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MINIREVIEW

Rational Development of Histone Deacetylase Inhibitors as Anticancer Agents: A Review

Milin R. Acharya, Alex Sparreboom, Jürgen Venitz, and William D. Figg

Clinical Pharmacology Research Core, National Cancer Institute, National Institutes of Health, Bethesda, Maryland (M.R.A., A.S., W.D.F.); and Department of Pharmaceutics, School of Pharmacy, Virginia Commonwealth University, Richmond, Virginia (M.R.A., J.V.)

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ABSTRACT

The epigenome is defined by DNA methylation patterns and the associated post-translational modifications of histones. This histone code determines the expression status of individual genes dependent upon their localization on the chromatin. The histone deacetylases (HDACs) play a major role in keeping the balance between the acetylated and deacetylated states of chromatin and eventually regulate gene expression. Recent developments in understanding the cancer cell cycle, specifically the interplay with chromatin control, are providing opportunities for developing mechanism-based therapeutic drugs. Inhibitors of HDACs are under considerable exploration, in part because of their potential roles in reversing the silenced genes in transformed tumor cells by modulating transcriptional processes. This review is an effort to summarize the nonclinical and clinical status of HDAC inhibitors currently under development in anticancer therapy.

In eukaryotic cells, DNA has been conserved over evolution in a condensed and densely packed higher order structure called chromatin. Chromatin, present in the interphase nucleus, is composed of regular repeating units of nucleosomes, which represent the principal protein-nucleic acid relationship. The major components of chromatin are nucleic acids (DNA and RNA), which are negatively charged; associated proteins, including histones, that are positively charged at neutral pH; and nonhistone chromosomal proteins, which are acidic at neutral pH. Within the nucleus, chromatin can exists in two different forms: heterochromatin, which is highly compact and transcriptionally inactive, or euchromatin, which is loosely packed and accessible to RNA polymerases for involvement in transcriptional processes and gene expression. A nucleosome is a complex of 146 nucleotide base pairs of DNA wrapped around the core histone octamer that helps organize chromatin. The histone octamer is composed of two copies each of H2A, H2B, H3, and H4 proteins, which are very basic, mainly because of positively charged amino-terminal side chains rich in the amino acid lysine. Post-translational and other changes in chromatin, such as acetylation/deacetylation at lysine residues, methylation at lysine or arginine residues, phosphorylation at serine resides, ubiquitylation at lysines, and/or ADP ribosylation, are mediated by chemical modification of various sites on N-terminal tail (Marks et al., 2000, 2003, 2004).

The structural modification of histones is regulated mainly by acetylation/deacetylation of the N-terminal tail and is crucial in modulating gene expression, because it affects the interaction of DNA with transcription-regulatory non-nucleo-

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ABBREVIATIONS: HAT, histone acetyltransferase; HDAC, histone deacetylase; SIR, silent information regulator; ER, estrogen receptor; PA, phenylacetate; PB, phenylbutyrate; AN-9, pivaloyl oxymethyl butyrate; HA, hydroxamic acid; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid; NVP-LAQ824, (2*E*)-*N*-hydroxy-3-[4-[[(2-hydroxyethyl)[2-(1*H*-indol-3-yl)ethyl]amino]methyl]-phenyl]-2-propenamide; TPX, trapoxin; FK228, depsipeptide; MS-275, *N*-(2-aminophenyl)-4-[*N*-(pyridine-3-ylmethoxy-carbonyl)aminomethyl]benzamide; CI-994, *N*-acetyldinaline; AZA, 5-aza-2′deoxycitidine; PKC412, *N*-benzoyl staurosporine; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MMP, matrix metalloproteinase; VA, valproic acid; siRNA, small interfering RNA; SB, sodium butyrate; A-161906, 7-[4-(4-cyanophenyl)phenoxy]-heptanohydroxamic acid; PDX101, *N*-hydroxy-3-[3-](phenylamino)sulfonyl]phenyl]-2-propenamide; JNJ16241199, 2-[4-(naphthalen-2-ylsulfonyl)piperazin-1-yl]pyrimidine-5-carbohydroxamic acid.

somal protein complexes. The balance between the acetylated/deacetylated states of histones is mediated by two different sets of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs preferentially acetylate specific lysine substrates among other nonhistone protein substrates and transcription factors, affecting DNA-binding properties and, in turn, altering gene transcription. HDACs restore the positive charge on lysine residues by removing acetyl groups and thus are involved primarily in the repression of gene transcription by compacting chromatin structure. Thus, open lysine residues attach firmly to the phosphate backbone of the DNA, preventing transcription. In this tight conformation, transcription factors, regulatory complexes, and RNA polymerases cannot bind to the DNA. Acetylation relaxes the DNA conformation, making it accessible to transcription machinery. High levels of acetylation of core histones are seen in chromatin-containing genes, which are highly transcribed genes; genes that are silent are associated with low levels of acetylation. Inappropriate silencing of critical genes can result in one or both hits of tumor suppressor gene inactivation in cancer; theoretically, therefore, the reactivation of affected TSGs could have an enormous therapeutic value in preventing and treating cancer (Thiagalingam et al., 2003).

Histone Acetylases and Deacetylases: Classification and Function

The equilibrium of steady state acetylation is tightly controlled by the antagonistic effect of both HATs and HDACs, which in turn regulates transcription status of not just histones but also of other substrates such as p53 (Grozinger and Schreiber, 2002). Several groups of proteins with HAT activity have been identified, including the GNAT (Gcn5-related *N*-acetyl transferase) family, the MYST (monocytic leukemia zinc finger protein) group, TIP60 (TAT-interactive protein), and the p300/CREB-binding protein family. HATs act as large multiprotein complexes containing other HATs, coactivators for transcription factors, and corepressors (Annunziato and Hansen, 2000; Chen et al., 2001; Gregory et al., 2001; Nakatani, 2001; Schreiber and Bernstein, 2002). HATs, which bind nonhistone protein substrates and transcription factors, have been called factor acetyltransferases. Acetylation of these transcription factors also affects their DNA binding properties and gene transcription (Roth et al., 2001; Gregoretti et al., 2004). HAT genes may be overexpressed, translocated, or mutated in both hematological and epithelial cancers (Mahlknecht and Hoelzer, 2000; Timmermann et al., 2001; Johnstone and Licht, 2003). Translocations of HATs, CREB-binding protein, and p300 acetyltransferases into genes have given rise to various hematological malignancies (Fenrick and Hiebert, 1998; Pandolfi, 2001).

Mammalian HDACs are divided into three major groups or classes based on their structural homologies to the three distinct yeast HDACs: Rpd3 (class I), Hda1 (class II), and Sir2/Hst (class III). Class III HDACs consist of the large family of sirtuins [silent information regulators (SIRs)] that are evolutionarily distinct, with a unique enzymatic mechanism dependent on the cofactor NAD^+, and are virtually unaffected by all HDAC inhibitors in current development (Imai et al., 2000; Gray and Ekstrom, 2001). Class I and II HDACs contain an active site zinc as a critical component of the enzymatic pocket, have been extensively described to have an association with cancers, and are believed to be comparably inhibited by all HDAC inhibitors in development. The Rpd3 homologous class I HDACs 1, 2, 3, and 8 are widely expressed in tissues and are primarily localized in the nucleus. Hda1 homologous class II HDACs 4, 5, 6, 7, 9a, 9b, and 10 are much larger in size, display limited tissue distribution, and can shuttle between the nucleus and cytoplasm, suggesting functions and cellular substrates different from Class I HDACs (Kao et al., 2001; Guardiola and Yao, 2002). HDACs 6 and 10 are unique in that they have two catalytic domains, whereas HDACs 4, 8, and 9 are expressed to greater extent in tumor tissues and have been shown to be specifically involved in differentiation (de Ruijter et al., 2003). There is some evidence that certain inhibitors display a variable degree of HDAC specificity; hence, it is imperative to identify differences in HDAC functions to better target and tailor specific drugs compounds (Jung, 2001; Grozinger and Schreiber, 2002; Miller et al., 2003; Heltweg et al., 2004). HDACs usually interacts with large protein complexes that down-regulate genes through association with corepressors [such as nuclear receptor corepressor (NcoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT)] transcription factors, estrogen receptors (ER), p53, cell cycle-specific regulators [such as retinoblastoma (Rb), E2F and other HDACs], as well as histones, but they can also bind to their receptor directly (Frye, 2000; Imai et al., 2000; Zhou et al., 2002).

