. [2005]
NDHF	Dermal fibroblast	NVP-LAQ824	10–1,000 nM	G1/G2 arrest	See reviews
NHBE	Bronchial epithelial	NVP-LAQ824	10–1,000 nM	G1/G2 arrest	See reviews
Transformed					
T24	Bladder	SAHA	2.5–20 μM	G1/G2 arrest	See reviews
MCF-7	Breast adenoca	SAHA	1.25–20 μM	Differentiation	See reviews
MEL	Murine erythroleukemia	Pyroxamide	1.25–20 μM	Differentiation	See reviews
VA-13	SV40-transformed fibroblast	SAHA	5 μM	Caspase independent death with ROS acc.	Ungerstedt et al. [2005]
CEM-CCRF	T cell leukemia	SAHA/Depsipeptide	1–2.5 μM	Caspase independent death	Peart et al. [2003]
MM.1S	Multiple myeloma	SAHA	5 μM	Caspase independent death	Mitsiades et al. [2003]
SNU-16	Gastric adenoca.	SK-7068	1 μM	Caspase mediated apoptosis	Park et al. [2004]
KCN	Neuroblastoma	CBHA	0.25–1 μM	Caspase mediated apoptosis	See reviews
HeLa	Cervical	ABHA	100 μg/ml	Aberrant mitosis; multinucleation	See reviews
MM96L	Melanoma	ABHA	100 μg/ml	Aberrant mitosis; multinucleation	See reviews

[a]See reviews cited for Table I.

Clinical Trials

SAHA, LAQ824, LBH589A, and PXD-101 are hydroxamate HDAC inhibitors that have moved forward in clinical trials [Lindemann et al., 2004; Marks et al., 2004; Rosato and Grant, 2004; Piekarz et al., 2004b; Drummond et al., 2005; Kelly and Marks, 2005]. An oral preparation of SAHA is phase I and phase II clinical trials [Kelly et al., In Press]. Results to date indicate that SAHA has good oral bioavailability, favorable pharmacokinetic profile, and anti cancer activity in a broad range of hematologic and solid tumors at doses that are well tolerated. Preliminary results of a phase II study with oral SAHA in patients with refractory cutaneous T-cell lymphoma (CTCL) included partial objective response to therapy in a number of patients. Symptomatic relief of the pruritus associated with cutaneous lymphoma occurred quickly in the majority of patients. The clinical results with SAHA are encouraging and there are multiple phase II studies ongoing or about to be initiated with SAHA as a single agent and in combination with other biologic or chemotherapeutic drug. No full reports of the efficacy results of clinical trials with LAQ-824, LBH-589A, or PXD101 have appeared.

The first generation of HDAC inhibitors to be in clinical trials were the short chain fatty acids, phenyl acetate, and phenylbutyrate. These agent showed modest anti-cancer activity and were associated with significant toxicities. Valproic acid, a common well tolerated anti-epileptic medication, is a short chain fatty acid that has recently been shown to be an inhibitor or HDACs. Phase I and phase II clinical trials are ongoing to evaluate this drug as an anti-tumor agent.

Depsipeptide completed phase I evaluation. Phase II studies alone or in combination with other anti-cancer agents are ongoing to determine the clinical efficacy in a range of solid and hematological malignancies [Piekarz and Bates, 2004; Piekarz et al., 2004a]. Preliminary results of phase II study in cutaneous T-cell and relapsed peripheral T-cell lymphoma reported significant responses in patients that have failed previous chemotherapies at well tolerated doses. Other phase II trials in solid and hematological tumors are continuing to explore the spectrum of clinical activity of depsipeptide.

MECHANISM OF ACTION OF HDAC INHIBITORS

HDAC inhibitors, such as SAHA (which was discovered and has been extensively studied in our laboratory [Richon et al., 1996, 1998; Kelly and Marks, 2005]), induce growth arrest, terminal differentiation and cell death of a broad variety of transformed cells in vitro and in vivo. The mechanism of HDACi is not fully understood. It is not clear why SAHA and other HDACi, induce different phenotypes in normal and in different transformed cells (Table II). The basis of the relative resistance of normal cells to SAHA and other HDACi compared to transformed cells is not adequately defined. The basis of HDACi selective alteration of gene transcription is not understood. The role of non-histone protein substrates of HDACi in the anti-cancer activity of these agents needs to be better elucidated. It also remains to be determined if HDAC specific inhibitors have potential therapeutic advantages over "pan-HDACi." The remainder of this review will focus on studies addressing some of these key questions.

