Chapter 13
Histone Deacetylase Inhibitors: Mechanisms and Clinical Significance in Cancer

HDAC Inhibitor-Induced Apoptosis

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Abstract Epigenic modifications, mainly DNA methylation and acetylation, are recognized as the main mechanisms contributing to the malignant phenotype. Acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. While histones represent a primary target for the physiological function of HDACs, the antitumor effect of HDAC inhibitors might also be attributed to transcription-independent mechanisms by modulating the acetylation status of a series of non-histone proteins. HDAC inhibitors may act through the transcriptional reactivation of dormant tumor suppressor genes. They also modulate expression of several other genes related to cell cycle, apoptosis, and angiogenesis. Several HDAC inhibitors are currently in clinical trials both for solid and hematologic malignancies. Thus, HDAC inhibitors, in combination with DNA-demethylating agents, chemopreventive, or classical chemotherapeutic drugs, could be promising candidates for cancer therapy. Here, we review the molecular mechanisms and therapeutic potential of HDAC inhibitors for the treatment of cancer.

Keywords HDAC inhibitors, HAT, SAHA, MS-275, TSA, TRAIL, apoptosis, caspase

1 Introduction

Recent years have seen major advances in elucidating the complexity of chromatin and its role as an epigenetic regulator of gene expression in eukaryotes. Epigenic modifications, mainly DNA methylation and acetylation, are recognized as additional

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mechanisms contributing to the malignant phenotype (Jones, 2002; Plass, 2002). Acetylation and deacetylation of histones play an important role in the regulation of gene expression (Grunstein, 1997). Histone acetylation is a reversible process whereby histone acetyltransferase (HAT) transfers the acetyl moiety from acetyl coenzyme A to the lysine; histone deacetylase (HDAC) removes the acetyl groups, reestablishing the positive charge in the histones. HATs and HDACs have recently been shown to regulate cell proliferation, differentiation, and apoptosis in various hematological and solid malignancies (Kouzarides, 1999). Altered HAT or HDAC activity is associated with cancer by changing the expression pattern of selected genes (Grignani et al., 1998; Lin et al., 1998). Hyperacetylation of histones correlates with gene activation, whereas deacetylation mediates eukaryotic chromatin condensation and gene expression silencing (Johnstone and Licht, 2003; Strahl and Allis, 2000). Recently, new roles of histone acetylation have been uncovered, not only in transcription, but also in DNA replication, repair, and heterochromatin formation (Kurdistani and Grunstein, 2003).

2 Histone Deacetylases

HDACs catalyze the removal of an acetyl group from the ε-amino group of lysine side chains of the core nucleosomal histones (H2A, H2B, H3, and H4), thereby reconstituting the positive charge on the lysine. Recent studies have revealed 12 human HDAC enzymes, HDAC1-11 (Emiliani et al., 1998; Gao et al., 2002; Grozinger et al., 1999; Taunton et al., 1996; Yang et al., 1996) and HDAC-A (Fischle et al., 1999). Based on the structural properties, HDACs can be divided into three classes (Gray and Ekstrom, 2001). Class I members (HDAC 1, 2, 3, 8, and 11) are transcriptional corepressors homologous to yeast RPD3 and have a single deacetylase domain at the N-termini and diversified C-terminal regions (de Ruijter et al., 2003). Class II members (HDAC 4, 5, 6, 7, 9, and 10) have domains similar to yeast HDA1 with a deacetylase domain at a C-terminal position (Verdin et al., 2003). In addition, HDAC 6 contains a second N-terminal deacetylase domain, which can function independently of its C-terminal counterpart. Class III HDACs are distinct from class I and II and are homologous of the yeast silent information regulator 2 (Sir2). All of these HDACs apparently exist in the cell as subunits of multiprotein complexes. Class II HDACs translocate from the cytoplasm to the nucleus in response to external stimuli, whereas class I HDACs are constitutively nuclear and play important roles in dynamic gene regulation (McKinsey and Olson, 2005).