Class III HDACs (sirtuins, SIR T1, 2, 3, 4, 5, 6, and 7) are not inhibited by class I and II HDAC inhibitors; instead, they are inhibited by nicotinamide (vitamin B_3). Nicotinamide inhibits a NAD-dependent p53 deacetylation induced by SIR2α and also enhances p53 acetylation levels in vivo (Luo et al., 2001). It has been shown recently that by restraining mammalian forkhead proteins, specifically foxo3a, SIRT1 also reduces apoptosis (Motta et al., 2004). The inhibition of forkhead activity by SIRT1 parallels the effect of this deacetylase on the tumor suppressor p53. These results have significant implications regarding an important role for Sirtuins in modulating the sensitivity of cells in p53-dependent apoptotic response and the possible effect in cancer therapy (Schwer et al., 2002; North et al., 2003).

Chromatin Modification and Cancer. DNA gene expression is controlled by an assembly of nucleoproteins that includes histones and other architectural components of chromatin, nonhistone DNA-bound regulators, and additional chromatin-bound polypeptides. Changes in growth and differentiation leading to malignancy seem to occur by alterations in transcriptional control and gene silencing. It is becoming increasingly apparent that imbalances of both DNA methylation and histone acetylation may play an important role in cancer development and progression (Marks et al., 2001, 2004; Timmermann et al., 2001; Jones and Baylin, 2002). Unlike normal cells, in cancer, changes in genome expression are associated with the remodeling of long regions of regulatory DNA, including promoters, enhancers, locus control regions, and insulators, into specific chromatin architecture. These specific changes in the DNA architecture result in a general molecular signature for a type of cancer and complement its DNA methylation-based component. The changes in the infrastructure of chromatin over a target promoter are more profound than those observed by these

enzymes acting independently (Wade, 2001; Davis and Brackmann, 2003). Apart from acetylation, histone tails undergo other modifications, including methylation, phosphorylation, ubiquitylation, and adenosine diphosphate ribosylation. These other areas of modifications ("histone code") have not yet been sufficiently explored to identify their roles in epigenetic modifications (Bhalla and List, 2004).

Disruption of HAT and HDAC function is associated with the development of cancer, and malignant cells target chromatin-remodeling pathways as a means of disrupting transcriptional regulation (Mahlknecht and Hoelzer, 2000). Of the various hypotheses describing deregulation mechanisms, three have been put forth frequently: 1) disordered hyperacetylation could activate promoters that are normally repressed, leading to inappropriate expression of proteins, 2) abnormally decreased acetylation levels of promoter regions could repress the expression of genes necessary for a certain phenotype, and 3) mistargeted or aberrant recruitment of HAT/HDAC activity could act as a pathological trigger. Even though no direct alterations in HDAC genes have been demonstrated in cancer, the association of HDACs with various oncogenes and tumor suppressor genes is now well established, as is the potential for HDAC involvement in tumorigenesis (Kristeleit et al., 2004).

Histone Deacetylase Inhibitors As Anticancer Agents. The discovery of recruitment of HDAC enzymes in cancer has provided a rationale for using inhibition of HDAC activity to release transcriptional repression as a viable option toward achieving eventual therapeutic benefit (Johnstone and Licht, 2003). Inhibition of HDAC function can release dysregulation of genes involved in cell cycle progression, differentiation, and apoptosis. HDAC inhibitors block deacetylation function, causing cell cycle arrest, differentiation, and/or apoptosis of many tumors (Pandolfi, 2001). Several HDAC inhibitors have exhibited potent antitumor activity in human xenograft models, suggesting their usefulness as novel cancer therapeutic agents. Several are currently in phase I/II clinical trials both in hematological malignancies and in solid tumors. Compared with agents used initially, some newer agents are effective at nanomolar concentrations and are relatively less toxic. A wide range of structures inhibits activity of class I/II HDAC enzymes; with a few exceptions, these can be divided into structural classes, including 1) carboxylates (short-chain fatty acids), 2) small-molecule hydroxamates, 3) electrophilic ketones (epoxides), 4) cyclic peptides, 5) benzamides, and 6) other hybrid compounds (Drummond et al., 2004). Table 1 describes the various compounds, their activities in cell lines and preclinical murine models, and their current clinical status.

Comprehensive reviews on structure, medicinal chemistry, and structure-activity relationships of more than 80 different HDAC inhibitors and analogs have been previously published or reviewed (Curtin et al., 2002; Kouraklis and Theocharis, 2002; Remiszewski, 2002, 2003; Remiszewski et al., 2002; Arts et al., 2003; Bouchain and Delorme, 2003; Bouchain et al., 2003; Curtin and Glaser, 2003; Kim et al., 2003; Miller et al., 2003; Drummond et al., 2004; Kristeleit et al., 2004; Plumb et al., 2004). Despite the variety of structural distinctiveness, all of these HDAC inhibitors can be broadly characterized by a common pharmacophore that in-

TABLE 1
Overview of HDAC inhibitors
Aliases can be found in the text and in the abbreviations list.

Class & Examples	In Vitro Cell Culture Activity (Concentration)	In Vivo Preclinical Activity (Murine or Human Xenograft Model)	Clinical Phase
Carboxylates (short-chain fatty acids)			
PA	Yes (μM)	Leukemia, glioblastoma	I/II
PB	Yes (μM)	Prostate, endometrial	I/II
VA	Yes (mM)	Brain, melanoma	I/II
AN-9	Yes (μM)	NSCLC, leukemia	I/II
Hydroxamic acids			
SAHA	Yes (nM)	Lung, prostate, melanoma	I/II
m-Carboxycinnamic acid bishydroxamic acid	Yes	Neuroblastoma	
Suberic bishydroxamic acid	Yes	Melanoma, sarcoma	
Pyroxamide	Yes (μM)		I
TSA	Yes (nM)	Cervical, hepatoma,	
Oxamflatin	Yes (μM)	Melanoma	
NVP-LAQ824	Yes (nM)	Colon, multiple myeloma	I
Electrophillic ketones (epoxides)			
TPX	Yes (nM)		
AOE			
Depudecin	Yes (mM)		
Cyclic peptides			
Apicidin	Yes (nM)	Melanoma, leukemia	
FK-228, FR901228	Yes (nM)	Melanoma, colon, sarcoma, fibrosarcoma, lung, gastric	I/II
Benzamides			
MS-275	Yes (μM)	Leukemia, colorectal, gastric, pancreatic, lung, ovarian	I/II
CI-994	Yes (indirect effect)	Colorectal, pancreatic, mammary, prostate, sarcoma, leukemia	I
Other hybrid compounds			
CHAPs	Yes (nM)	Melanoma, lung, stomach, breast	
Scriptaid	Yes (nM)		
Tubacin			
JNJ16241199			
A-161906	Yes (nM)		
6-(3-Chlorophenylureido)caproic hydroxamic acid			
PXD101	Yes (nM)	Breast, prostate, ovarian, colon, NSCLC	

cludes key elements of inhibitor-enzyme interactions (Miller et al., 2003). Most of these compounds were designed to have three basic components: a hydrophobic cap that blocks the entrance to active site, a polar site, and a hydroxamic acid type zinc-binding active site separated by a hydrophobic spacer that has optimal length spanning the hydrophobic pocket on the enzyme (Drummond et al., 2004).