The phenotype response in transformed cells to an HDAC inhibitor appears to be determined primarily by the cell type (the cell "context") and to a lesser extent by the structure of the HDACi or the concentration of the HDACi. HDACi can have cytotoxic effects on both proliferating and arrested tumor cells, while normal cells may be 10 fold or more resistant to HDACi induced cell death [Ungerstedt et al., 2005].

HDACi cause different phenotypes in different transformed cells including G1 arrest, terminal differentiation, mitochondria mediated caspase dependent apoptosis; caspase independent cell death associated with ROS or polyploidy, with failure of cytokineses and cell death (Table II). The cytotoxic effects on transformed compared to normal cells are not due to differences in the ability to inhibit HDAC activity, since accumulation of acetylated histone occurs in both normal and transformed cells [Marks et al., 2001].

We recently provided insight into a possible basis for the relative resistance of certain normal cells and the sensitivity of certain transformed cells to HDACi [Ungerstedt et al., 2005] (Fig. 1). SAHA and MS-275, arrested the growth of both normal (WI38- human embryonic lung fibroblast and Hs578Bst, human breast fibroblast) and transformed cells

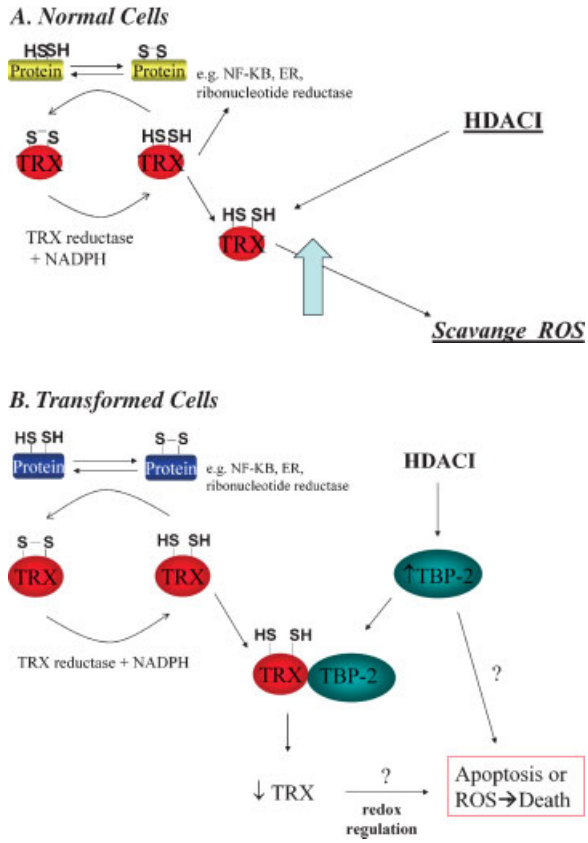


Fig. 1. Thioredoxin–redox system and histone deacetylase inhibitor action: (**A**) model for the effect of histone deacetylase inhibitor (HDACi) in normal cells. HDACi increases the level of reduced thioredoxin (Trx-SH-SH) which act to scavenger free radical oxygen species (ROS). Trx-SH-SH has several targets including ribonucleotide reductase required DNA synthesis, transcription factors, such as NF-kB and receptors such as the estrogen receptor (ER). **B**: Model for the effect of HDACi in transformed cells. HDACi induced thioredoxin binding protein (TBP-2) which binds to and inactivates TRX-SH-SH, and, inturn, facilitates apoptosis or ROS induced cell death.

(ARP-1, human multiple myeloma and VA13-SV40 transformed WI38 cells) but induced rapid cell death of only the transformed cells. Both SAHA and MS-275 caused an accumulation of reactive oxygen species (ROS) and caspase activation in transformed but not normal cells. Completely blocking the increase in caspase activity with the pan-caspase inhibitor, Z-VAD-fmk, did not inhibit HDACi induced transformed cell death. In normal cells cultured with SAHA or MS-275, the level of thioredoxin protein was consistently higher than in transformed cells. SAHA induced increased expression of TBP2 with an associated decrease in the Trx level in transformed cells [Butler et al., 2002]. TBP2 specifically binds to reduced thio-

redoxin (Trx), which is a major reducing protein with many targets including ribonucleotide reductase (required for DNA synthesis) and is an active scavenger of ROS [Arner and Holmgren, 2000]. Further, transfection of the transformed cells with thioredoxin siRNA decreased proliferation of cells and increased their sensitivity to SAHA induced cell death. Reduced Trx appears to play an important role in the resistance or sensitivity of at least some normal and transformed cells to these agents. There are reports that transformed cells with higher levels of Trx are relatively resistant to cytotoxic agents [Arner and Holmgren, 2000].