Sir2 enzymes (or sirtuins) are NAD(+)-dependent deacetylases that modulate gene silencing, aging, and energy metabolism. Previous work has implicated several transcription factors as Sir2 targets. Sir2 silences transcription at silent mating loci, telomerese, and ribosomal DNA (rDNA), and this also suppresses recombination in rDNA. Earlier experiments have shown that the overexpression of Sir2 in yeast induced the global deacetylation of histones, indicating that Sir2 was an

HDAC (Braunstein et al., 1993). Later, it was shown that *cobB*, a bacterial homologue of Sir2, had ribosyltransferase activity, leading to experiments showing that Sir2 was also able to transfer adenosine diphosphate-ribose (ADP-ribose) from nicotinamide adeninedinucleotide (NAD) (Frye, 1999). Subsequently, it was confirmed that Sir2 was an NAD-dependent HDAC (Imai et al., 2000). The ADP-ribosylation of an acetylated lysine residue is an intermediate state of the enzymatic reaction catalyzed by Sir2. Only class III enzymes use NAD as a cofactor. Therefore, they are known as NAD-dependent HDACs.

Recently, Sir2 has attracted much attention, because it is related to longevity (Bordone and Guarente, 2005). The overexpression of Sir2 extends the life span of budding yeast, while its knockout shortens the life span by about 50% (Kaeberlein et al., 1999). Sir2 is conserved from bacteria to humans. In the nematodes, the gene most homologous to yeast *Sir2* gene is Sir-2.1. A duplication containing the *Sir-2.1* gene confers a life span that is extended by up to 50% (Tissenbaum and Guarente, 2001). The mammalian homologues consist of seven members, Sirt1–Sirt7. In mammalian cells, Sirt1 downregulates stress-induced p53 and FOXO pathways for apoptosis, thus favoring survival under stress. In the absence of applied stress, Sirt1 silencing induces growth arrest and/or apoptosis in human epithelial cancer cells (Ford et al., 2005). In contrast, normal human epithelial cells and normal human diploid fibroblasts seem to be refractory to Sirt1 silencing. Further studies have revealed that the Sirt1-regulated pathway is independent of p53, Bax, and caspase-2. Alternatively, Sirt1 may suppress apoptosis downstream from these apoptotic factors. FOXO4 (but not FOXO3) is required as proapoptotic mediator. Caspase-3 and caspase-7 act as downstream executioners of Sirt1/FOXO4-regulated apoptosis. These data suggest that Sirt1 as a novel target for selective killing of cancer vs noncancer epithelial cells. Upregulation of Sirt1 may be a double-edged sword that both promotes survival of aging cells and increases cancer risk in mammals.

Histones are part of the core proteins of nucleosomes. The recruitment of HATs and HDACs plays an important role in proliferation, differentiation and apoptosis (Glass and Rosenfeld, 2000; Kouzarides, 1999). Altered HAT or HDAC activity is associated with the development of cancer by changing the expression of several genes (Grignani et al., 1998; Lin et al., 1998). Treatment of malignant cells with HDAC inhibitors regulates only a small number (1–2%) of genes, as examined by DNA microarray studies (Van Lint et al., 1996). HDAC1 interacts directly with other transcription repressors, including all three of the pocket proteins, Rb, p107 and p130, and YY1. HDAC1 causes transcription repression by locally deacetylating histones, leading to a compact nucleosomal structure that prevents transcription factors from accessing DNA to promote transcription. Furthermore, HDAC1 knockout mice were embryonic lethal, possibly due to a proliferative defect upon unrestricted expressions of the cell cycle inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (Lagger et al., 2002). Overexpression of HDAC I confers resistance to sodium butyrate-mediated apoptosis in melanoma cells through a p53-mediated pathway (Bandyopadhyay et al., 2004). We and others have shown that inhibition of HDAC activity induces apoptosis in various types of cancer (Fandy et al., 2005; Fang, 2005; Marks et al., 2003; Rosato et al., 2001; Singh et al., 2005).