Short-Chain Fatty Acids

Dimethyl sulfoxide was one of the first compounds identified as active in transformed cell differentiation. Because of this, several compounds were synthesized and screened for activity in differentiation, growth arrest, and/or apoptosis (Marks et al., 2001). Valproic acid is effective in vitro as an HDAC inhibitor at relatively high (millimolar) concentrations and has much weaker affinity. It has been shown to selectively induce proteasomal degeneration of HDAC2 and is antiangiogenic in vitro and in vivo (Kramer et al., 2003; Eyal et al., 2004; Michaelis et al., 2004). Valproic acid, a well established anticonvulsant for seizures and bipolar disorders, has been shown to have antigrowth activity of human endometrial cells and to inhibit proliferation and induce apoptosis in acute myeloid leukemia cells expressing ABCB1 (P-glycoprotein) and the multidrug resistance protein ABCC1 (MRP1) (Gurvich et al., 2004; Takai et al., 2004; Tang et al., 2004). Valproic acid has recently been shown to inhibit angiogenesis in vitro and in vivo and markedly effects genes relevant in proliferation and apoptosis (Michaelis et al., 2004; Thelen et al., 2004).

Phenyl acetate (PA) can penetrate the central nervous system; when tested in solid tumors, it showed antitumor effects mediated by histone acetylation. PA is a metabolite of phenylbutyrate (PB) after B-oxidation in the liver and kidney (Piscitelli et al., 1995; Carducci et al., 1996). PB, a well studied member of the short-chain fatty acids, can arrest cells in G_1-G_0 by inducing $p21^{WAF1}$ and other cdk-2-associated cell cycle proteins, alter levels of expression and activation of chemotaxis proteins, such as urokinase-plasminogen activator, induce apoptosis, inhibit telomerase, and increase major histocompatibility complex class I expression in various tumor models (Gore and Carducci, 2000). However, the short-chain fatty acids have a low potency because of their short side chains, limiting their contact with the catalytic pocket of HDACs (Johnstone, 2002). In human CCRF-CEM cells, acute T-lymphoblastic leukemia cells, butyrate and other HDAC inhibitors caused G_2/M cell cycle arrest and apoptotic cell death (Bernhard et al., 1999). Butyrates induce histone acetylation and granulocyte maturation in acute myeloid leukemia (AML) and selectively inhibit growth in human prostate cancer and cervical carcinoma cells (Finzer et al., 2003; Gozzini et al., 2003; Kuefer et al., 2004). Butyrates have been under extensive clinical evaluation in both hematological malignancies and solid tumors. Butanoic acid or its prodrug pivaloyl oxymethyl butyrate (AN-9) is currently undergoing clinical trials after it showed 10-fold more potent activity than SB in leukemia tumor cell lines (Batova et al., 2002; Patnaik et al., 2002; Reid et al., 2004). The antineoplastic activity of AN-9 stems from rapid hydrolysis and release of butyrate, permitting efficient delivery to subcellular targets (Zimra et al., 1997, 2000). Despite the overall weak activity of short-chain fatty acids, several agents have been studied clinically because of their use for alternative medical conditions (Melchior et al., 1999; Gore et al., 2001, 2002).

Hydroxamic Acids

This is the broadest class of inhibitors with high affinity for HDAC that has been shown to inhibit both class I and II HDACs. Inhibitors containing hydroxamic acid (HA) residues bind with high affinity to the HDAC catalytic site, blocking the access of the substrate to the zinc ion (Finnin et al., 1999). The general structure of these substances consists of a hydrophobic linker that allows the hydroxamic acid moiety to chelate the cation at the bottom of the HDAC catalytic pocket while the bulky part of the molecule acts as a cap for the tube. Most of the chemicals in this group are very potent (functioning at nanomolar to micromolar concentrations in vitro) but are reversible inhibitors of class I/II HDACs.

Trichostatin A (TSA) was one of the first HDAC inhibitors to be described and is widely used as a reference in research in this field (Yoshida et al., 1995, 2001). It was developed as an antifungal agent but is relatively unstable; its toxicity to patients and lack of specificity for certain HDACs motivated the search for other substances (Jung et al., 1999; Jung, 2001). The design of many synthetic drugs has been inspired by the TSA structure (the aromatic "cap", hydroxamic acid functionality, and hydrophobic linker between them). TSA blocks proliferation and triggers apoptosis in hepatocellular carcinoma cells, blocks cell cycle progression in HeLa cells, and differentiation in ovarian cancer cells by changing p21 tumor suppressor gene and DNA-binding Id1 protein (Hoshikawa et al., 1994; Herold et al., 2002; Strait et al., 2002). TSA has also been shown to suppress growth of pancreatic adenocarcinoma cells and ACHN renal cell carcinoma via cell cycle arrest in association with p27 or apoptosis (Donadelli et al., 2003; Park et al., 2003). TSA is more sensitive in estrogen receptor α-positive breast cancer cells in inhibiting HDAC (Margueron et al., 2003).

Simple hydroxamic acid derivatives such as suberoylanilide hydroxamic acid (SAHA) and pyroxamide have activity at submicromolar concentrations (Richon et al., 1998, 2001; Marks, 2004). SAHA is a second-generation polar-planar compound that induces growth arrest, differentiation, and/or apoptosis and is under clinical investigation in both hematological and nonhematological malignancies (Richon et al., 1996, 1998; Coffey et al., 2000; Munster et al., 2001). In studies with breast cancer cells, SAHA inhibited clonogenic growth and induced apoptosis, whereas in malignant human hemotopeoitic cells, SAHA induced marked toxicity but showed relatively minor maturation activity (Vrana et al., 1999; Huang and Pardee, 2000). SAHA also showed antiproliferative and pro-apoptotic actions in several mouse xenografts and cancer cells, including prostate, bladder carcinoma, and myeloma. SAHA also induced the CDK inhibitor $p21^{WAF1/Cip1}$, and the inhibitory activity was independent of p53 status (Butler et al., 2000, 2002; Richon et al., 2000; Cohen et al., 2002; Gui et al., 2004). Pyroxamide is another compound in this class that induced terminal differentiation in murine erythroleukemic cells and caused growth inhibition in prostate carcinoma, bladder, and neuroblastoma cells via apoptosis (Butler et al., 2001; Kouraklis and Theocharis, 2002; Kutko et al., 2003). In experiments with SAHA and

butyrates, a model was proposed in which induction of apoptosis in Bcr/Abl+ cells by HDAC inhibitors involves coordinate inactivation of the cytoprotective Raf/MEK/ERK pathway in conjunction with the reactive oxygen species-dependent activation of JNK (Yu et al., 2003c).

Oxamflatin is another compound in the same class that induces transcriptional activation of junD, causing cell cycle arrest and morphological changes similar to those caused by TSA (Kim et al., 1999). Scriptaid was found to be one of the most potent analogs in a random search for substances that augment signal transduction pathways and, when screened in human and animal tumor cells, showed antiproliferative effects similar to those of SAHA (Su et al., 2000; Bouchain and Delorme, 2003). NVP-LAQ824, a cinnamic HA, has been shown to inhibit HDAC in vitro and cause transcriptional activation of p21 promoter in reporter gene assays at submicromolar concentrations in multiple myeloma (Catley et al., 2003). NVP-LAQ824, like most other HDAC inhibitors, was selective in its action because it required longer exposure and higher concentrations to retard growth of normal human fibroblasts (Atadja et al., 2004a). Another HA analog, suberic bishydroxamate, was shown to regulate expression of multiple apoptotic mediators and to induce mitochondria-dependent apoptosis in melanoma cells (Zhang et al., 2004b). PXD101 is a novel hydroxamate-type inhibitor of HDAC activity in nanomolar ranges in leukemia cells. It was shown to delay growth for xenografts of cisplatin-resistant ovarian tumor cells and had marked increase in acetylation of histone and showed good antitumor activity (Plumb et al., 2003).