The TBP2-Trx oxidation–reduction pathway is not the only determinant of the HDACi induced phenotype of transformed cells. For example, the HDACi, depsipeptide, induced apoptosis and loss of cell proliferation of human glioblastoma cells in in vitro and in vivo associated with a decrease in anti-apoptotic protein BcL-x1 and increased expression of BAD which is a pro-apoptotic factor [Piekarz and Bates, 2004]. LAQ824 significantly inhibits the proliferation of leukemic lymphoblastic cells, by inducing apoptosis which is independent on caspase activation. MS-275 can induce a caspase dependent apoptosis in T cell chronic lymphatic leukemia cells, but can also induce caspase independent transformed cell death of other types of transformed cells. SAHA, oxamflatin, and depsipeptide induce apoptosis in certain transformed cells that can be inhibited by over-expression of Bc12, but not by the polycaspase inhibitor, Z-VAD-fmk. These studies clearly indicate that there are differences in the mechanism of cell death induced by HDACi in different transformed cells.

We have investigated the possible basis of HDACi selectivity in altering gene expression. One of the most commonly induced genes by HDAC inhibitors is the cell cycle kinase inhibitor $p21^{waf1}$. The increase in the level of p21 protein can lead to arrest of cells in G1. It has been shown that the HDAC inhibitor induced expression of $p21^{waf1}$ correlates with an increase in the acetylation of histone associated with the p21 promoter region [Gui et al., 2004]. This suggests that the p21 gene promoter is a direct target for SAHA. We found that SAHA causes specific modifications in the pattern of acetylation and methylation of lysines in histones H3 and Histone H4 associated with the proximal promoter. These changes in histones

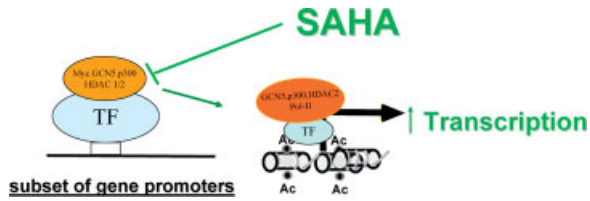


Fig. 2. Model for the mechanism of SAHA selectivity in altering gene transcription. The protein composition of the transcription factor complex of a subset of gene promotors include HDACs, HATs and other proteins—whose composition and configuration are the basis of the selectivity of SAHA action.

did not occur in the proteins associated with the promoter region of the $p27^{KIP1}$ or of the epsilon globin genes. The $p27^{KIP}$ gene is expressed in the transformed cells (ARP-1) and the epsilon globin gene is not expressed in these cells and neither gene is altered in its transcription by SAHA. The protein complex associated with the proximal promoter region of the p21 gene contained HDAC1, HDAC2, Myc, BAF155, Brg-1, GCN5, P300, and Spl (Fig. 2). SAHA caused a marked decrease in HDAC1 and Myc and recruited RNA polymerase II in the protein complex bound to the p21 promoter region, with no detectable changes in HDAC2 or any of the other proteins in the complex. Further, the loss of HDACi from the complex was not associated with a decrease in this protein in the nuclear extract. These findings suggest a basis for the HDACi selective alteration of transcription of the p21 gene. The composition and configuration of the proteins in the transcription factor complex containing the HDAC(s) determines the selective targeting of SAHA.

CONCLUSIONS AND PERSPECTIVES

HDACi are promising new targeted anticancer agents. These agents can cause transformed cells to undergo growth arrest, differentiation, apoptosis, or ROS associated cell death. Normal cells are much less sensitive to HDACi than transformed cells. The pattern of the altered gene expression and altered activity of regulatory proteins caused by HDACi appears to determine the phenotypes induced in transformed cells. This is not surprising—since different transformed cells generally have different (and multiple) molecular defects that lead to unregulated cell proliferation and survival.

The selectivity of HDAC inhibitors in altering transcription of genes may reflect in part, the proteins composing the transcription factor complex to which HDACs are recruited. The relative importance of altered gene expression and of changes in non-histone regulatory proteins caused by acetylation is not clear-but the accumulated evidence indicates that both types of effects play a role in the anti-proliferative activity of HDACi. There is evidence that Class I rather than Class II HDACs are primarily involved in cell proliferation and possibly survival of transformed cells. An important question is whether HDAC isotype specific inhibitors can be developed and whether selective inhibition of an HDAC will have therapeutic advantages compared to a pan HDAC inhibitor such as SAHA.

The answers to these questions-as they emerge-will undoubtedly have therapeutic importance, since we may develop strategies to target different factor that could enhance the efficacy and safety of HDAC inhibitors. The marked increase in research on HDACs, HATs, and HDAC inhibitors should lead to answering these and other questions related to the development of these agents as effective therapeutics for cancers and other diseases.

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