Stability of HDACs is an important factor in determining the biological activity. HDAC4 is unusually unstable, with a half-life of less than 8 h (Liu et al., 2004). Consistent with the instability of HDAC4 protein, its mRNA was also highly unstable (with a half-life of less than 4 h). The exposure of cells to ultraviolet (UV) irradiation resulted in the degradation of HDAC4. This degradation was not dependent on proteasome or CRM1-mediated export activity but instead was caspase-dependent and was detectable in diverse human cancer lines. Of two potential caspase consensus motifs in HDAC4, both lying within a region containing proline, glutamic acid-, serine-, and threonine-rich (PEST) sequences, Asp-289 as the prime cleavage site was identified by site-directed mutagenesis (Liu et al., 2004). Notably, this residue is not conserved among other class IIa members, HDAC5, HDAC7, and HDAC9. Finally, the induced expression of caspase-cleavable HDAC4 led to markedly increased apoptosis. These results therefore link the regulation of HDAC4 protein stability to caspases, enzymes that are important for controlling cell death and differentiation.

3 Histone Deacetylase Inhibitors

It is well established that hyperacetylation of the N-terminal tails of histones H3 and H4 correlates with gene activation, whereas deacetylation mediates transcriptional repression (Strahl and Allis, 2000). Revived interest in these enzymatic pathways and how they modulate eukaryotic transcription has led to the identification of multiple cofactors whose complex interplay with HDAC affects gene expression. Concurrent with these discoveries, screening of natural product libraries yielded new small molecules that were subsequently identified as potent inhibitors of HDAC. While predominantly identified by using antiproliferative assays, the biological activity of these new HDAC inhibitors also encompasses significant antiprotozoal, antifungal, phytotoxic, and antiviral applications. During the past decade, a number of HDAC inhibitors have been shown to induce growth arrest, differentiation, and/or apoptosis in cancer cells (Boyle et al., 2005; Fandy et al., 2005; Kwon et al., 2002b; Marks et al., 2004; Singh et al., 2005), and inhibit tumor growth in various xenograft models (Bordin et al., 2004; Butler et al., 2000; Park et al., 2004; Sakajiri et al., 2005; Shao et al., 2004; Takimoto et al., 2005; Tang et al., 2004; Zhang et al., 2004c). HDAC inhibitors induce expression of cell cycle regulatory (e.g., $p21^{/WAF1/CIP1}$) and apoptotic proteins (e.g., Bax, PUMA, and Noxa), downregulate survival signaling pathways (e.g., Raf/MAPkinase/ERK), and disrupt cellular redox state (e.g., reactive oxygen species, ROS). Therefore, HDAC inhibitors are considered candidate drugs in cancer therapy (Johnstone, 2002; Marks et al., 2001b; McLaughlin and La Thangue, 2004).

Seven classes of HDAC inhibitors have been characterized and include short-chain fatty acids (e.g., sodium butyrate and phenylbutyrate); hydroxamic acids (e.g., suberoylanilide hydroxamic acid [SAHA], LAQ824, and trichostatin A [TSA]); benzamides (e.g., MS-275, CI994); cyclic tetrapeptide containing a 2-amino-8-oxo-9,

10-epoxy-decanoyl (AOE) moiety (e.g., trapoxin A); cyclic peptides without the AOE moiety (e.g., FK228/depsipeptide, apicidin); and epoxides (e.g., depudecin). These inhibitors induce a dose-dependent inhibition of either class I or class II HDACs, or both. Newly characterized HDAC inhibitors are now available that preferentially inhibit specific HDAC classes, including SK7041 (inhibits class I HDACs) and splitomicin (inhibits class III HDACs). A wide variety of HDAC inhibitors of both natural and synthetic origin has been reported. Except for depsipeptide (FK228), natural HDACs (TSA, depudecin, trapoxins, and apicidins), as well as sodium butyrate, phenylbutyrate, and SAHA, while effective in vivo, are marked by instability and low retention. Subsequently, synthetic analogs isolated from screening libraries (oxamflatin, scriptaid) were discovered as having a common structure with TSA and SAHA: a hydroxamic acid zinc-binding group linked via a spacer (5 or 6 CH2) to a hydrophobic group. Second-generation HDAC inhibitors such as LAQ824 and PDX101 are currently under clinical trials. Synthetic benzamide-containing HDAC inhibitors (e.g., MS-275 and CI-994) are also being evaluated in the clinics.