Newer compounds, such as cyclic HA peptides, structural combinations of a HA (such as TSA) and a cyclic tetrapeptide (such as trapoxin), inhibit isoform selective HDACs at nanomolar concentrations (Furumai et al., 2001; Nishino et al., 2003). One of the cyclic HA peptide derivatives inhibited growth in four of five human tumor lines implanted into nude mice and showed great promise as a therapeutic agent with higher selective inhibition of HDAC (Komatsu et al., 2001).

Cyclic Peptides

Cyclic peptides having epoxyketone (epoxides) may act by chemically modifying an active site nucleophile with the epoxy group and forming H-bonds with ketone. These chemicals are supposed to trap HDACs through the reaction of the epoxide moiety with the zinc cation or an amino acid (forming a covalent attachment) in the binding pocket. However, the lability of the epoxide functionality prevents significant in vivo activity, which makes them of little pharmacologic interest. The only HDAC inhibitors in this set of compounds are a number of natural products with significant in vitro activity, such as trapoxin (TPX) A and B, depudecin, and 2-amino-8-oxo-9,10-epoxydecanoic acid. TPX is a hybrid molecule containing cyclic peptide (acts as hydrophobic cap) and epoxyketone moiety that has shown irreversible inhibition of mammalian HDACs at nanomolar ranges (Kijima et al., 1993; Kosugi et al., 1999; Komatsu et al., 2001). Cyclic tetrapeptides such as apicidin, which has an ethyl ketone moiety, and FK228 (FR901228, also referred to as depsipeptide) inhibit HDACs at nanomolar concentrations. Apicidin is a fungal metabolite that is able to inhibit HDACs and proliferation of tumor cells via induction of $p21^{WAF1/Cip1}$ and gelsolin (Han et al., 2000). It is postulated that apicidin interacts with the catalytic site and has been shown to inhibit cell proliferation in several human cancer cell lines because of its anti-invasive and antiangiogenic activity (Meinke et al., 2000; Singh et al., 2001, 2002; Hong et al., 2003; Kim et al., 2004). FK228 is a natural product derived from *Chromobacterium violaceum* that exhibits potent antitumor activity through currently unknown mechanism of action (Piekarz and Bates, 2004). One hypothesis proposes that the disulfide bridge is reduced inside the cell or organism and the 4-mercaptobut-1-enyl residue then fits inside the HDAC catalytic pocket, chelating Zn^{2+} in a manner similar to that of other inhibitors. In cultured cells, it is able to induce histone hyperacetylation and growth arrest at nanomolar concentrations. In human leukemia cells, FK228 had an IC_{50} at nanomolar concentrations and induced apoptosis in cells from patients with chronic lymphocytic leukemia (Byrd et al., 1999; Sasakawa et al., 2002, 2003a,b). In addition, depsipeptide has been shown to be antiangiogenic by modulating expression of c-myc and other regulatory genes (Kwon et al., 2002). FK228 is currently undergoing extensive evaluation in clinical trials (Kwon et al., 2002; Marshall et al., 2002; Sandor et al., 2002; Byrd et al., 2004).

Benzamides

The synthetic benzamide derivatives include a structurally diverse group of compounds such as MS-275 and CI-994. CI-994 has shown efficacy in solid tumors in murine models but did not inhibit HDAC directly. The mechanism of its action is unknown, but it seems to inhibit both histone deacetylation and cellular proliferation at the G_1-S phase transition (LoRusso et al., 1996; Graziano et al., 1997; Prakash et al., 2001). MS-275 and some of its derivatives inhibit HDACs in vitro at micromolar concentrations, but the mechanism is not clearly understood. It is believed that the diaminophenyl group is very important for the inhibitory behavior; probably, both amino functionalities chelate the metallic ion in the catalytic site. MS-275–associated HDAC-inhibitory activity is accompanied by an increase in expression of cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ and accumulation in G_1 phase (Saito et al., 1999; Suzuki et al., 1999). MS-275 displays antiproliferative activity in several human cancer cell lines, including breast, colorectum, leukemia, lung, ovary, and pancreas. MS-275 suppressed growth of several pediatric cancer cell lines in a dose-dependent manner, as well as tumors transplanted in nude mice (Jaboin et al., 2002). MS-275 and CI-994 are undergoing clinical trials. There are reports of novel nonhydroxamate sulfonamide anilides similar in structure to MS-275 being synthesized that have shown lower toxicity and comparable antiproliferative activity (Fournel et al., 2002; Bouchain et al., 2003). Focus is on the development of novel compounds based on core structures of HA or benzamide platform, which may have a better HDAC inhibitory profile and lower toxicity compared with parent compounds.

Mode of Action of HDAC Inhibitors in Cancer Cells. Even though a number of HDAC inhibitors have shown considerable promise in preclinical models, the mechanism of action has not been fully evaluated. HDAC inhibitors are effective in affecting cell cycle arrest, apoptosis, antiangiogenesis, and differentiation in cultured and transformed cells from both hematological (leukemias, lymphomas, and myelo-

mas) and epithelial (breast, bladder, ovary, prostate, and lung) tumor sources. The change that occurs after treatment with HDAC inhibitors (growth arrest, terminal differentiation, or apoptosis) seems to be dependent upon the tumor cell rather than on the specific HDAC inhibitors used (Bhalla and List, 2004). The HDAC family is divided into Zn-dependent (class I and II) and Zn-independent/NAD-dependent (class III) enzymes. The Zn-dependent enzymes have been the focus of intense research, whereas class III has been recently implicated in acetylation and regulation of key cell cycle proteins such as p53 (Cheng et al., 2003; McLaughlin and La Thangue, 2004). It is interesting that a number of studies have showed that HDAC inhibitors are relatively nontoxic to normal cells or tissues but exhibit selective cytotoxicity against a wide range of cancer cells (Zhu et al., 2001a; Rosato and Grant, 2003). It has been postulated that defective cell cycle checkpoint regulation of neoplastic cells may render them susceptible to HDAC inhibition-induced apoptosis (Johnstone and Licht, 2003; Warrener et al., 2003).

As noted above, histone acetylation is known to precede gene transcription; among the genes that are consistently up-regulated because their promoters are associated with acetylated histones is the cell cycle gene *CDKN1A*, which encodes cyclin-dependent kinase inhibitor $p21^{WAF1}$. Cyclin-dependent kinase inhibitor WAF1 inhibits cell-cycle progression by blocking cyclin-dependent kinase activity and the arrest of the cell cycle in G_1 stage. Most HDAC inhibitors (i.e., butyrates, TSA, depsipetide, oxamflatin, MS-275, and SAHA) induce expression of p21 (Archer et al., 1998; Saito et al., 1999; Sowa et al., 1999a,b; Vrana et al., 1999; Chai et al., 2000; Han et al., 2000; Huang et al., 2000; Sandor et al., 2000; Siavoshian et al., 2000; Lavelle et al., 2001; Blagosklonny et al., 2002; Wang et al., 2002). Some cDNA microarray studies have shown that treatment with TSA or SAHA alters the expression of a selective subset of approximately 2% of cellular genes that are either up- or down-regulated (Mitsiades et al., 2003; Chiba et al., 2004a,b). The genes that are usually affected by these inhibitors are *CDKN1A* and *CDKN2A*; the latter encodes genes of cell cycle regulation, such as p16, cyclin E, and thioredoxin binding protein 2 (Huang and Pardee, 2000; Kim et al., 2000). Thus, gene promoters have specific sites, such as SP1, that bind HDAC-containing transcription complexes and repress gene transcription (Li and Wu, 2004; Yokota et al., 2004). Inhibition of HDACs will activate these silenced genes, contributing to growth arrest, differentiation, and/or apoptosis of transformed cells. Treatment with HDAC inhibitors triggers both the intrinsic and sensitizes tumor cells to the death ligands that initiate the extrinsic pathway of apoptosis (Bhalla and List, 2004). Several HDAC inhibitors, including SB, SAHA, and MS-275, induce mitochondrial permeability transition, in which pro-apoptotic molecules, such as cytochrome *c*, are released into the cytosol, resulting in eventual activation of caspase-dependent apoptotic cascades (both receptor- and mitochondria-mediated) (Rosato et al., 2001; Aron et al., 2003; Nguyen et al., 2003; Guo et al., 2004). Up-regulation and induction of a conformational change of the pro-apoptotic proteins are some of the HDAC inhibitor-induced upstream events that may trigger the mitochondrial pathway of apoptosis, as described for MS-275 and SB or, as proposed in case of SAHA, may not require key caspases such as caspase-8 and caspase-3 (Ruefli et al., 2001; Lucas et al., 2004). Reactive oxygen species have recently been identified as a major cell death mechanism of several HDAC inhibitors (Ruefli et al., 2001; Rosato et al., 2003). There is some evidence that HDAC inhibitors may induce acetylation of nonhistone proteins, such as the 90-kDa heat shock protein (hsp90). Depsipeptide, SAHA, and LAQ824 lower the threshold for apoptosis by inducing the acetylation hsp90 and thus affect oncoproteins such as Bcr-Abl and FLT-3 (Yao et al., 2003; Atadja et al., 2004b). This eventually results in the inhibition of its chaperone association with important pro-survival client proteins such as Erk, Akt, and c-Raf (Yu et al., 2003c). SAHA and oxamflatin were also shown to kill both ABCB1-positive and -negative cells, whereas FK228 was shown to be substrate for ABCB1 (Peart et al., 2003). These data may provide insight into defining rational approaches to chemotherapy in which the genetic profile of the tumor is matched with a functional profile to promote favorable clinical response.

Induction of the cell cycle inhibitor plays an important role in the induction of differentiation by HDAC inhibitors. SAHA and sodium butyrate were shown to induce differentiation of leukemia and breast cancer cells (Gore et al., 2001, 2002). Induction of the expression of other molecules involved in differentiation (such as gelsolin, an actin-binding protein involved in cell morphology) and structural changes were observed during treatment with HDAC inhibitors (Hoshikawa et al., 1994; Mielnicki et al., 1999; Han et al., 2000; Kamitani et al., 2002). In addition to pro-apoptotic and cytostatic activities, another mode of tumor regression after treatment with HDAC inhibitors may be by indirect inhibition of angiogenesis. In in vitro models, depsipeptide potently blocked the hypoxia-stimulated proliferation, invasion, migration, adhesion, and tube formation of bovine aortic endothelial cells (Kwon et al., 2002). Effective concentrations were comparable with cytotoxic concentrations, and there was an indication of possible modulation of gene transcription as evidenced by the expression of angiogenic-inhibiting factors such as von Hippel Lindau and neurofibromin 2 and the suppression of angiogenic-stimulating factors such as vascular endothelial growth factor (Mie Lee et al., 2003; Sasakawa et al., 2003b). Other HDAC inhibitors, such as apicidin, TSA, butyrate, and newer analog LAQ824 were all shown to inhibit angiogenesis through vascular endothelial growth factor inhibition (Williams, 2001; Sawa et al., 2002; Kim et al., 2004; Michaelis et al., 2004; Qian et al., 2004). Such insights into the mechanisms by which HDAC inhibitors interfere with cancer cell growth and survival have prompted the search for combination strategies to optimize therapy.

Combination Therapy of HDAC Inhibitors with Other Drugs. Silencing of genes that affect growth and differentiation has been shown to occur by aberrant DNA methylation in the promoter region and by changes in chromatin structure that involve histone deacetylation (Baylin et al., 2001; Herman and Baylin, 2003). DNA methylation and histone deacetylation seem to act as synergistic layers for the transcriptional silencing of genes in cancer (Cameron et al., 1999; Zhu et al., 2001; Zhu and Otterson, 2003). Such findings have great implication in development of combination therapies.

Epigenetic mechanisms, such as DNA methylation and histone deacetylation, may also play a role in loss of ERα

expression in ER-negative human breast cancer cells. Previous studies showed that pharmacologic inhibition of these mechanisms using the DNA methyltransferase inhibitor 5-aza-2′deoxycitidine (AZA) and TSA resulted in expression of functional ER mRNA and protein (Yang et al., 2001). Scriptaid, a novel TPX-HA analog, inhibits tumor growth in vitro and in vivo and, in conjunction with AZA, acts to re-express functional ER (Keen et al., 2003). In another study, TSA was shown to sensitize ERα-negative antihormone-unresponsive breast cancer cells to tamoxifen treatment by up-regulating tamoxifen's activity (Jang et al., 2004). The in vitro antineoplastic activity of AZA, in combination with TSA or depsipeptide, on the human myeloid leukemic cell lines produced a greater inhibition of growth and DNA synthesis and a greater loss of clonogenicity than that caused by either agent alone (Shaker et al., 2003). Similar results were noted with PB and AZA combination in lymphoid leukemic cells (Lemaire et al., 2004). Another study found that when AZA was combined with PB, murine lung tumor development was significantly reduced >50%, whereas no effect was observed with PB alone (Belinsky et al., 2003).

Chromatin DNA is tightly packed; hence, accessibility to the drug target may reduce the efficiency of these anticancer drugs. When six cancer cell lines were pretreated with TSA or SAHA followed by exposure to anticancer drugs that target chromatin DNA, such as etoposide (VP-16), camptothecin, cisplatin, doxorubicin, 5-fluorouracil, cyclophosphamide, or ellipcitine, there was >10-fold sensitization of cells for VP-16. The data suggested that loosening up the chromatin structure by histone acetylation can increase efficiency of several anticancer agents (Kim et al., 2003). SAHA significantly potentiated the DNA damage by topoisomerase II inhibitors; however, synergy was dependent on the sequence of drug administration and expression of target. Pre-exposure of cells to SAHA for 48 h was synergistic, whereas shorter periods of exposure abrogated synergy, and pretreatment with topoisomerase II inhibitor showed antagonistic effects (Marchion et al., 2004).

Inhibition of cell survival signals and proliferation by using inhibitors of tyrosine kinase activity, in combination with HDAC inhibitors is another mechanism to induce differentiation and/or apoptosis (Nimmanapalli and Bhalla, 2002). The cytotoxic effects that occurred after the introduction of SAHA with imatinib mesylate showed accumulation of acetylated histones H3 and H4 and induction of p21 and p27; after SAHA treatment, there was a decline in the mRNA and protein levels of Bcr-Abl, resulting in G_1 arrest and apoptosis of leukemic cells. Cotreatment with imatinib mesylate and SAHA caused significantly more down-regulation of tyrosine kinase activity of Bcr-Abl and apoptosis of these cells compared with treatment with SAHA alone. These findings suggested that cotreatment with SAHA and imatinib mesylate or arsenic trioxide are cytotoxic to Bcr-Abl–positive acute leukemia cells, and these agents may be promising therapy against imatinib mesylate-refractory Bcr-Abl positive acute leukemia (Nimmanapalli et al., 2003a; Yu et al., 2003a). Similar results were achieved on combined exposure of Bcr/Abl-positive human myeloid leukemia cells to imatinib (STI571) and SAHA, leading to diverse perturbations in signaling and cell cycle-regulatory proteins associated with a marked increase in mitochondrial damage and cell death (Yu et al., 2003a). SAHA and PB were also shown to synergistically induce apoptosis in human leukemic cells when cotreated with the hsp90 antagonist 17-allylamino-17-demethoxygeldenamycin (Rahmani et al., 2003). Similar cumulative inhibitory effects were noted on combined treatment of SB and flavopiridol, in which interruption of HDAC-mediated $p21^{WAF1/Cip1}$ induction by flavopiridol potentiated apoptosis (Rosato et al., 2002, 2004). The same investigators showed recently that MS-275 acts synergistically with fludarabine to increase the apoptotic activity in leukemia cells (Maggio et al., 2004). Moreover, the proteasome inhibitor bortezomib interacts synergistically with SB or SAHA to cause oxidative injury and apoptosis in Bcr/Abl-positive multiple myeloma and leukemia cells that are sensitive and resistant to imatinib (Yu et al., 2003b; Pei et al., 2004).

LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib-sensitive or refractory chronic myelogenous leukemia-blast crisis cells (Nimmanapalli et al., 2003b). Recent studies show that LAQ824 can also promote degradation of mutant FLT-3 and induce apoptosis of AML cells carrying the mutated FLT-3. The addition of the Flt-3 kinase inhibitor PKC412 had a synergistic effect on apoptosis in AML cells with mutant FLT-3 (Bali et al., 2004). The combination of SAHA or LAQ824 with various cytotoxic agents such as taxotere, trastuzumab, gemcitabine, and epothilone B enhanced the cytotoxic effects in breast cancer cells, whereas the combination of 5-fluorouracil and other chemotherapy agents with PB also enhanced the cytotoxic effects in colorectal cancer cells (Huang and Waxman, 1998; Huang et al., 2000; Fuino et al., 2003). In two separate studies, SAHA also potentiated sensitizing melanoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by simultaneous activation of intrinsic and extrinsic pathways (Rosato et al., 2003; Zhang et al., 2003). In another study, VA was shown to increase cellular sensitivity to estrogens, progestins, and other hormone nuclear ligands by functioning as an activator of p42/p44 mitogen-activated protein kinase (Jansen et al., 2004). TSA up-regulated RECK glycoprotein, which negatively regulates matrix metalloproteinases (MMPs) and inhibits tumor metastasis and angiogenesis by specifically inhibiting MMP-2 (Liu et al., 2003). Radiotherapy is an effective treatment for several cancers but causes cutaneous radiation syndrome. PB, TSA, and VA were shown to decrease skin fibrosis and tumorigenesis by suppressing aberrant expression of transforming growth factor-β and tumor necrosis factor-α (Chung et al., 2004). In human gastric and colorectal cancer cells, depsipeptide, MS-275, and *m*-carboxycinnamic acid bishydroxamide augmented radiation-induced cell death (Zhang et al., 2004c). Moreover, HDAC inhibitors have shown synergism when combined with all-*trans*-retinoic acid to overcome the block in differentiation due to specific translocations associated with acute promyelocytic leukemia (Coffey et al., 2000, 2001; He et al., 2001).

Histone Deacetylase Inhibitors in Clinical Trials. Based on promising preclinical data, several HDAC inhibitors are currently being investigated in early phase trials in humans, both as single agents and in combination with known cytotoxic compounds. HDAC inhibitors such as PA, PB, VA, AN-9, SAHA, LAQ824, pyroxamide, FK228, MS-275, and CI-994 are evaluated in patients with various metastatic or refractory solid tumors in advanced stages and those with hematological malignancies such as AML, acute lymphocytic

TABLE 2
HDAC inhibitors in clinical trials as single agents

Name (Ref)	Phase	*N*	Tumor Type	Route of Administration/Dosing Regimen	DLT and Adverse Events	PK Results	Clinical Response/Outcome
PA (Thibault et al., 1994)	I	17	Solid tumors	IV bolus (60–150 mg/kg), target level 200–400 g/ml × 2 weeks	CNS depression, emesis, confusion, lethargy	Nonlinear PK, evidence of drug induction, 99% PA converted to PG and eliminated in urine, CNS penetration	3/9 SD × 2 months in HRPC, 1/6 SD > 9 months in glioblastoma
PA (Thibault et al., 1995)	I	18	Solid tumors	IV 1-h infusion b.i.d. 125 and 150 mg/kg × 2 weeks every 4 weeks	CNS depression	PA induced own clearance (27%), MTD 125 mg/kg, C_{max} 2500 g/ml	1 PR glioblastoma, 1 hormone-refractory prostate cancer with 50% post-therapy PSA decline
PA (Chang et al., 1999, 2003)	II	9 & 43	Recurrent malignant gliomas	IV infusion 400 mg/kg/day, compared 2 schedules, 2 weeks every 2 weeks or 12-day every 2 days, max 450 mg/mg/day	Fatigue, somnolence, lethargy, disorientation, malaise, weakness, N/V & granulocytopenia	No differences in plasma concentration between 2 treatments, no apparent induction of PA metabolism	For schedule 1, PR 3/40 (7.5%), SD in 7/40 patients (17.5%), PD < 2 months 30/40 patients, For schedule 2, 1/7 SD, 6/7 PD
PB (Carducci et al., 2001)	I	24	Refractory solid tumors	IV infusion 120 h every 3 weeks, dose 150–515 mg/kg/day	Neurocortical somnolence, confusion, hypokalemia, hyponatremia, fatigue, nausea	MTD = 410 mg/kg/day, plasma CL increased continuously after 24 h, PA accumulated when V_{max} was less than dosing rate	No CR, 2 SD, reduction in bone pain
PB (Gilbert et al., 2001)	I	28	Refractory solid tumors	Oral dose t.i.d. 9–45 g/day in 5 dose levels	Grade 1–2 dyspepsia, fatigue, neurocortical nausea, vomiting, hypocalcemia	MTD 27 g/day, bioavailability 78%, biologically active concentrations (0.5 mM)	No CR, PR, 7 patients (25%) with SD > 6 months
PB (Gore et al., 2001)	I	27	Myeloid dysplasia, AML	IV infusion for 7 days every 28 days	Neurocortical somnolence, confusion, slurred speech, hyperammonemia	MTD 375 mg/kg/day	No CR, PR, hematological improvements, increased neutrophils in 3, decreased blasts in 3
AN-9 (Patnaik et al., 2002)	I	28	Advanced solid tumors	IV infusion, 6 h × 5 days every 21 days at doses 0.047–3.3 g/m^2/day	No DLT, nausea, vomiting, fatigue, vision disturbance, anorexia, fever	MTD 3.3 g/m^2/day based on volume of maximum lipid formulation administrable	1 PR, no increase in fetal hemoglobin
AN-9 (Reid et al., 2004)	II	47	Refractory NSCLC	IV infusion, 2.34 g/m^2/day over 6 h × 3 days every 21 days	Grade 1–2 fatigue (34%), nausea (17%), dysgeusia (11%)		3/47 PR, 14 patients with SD > 12 weeks (30%), median survival 6.2 months, 1-year survival of 26%
VA (Atmaca, 2004)	I	26	Progressive cancers	IV infusion 1 h split twice daily × 5 days every 2 weeks at 30–120 mg/kg/day	Grade 3/4 neurotoxicity, no severe hematological	MTD 60 mg/kg, PBMC showed hyperacetylation	Neurotoxicity is dose-limiting
SAHA (Kelly et al., 2003)	I	37	Solid tumor and hematologic malignancy (B)	IV infusion, (A) 2 h × 3 days every 3 weeks, at 75–900 mg/m^2/day (B) 2 h × 5 days every 1–3 weeks 300–900 mg/m^2/day for 3–15 days	(A) No DLT in 8/8, (B) Grade 3/4 thrombocytopenia and neutropenia in hematological patients	MTD on (B), 300 mg/m^2/day $t_{1/2}$ = 21–58 min, AUC increased with dose, accumulation of acetylated histones in PBMC after 4 h at all dose levels	1 PR in refractory Hodgkin's disease & SD > 6 months in 2 patients with bladder cancer
SAHA (Garcia-Manero, 2004)	I	15	Advanced refractory leukemias or MDS	Orally t.i.d. × 14 days every 21 days at 100–250 mg	No DLT, nausea, vomiting, diarrhea, anorexia, headache, fatigue, dyspepsia	Histone hyperacetylation at all dose levels	1 CR at dose level 3 after 2 courses, 2 AML, 1 MDS patient had decrease in marrow blasts to <10%