3.1 Short-Chain Fatty Acid

Butyrate inhibits HDAC activity at micromolar concentrations. It is generated by the fermentation of dietary fibers in the lumen of the large intestine. The aromatic fatty acids phenylbutyrate and phenylacetate, which has been used to treat patients with disorders of urea metabolism, also inhibits HDAC activity and possess anticancer activity (Appelskog et al., 2004; Boivin et al., 2002; Pili et al., 2001; Sowa and Sakai, 2000; Warrell et al., 1998; Zhang et al., 2004a). Valproic acid (VPA), an anticonvulsant, has been shown to have HDAC inhibitory activity at relatively high concentrations (Catalano et al., 2005; De Felice et al., 2005; Facchetti et al., 2004; Sakajiri et al., 2005; Shen et al., 2005; Takai et al., 2004a). VPA also inhibits angiogenesis, but displays no toxicity in endothelial cells (Michaelis et al., 2005). VPA increases extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation in human umbilical vein endothelial cells. Moreover, the combination of VPA with PD98059, a pharmacological inhibitor of the mitogen-activated protein kinase kinase 1/2, synergistically inhibited angiogenesis in vitro and in vivo.

3.2 Hydroxamic Acids

Essential characteristics of hydroxamic acid-based inhibitors are the polar hydroximic group – a six-carbon hydrophobic methylene spacer, a second polar site, and a terminal hydrophobic group. TSA from *Streptomyces hygroscopicus* was initially identified as an antifungal agent (Tsuji et al., 1976). TSA and SAHA act as noncompetitive inhibitor of HDAC by mimicking the lysine substrate as well as chelating a

zinc atom crucial for enzymatic activity (Yoshida et al., 1990b). TSA and SAHA inhibit both class I and II HDACs. Simple analogs of cyclic tetrapeptides that contain suberic acid linkers and hydroxamate, instead of epoxyketone or ketone functional group, inhibit HDAC activity (Hoffmann et al., 2000). The structurally related hybrid polar compounds (HPCs) were shown to induce differentiation in a wide variety of transformed cells (Marks et al., 1996). The first representative was hexamethylene bisacetamide (HMBA) which induced differentiation of transformed cells in millimolar range (Marks and Rifkind, 1988). HMBA regulates genes that control G1-to-S phase transition, leading to G1 arrest and inhibition of DNA synthesis. Among the inducer-mediated changes, suppression of cyclin-dependent kinase cdk4, which may be required for phosphorylation of the retinoblastoma protein pRB and perhaps p107, is critical in the pathway of terminal differentiation. HMBA induces an increase in the level of $p21^{WAF1/CIP1}$ which inhibits cyclin-dependent kinase activity and, in turn, may cause cells to arrest in G1. p107 complexes with transcription factor E2F, which may alter E2F-dependent gene transcription. HMBA has also been shown to induce differentiation of neoplastic cells in patients. Furthermore, a second generation of HPCs have been synthesized which are up to 1,000-fold more potent than HMBA. Second-generation HPCs such as oxamflatin, SAHA, suberic bishydroxamic acid (SBHA), and m-carboxycinnamic acid bishydroxamide (CBHA) inhibited HDAC activity and induced cancer cell differentiation and apoptosis (Richon et al., 1998; Shankar et al., 2005b). Polyaminohydroxamic acids (PAHAs) represent an important new chemical class of HDAC inhibitors and appear to be more specific than SAHA, TSA, and MS-275, because they are selectively directed to chromatin and associated histones by the positively charged polyamine side chain. Several other analogs of hydroxamic acids are being developed (Hoffmann et al., 2000; Qiu et al., 2000).