TABLE 2—*Continued.*

Name (Ref)	Phase	*N*	Tumor Type	Route of Administration/Dosing Regimen	DLT and Adverse Events	PK Results	Clinical Response/Outcome
SAHA (Kelly, 2002)	I	39	Advanced cancers	Oral, daily or BID at 200–600 mg	Thrombocytopenia, fatigue	Prolonged plasma concentrations <10 h with single dose	Prolonged duration of acetylated histones in peripheral blood mononuclear cells (>10 h), objective response in patients with larynx, renal cancer and lymphoma
SAHA (Blumenschein, 2004)	II	13	SCCHN (metastatic head and neck cancers)	Oral, daily at 400 mg	No DLT, grade 3–4 thrombocytopenia, anemia, anorexia		No PR or CR, 1 MR based on tumor shrinkage,
FK-228 (Marshall et al., 2002)	I	33	Advanced cancers	IV infusion 4 h, weekly × 3 every with 1 week off at 1–17.7 mg/m^2	Grade 3 thrombocytopenia, fatigue, nausea, vomiting, anorexia at dose above 5 mg/m^2, subtle electrocardiographic changes	MTD 13.3 mg/m^2/day	
FK-228 (Piekarz et al., 2001; Sandor et al., 2002)	I	37	Advanced or refractory cancers	IV infusion 4 h on days 1 and 5 every 21 days at dose 1–24.9 mg/m^2	Grade 3 fatigue, nausea, vomiting, grade 4 thrombocytopenia, cardiac arrhythmia	MTD 17.8 mg/m^2 over 4 h over 4 h $t_{1/2}$ (a) =0.42 h; elimination $t_{1/2}$ (b) =8.1 h, mean CL = 11.6 L/h/m^2, inhibition of cell cycle in PC-3 cells	Increased acetylation of histones in Sezary cells, variable effect on histones after 7 h, 1 PR in colon cancer × 6 months, 1 CR in peripheral T-cell lymphoma, 3 PR in CTCL
FK-228 (Byrd et al., 2004)	I	20	CLL and AML	IV infusion on days 1, 8, 15 at 13 mg/m^2	Fatigue, nausea, progressive constitutional symptoms	Increases in histone acetylation by 100%, p21 promoter H4 acetylation, p21 protein	No cardiotoxicity, need to explore other schedules due to progressive toxicity
CI-994 (Prakash et al., 2001)	I	53	Solid tumors	Orally on schedule (A) × 2 weeks, (B) × 8 weeks followed by 2 weeks' rest	Schedule (A) thrombocytopenia, neutropenia, increased liver function tests, creatinine, (B) thrombocytopenia, nausea, vomiting	Schedule (A) MTD 15 mg/m^2/day, no cumulative toxicities, (B) MTD 8 mg/m^2/day, $t_{1/2}$ = 7.4–14.1 h, inverse relationship between platelet nadir and AUC, low effect of food on absorption	Both schedules, 1 PR in NSCLC × 2 years, 3 SD in NSCLC, colorectal and renal cancer
MS-275 (Ryan, 2003)	I	30	Solid tumors and lymphomas	Orally on schedule (A) daily × 28 days every 6 weeks, (B) weekly × 4, every 6 weeks at 2–12 mg/m^2	Schedule (A) severe GI toxicity, (B) and (C) fatigue, nausea, vomiting, anxiety thrombocytopenia, headache	MTD on (A) 2 mg/m^2, (B) 10 mg/m^2, histone acetylation at all dose levels	Schedule (A) intolerable, 15 SD on (B),
MS-275	I		Hematologic malignancy	Orally every 7 days for 4 weeks at 4–10 mg/m^2	Severe infections at 10 mg/m^2, thrombocytopenia, gastrointestinal toxicity	MTD at 8 mg/m^2	
MS-275 (Gore, 2004)	I	17	Solid tumors and lymphomas	Orally on schedule (A) 2–6 mg/m^2 biweekly, (B) 2 mg/m^2 twice weekly × 3 weeks with 1 week off, (C) 4 mg/m^2 weekly for 3 weeks with 1 week off	No drug related DLT, grade 1–3 hypophosphatemia, asthenia, nausea, anorexia	MTD not reached on (A), (B) not pursued, rapid absorption with T_{max} 0.5- 2 h, dose-dependent increase in exposure, biphasic elimination with $t_{1/2}$ = 100 h	1 PR on (A) in melanoma, 3 SD in Ewing's sarcoma, rectal carcinoma and melanoma

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TABLE 2—*Continued.*

Name (Ref)	Phase	*N*	Tumor Type	Route of Administration/Dosing Regimen	DLT and Adverse Events	PK Results	Clinical Response/Outcome
LAQ824 (Ottmann, 2004)	I	21	ALL, AML, CLL, CML, MDS	IV infusion, 3 h on days 1–3 of 21-day cycle at 6–80 mg/m^2 in 6 dose levels	Thrombocytopenia (cerebral bleeding), grade 2 hyperbilirubinemia	MTD 36 mg/m^2, dose-proportional increase in exposure, $t_{1/2}$ = 9–18 h, 1.5 fold accumulation at day 3, C_{max} after 1.5 h, not at end of infusion in >50% patients, indicates nonlinear PK	No QTc prolongation, ECG <400 ms, 1 CR in M1 AML, 6 SD, histone acetylation at >12 mg/m^2 doses
LAQ824 (Rowinsky, 2004)	I	28	Advanced solid tumors	IV infusion, 3 h on days 1–3 of 21-day cycle at 6–100 mg/m^2 in 7 dose levels	Grade 3/4 transient transaminitis, fatigue, hyperbilirubinemia, nausea, thrombocytopenia,	Dose-proportional increase in exposure, $t_{1/2}$ = 8–14 h, C_{max} after 1.5 h, not at end of infusion in >50% patients, indicates nonlinear PK	1 ECG > 500 ms, 3 SD, histone acetylation at >12 mg/m^2 doses FK-228 (Piekarz et al., 2001; Sandor et al., 2002)
LBH589 (Beck, 2004)	I	13	Advanced solid tumors	IV infusion, 30 min either on (A) days 1–3 and 8–10 of 21-day cycle at 1.2 – 7.2 mg/m^2, (B) days 1–3 or 15–17 of 28 day cycle at 2.4–4.8 mg/m^2	Prolonged grade 2 thrombocytopenia in (A), grade 3 neutropenia, anemia, hypoglycemia	Exposure increased proportionally with dose, $t_{1/2}$ = 15–20 h	6 SD, increased histone acetylation after first dose

MTD, maximum tolerated dose; DLT, dose limiting toxicity; PK, pharmacokinetics.

leukemia, chronic myeloid leukemia, chronic lymphoid leukemia, or lymphomas. Details about phase of development, major toxicities, pharmacokinetics, and preliminary data on clinical response of various HDAC inhibitors used as single agent or given in combination with cytotoxic agents that are undergoing clinical development are summarized in Tables 2 and 3, respectively.