These HDAC inhibitors inhibits proliferation, causes cell cycle arrest, and induces differentiation and/or apoptosis in numerous models of lymphoma, leukemia, multiple myeloma, and solid tumors (Fandy et al., 2005; Fronsdal and Saatcioglu, 2005; Inoue et al., 2002; Monneret, 2005; Shankar et al., 2005b; Taghiyev et al., 2005; Toth et al., 2004; Tsatsoulis, 2002; Vanhaecke et al., 2004a; Vanhaecke et al., 2004b; Wang et al., 2002; Yamashita et al., 2003). TSA is also effective in xenograft models (Canes et al., 2005; Touma et al., 2005). TSA attenuates the development of allergic airway inflammation by decreasing expression of the Th2 cytokines, IL-4 and IL-5, and IgE, which results from reduced T-cell infiltration, suggesting that HDAC inhibition may attenuate the development of asthma by a T-cell suppressive effect (Choi et al., 2005). Other analogs of TSA such as oxamflatin, scriptaid, and amide derivatives have been reported to have anticancer activity (Jung et al., 1999; Kim et al., 1999c; Monneret, 2005; Su et al., 2000). Scriptaid induces reticulocytosis and human gamma-globin synthesis (Johnson et al., 2005), suggesting its potential as a treatment option for sickle cell disease. The suppressed RARβ expression in head and neck carcinoma (HNSCC) can be reactivated by TSA (Wang et al., 2005). Additionally, TSA alone or in combination with 5-aza-2′-deoxycytidine (5-AzaC) increases lysine-9 (Lys-9) acetylation and Lys-4 methylation of the first exon at the *RAR*β gene, while decreasing the methylation

of Lys-9. Similarly, treatment of gastric carcinoma with 5-aza-C, and/or TSA resulted in reexpressed caspase-1 mRNA (Jee et al., 2005). DNA methylation-mediated repression of eNOS promoter activity was partially reversed by TSA treatment, and combined treatment of TSA and 5-AzaC synergistically induced eNOS expression in nonendothelial cells (Gan et al., 2005). Furthermore, TSA downregulates DNMT3B mRNA and protein expression in human endometrial cancer cells (Xiong et al., 2005). This decrease in DNMT3B mRNA results in a significant reduction in de novo methylation activities, suggesting that TSA may not only modify histone acetylation, but also potentially alter DNA methylation. The above findings suggest that epigenetic events such as DNA methylation and histone deacetylation play important roles in the regulation of cancer-related genes.

3.3 Benzamides

Several benzamides have been found to inhibit HDAC activity in the low micromolar range. A 2′-hydroxy or amino function seems to be essential for the optimum activity (Suzuki et al., 1999). A newly synthesized benzamide derivative with HDAC inhibitory activity, MS-275 is believed to enter the catalytic site and bind the active zinc, inhibits HDAC at micromolar concentrations. MS-275 is the first HDAC inhibitor discovered with oral anticancer activity in several animal models. Pretreatment of human leukemic cells with MS-275 significantly enhances the abrogative capacity of an established nucleoside analogue, fludarabine (Maggio et al., 2004). The study indicates that apart from promoting acetylation of histones and regulation of genes involved in differentiation and apoptosis, MS-275 also induces multiple perturbations in signal transduction, survival and cell cycle regulatory pathways that increase the fludarabine-mediated cell death. CI-994 (*N*-acetyl dinaline), originally synthesized as an anticonvulsant, does not seem to directly inhibit HDAC, but causes accumulation of acetylated histones by an unknown mechanisms. MS-275, acetyldinaline, and CI-994 are in clinical trials for the treatment of several cancers (Monneret, 2005; Ryan et al., 2005).

3.4 Cyclic Tetrapeptides Containing AOE Moiety

Hydrophobic cyclotetrapeptides contain common amino acid (*S*)-2-amino-9,10-epoxy-8-xodecanoic acid (L-Aoe) and have been reported to inhibit HDACs (Brosch et al., 1995; Kijima et al., 1993). The epoxyketone was first thought to be essential for activity, as reduction or nucleophoilic attack resulted in inactivation of compounds (Brosch et al., 1995; Kijima et al., 1993). Trapoxin A, a microbially derived cyclotetrapeptide, is an irreversible inhibitor in the low nanomolar range (Kijima et al., 1993). Trapoxin A irreversibly inhibits histone deacetylation in vivo

and causes mammalian cells to arrest in the cell cycle (Taunton et al., 1996). On the other hand, related HC toxin (host-selective toxin of *Cochliobolus carbonum*) inhibits maize enzyme activity reversibly (Brosch et al., 1995). K-trap (an analogous of trapoxin A) inhibited HDAC1 activity. A number of derivatives, such as 9-acyloxyapicidins and 9-hydroxy, have been prepared and are under investigation. Trapoxin analogs that combine cyclotetrapeptide and hydroxamic acid moieties have been prepared. The inhibitors of quinolone analogs and the hydroxamic acid analogs of apicidin yielded promising results (Meinke et al., 2000; Meinke and Liberator, 2001). Depudecin, a natural epoxide derivative isolated from the fungus *Alternaria brassicicola*, induces hyperacetylation of histones and morphological reversion in v-ras-transformed NIH 3T3 cells (Kwon et al., 1998).