Future Directions

The concept of mechanism-based therapeutic development of novel anticancer agents is now being recognized, because better targeting of abnormalities has been shown to offer new directions. The HDAC inhibitors in clinical trials have shown encouraging antitumor effects and well tolerated safety profiles. There may be significant repercussions in success or failure of an anticancer agent when targeting a specific subtype of HDAC without having a broader understanding of mechanism of action and the differential role each enzyme plays in chromatin remodeling in cancer cells. Although none of these agents in clinical trials were developed to be selective inhibitors of individual HDAC subtype, they do show some target selectivity (McLaughlin and La Thangue, 2004). For example, MS-275 showed selective inhibition of HDAC1 and HDAC3 but was inactive against HDAC8 (Hu et al., 2003). Likewise, FK228 has activity against class I (HDAC1 and HDAC2) enzymes but not against class II (HDAC4 and HDAC6) (Furumai et al., 2002). It remains a challenge to develop specific inhibitors of class I HDACs that are primarily located within the nucleus and class II HDACs that are known to shuttle between nucleus and cytoplasm (Kao et al., 2001; Johnstone, 2002). Recent findings using siRNA techniques to understand HDAC isotypes as potential targets suggested that class I HDAC enzymes may be more relevant targets for intervention in oncology (Curtin and Glaser, 2003). In any case, chromatin-modifying enzymes have provided an increasingly validated therapeutic target, and there is now compelling evidence that these compounds exhibit efficacy in human diseases.

Phase I and II clinical trials with HDAC inhibitors have been completed, and others are being initiated. Most of these have been able to identify suitable doses for treatment with relatively less toxicity and reasonable efficacy in various cancers. Remission seemed to be transient in some of the patient trials, suggesting a need for determination of dosing parameters (Bhalla and List, 2004). Based on preliminary clinical data and the apparent cytostatic mechanism of action, most HDAC inhibitors, with the possible exception of FK228 in the treatment of renal cell carcinoma, seem to be more suited to combination treatment with existing chemotherapy regimens and to being used in other mechanism-based agents. Nonetheless, various questions still remain to be answered: 1) what role do altered HAT or HDAC activities have in conjunction with tumorigenesis? Is it a direct effect or is an epigenetic adaptive phenomenon? 2) Why are tumor cells more sensitive to HDAC inhibitors than normal cells, and is there a possibility that there may be increased HAT/HDAC activity in tumors? 3) Is modification of histone(s) the only mechanism leading to antineoplastic effects, or are there targets responsible that are as-yet undefined? and 4) What is the target specificity of HDAC inhibitors? (Piekarz and Bates, 2004). Unraveling specific roles of HDAC isozymes

TABLE 3
HDAC inhibitors in combination therapy with other agents

Name (Ref)	Phase	*N*	Tumor Type	Route of Administration/Dosing Regimen	DLT and Adverse Events	PK Results	Clinical Response/Outcome
CI-994 + gemcitabine (Nemunaitis et al., 2003)	I	20	Advanced cancers	Gemcitabine IV infusion weekly × 3 with 1 week off at 1000 mg/m^2, CI-994 orally daily × 21 days escalating at 2–8 mg/m^2	Grade 4 thrombocytopenia (30%) at 8 mg/m^2	MTD 6 mg/m^2 oral × 21 days with 1000 mg/m^2 gemcitabine, rapid absorption, C_{max} within 2 h of dosing	2 MR, 12 SD with median 105 days, 4 PD
CI-994 + capecitabine (Undevia et al., 2004)	I	54	Advanced cancers	Schedule (A) IV capecitabine twice daily at 1650 mg/m^2/day, CI-994, 2–10 mg/m^2 orally × 2 of 3 weeks, (B) CI-994 × 5 of 6 weeks, (C) capecitabine 2000 mg/m^2/day, CI-994 orally × 2 of 3 weeks	Thrombocytopenia	MTD 6 mg/m^2 (10 mg) with capecitabine 2000 mg/m^2/day, PK of CI-994 unaltered by capecitabine	No correlation between BSA and PK parameters, platelet nadir best predicted by C_{max}
CI-994 (Wozniak, 1999)	II	32	NSCLC	Orally, daily at 8 mg/m^2	Thrombocytopenia, fatigue, anorexia, nausea, vomiting, paresthesia		2 PR, 8 SD > 8 weeks, median survival 30 weeks
CI-994 (O'Shaughnessy, 1999)	II	48	Renal cell carcinoma	Orally, daily at 8 mg/m^2	Thrombocytopenia, fatigue, anorexia, nausea, vomiting, paresthesia		26 SD for >8 weeks, median survival = 48 weeks
CI-994 (Zalupski, 2000)	II	17	Advanced pancreatic cancer	Orally, daily at 8 mg/m^2	Thrombocytopenia, asthenia, anorexia		2 SD for 6 weeks, no objective response, cytostatic mechanism
CI-994 + carboplatin or paclitaxel (Olivares, 2001)	I	21	Refractory solid tumors	Oral CI-994 daily × 7 or 14 days every 21 days (4–6 mg/m^2/day), Carboplatin every 21 days; paclitaxel 175–225 mg/m^2 every 21 days	DLT = neutropenia, thrombocytopenia diarrhea & weakness	MTD; CI-994 4 mg/m^2/day with paclitaxel 200 mg/m^2. carboplatin	1 CR bladder, 2 PR NSCLC, 6 SD
PB + AC	I	6	Solid tumors	AC 25 mg/m^2 o.d. days 1–14 PB 400 mg/kg/day Cl days 6 and 13 every 5 weeks	N.D.	N.D.	No change in pre- or post-tumor specimens for methyltransferase or GST
PB + RA		5	APL	RA (30–90 mg/m^2/day)+PB (150–400 mg/kg/day)	N.D.	N.D.	1/5 cytological CR
AN-9 + docetaxel (Reid, 2004)	II	12	Advanced NSCLC	AN-9 IV infusion 6 h/day for days 1–3 at 1.5–2.5 g/m^2, docetaxel on day 4 at 75 mg/m^2, regimen repeated every 3 weeks	No DLT, adverse events unrelated to AN-9, grade 3 neutropenia due to docetaxel in 9 (75%) patients	MTD 2.5 g/m^2 with 75 mg/m^2 docetaxel	3 PR, decrease in tumor size

N.D., not determined; NSCLC, non–small-cell lung cancer.

during human tumorigenesis will provide further incentive for the development of more specific HDAC inhibitors, potentially those enhancing clinical activity as well as decreasing nonspecific toxicities. In addition, optimizing potential interactions with other rationally designed and integrated therapeutic agents remains a promising premise for exploration. In addition, there is a general current lack of knowledge on the pharmacokinetics and biodistribution of various HDAC inhibitors studied clinically. Current evidence suggests that novel formulations and drug delivery strategies that allow better targeting may significantly enhance the therapeutic potential of HDAC inhibitors (Drummond et al., 2004).

Conclusion

A wealth of recent data has become available suggesting that histone modification is a promising therapeutic strategy affecting many of the hallmark traits of cancer (Hanahan and Weinberg, 2000). Drugs such as HDAC inhibitors that have pleiotropic actions in modulating multiple genes, pathways, and biological features of malignancy might prove to be suited for dealing with combinatorial oncogenic abnormalities seen with most cancer types (Kristeleit et al., 2004). In addition to applications in oncology, manipulation of histones involved in other diseases, such as Huntington's disease and hepatic fibrosis, may be avenues for further explorations in other therapeutic areas (Penner et al., 1987; Ferrante et al., 2003; Hockly et al., 2003). Although the clinical development of novel HDAC inhibitors seems certain, their actual value will greatly depend on identification of molecular and cellular predictors and elucidation of their mechanism of action as anticancer agents.

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Address correspondence to: Dr. William D. Figg, Clinical Pharmacology Research Core, National Cancer Institute, 9000 Rockville Pike, Building 10/Room 5A01, MSC1910, Bethesda, MD 20892. E-mail: wdfigg@helix.nih.gov