3.5 Cyclic Peptides that do not Contain an AOE Moiety

Cyclic peptides such as depsipeptide (FR901228/FK228) isolated from *Chromobacterium violaceum* inhibits HDAC activity at nanomolar concentrations. Depsipeptide induces differentiation, growth arrest and apoptosis, and inhibits metastasis and angiogenesis (Aron et al., 2003; Doi et al., 2004; Khan et al., 2004; Klisovic et al., 2003a, b, 2005; Kwon et al., 2002a; Mie Lee et al., 2003; Sasakawa et al., 2002, 2003; Sato et al., 2004; Sawa et al., 2004; Vanoosten et al., 2005). Depsipeptide is also very promising antitumor agent against osteosarcoma, inducing apoptosis by the activation of the Fas/FasL system (Imai et al., 2003). A novel fungal metabolite, apicidin (cyclo(*N*-*O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)), exhibits potent, broad spectrum antiprotozoal activity in vitro against apicomplexan parasites (Darkin-Rattray et al., 1996). Apicidin's antiparasitic activity appears to be due to low nanomolar inhibition of HDAC, which induces hyperacetylation of histones in treated parasites. Since apicidin and apicidin A possess only a ketone functional group and are active in the low nanomolar concentrations, it appears that the presence of the epoxy group is not essential for activity. Apicidin induces differentiation, cell cycle arrest and apoptosis, and inhibits metastasis and angiogenesis in several cancer models (Cheong et al., 2003; Han et al., 2000, 2001; Hong et al., 2003; Kim et al., 2001a, 2004b, c; Kouraklis and Theocharis, 2002; Kwon et al., 2002b). It promotes histone acetylation and gene transcription. Its activity is enhanced by DNA methyltransferase inhibitors in AML1/ETO-positive leukemic cells (Khan et al., 2004). Preclinical studies with depsipeptide in chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) have demonstrated that it effectively induces apoptosis at concentrations at which HDAC inhibition occurs. A dose-dependent increase in H3 and H4 histone acetylation was noted in depsipeptide-treated AML1/ETO-positive Kasumi-1 cells and blasts from a patient with t(8;21) AML (Klisovic et al., 2003b). A phase I and pharmacodynamic study of depsipeptide in CLL and AML have yielded promising results (Byrd et al., 2005).

Opening of the disulfide bridge leads to a thiol that may be able to enter the active site and complex the zinc ion. In this regard, garlic constituents and their metabolites such as diallylsulfide and allylmercaptan inhibited HDAC activity. Diallyl disulfide caused increased acetylation of H3 and H4 histones in DS19 mouse erythroleukemic cells and K562 human leukemic cells (Lea et al., 1999), suggesting that differentiation in erythroleukemic cells by diallyl disulfide and allyl mercaptan may be mediated through induction of histone acetylation. Acetylation was also induced in rat hepatoma and human breast cancer cells by diallyl disulfide or its metabolite, allyl mercaptan. Diallyl disulfide increased histone acetylation and $p21^{WAF1/CIP1}$ expression in human colon tumor cell lines (Druesne et al., 2004).

3.6 Epoxides

The naturally occurring epoxide depudecin (a microbial metabolite containing two epoxide groups) irreversibly binds to HDAC and inhibits its activity at micromolar concentration. Depudecin inhibited embryonic angiogenesis, involving the chorio-allantoic membrane of growing chick embryo (Oikawa et al., 1995). It also affected the growth of vascular endothelial cells, a key event in the process of angiogenesis in vivo. Depudecin reverts the rounded phenotype of NIH 3T3 fibroblasts transformed with v-ras and v-src oncogenes to the flattened phenotype of the nontransformed parental cells (Kwon et al., 1998). These data suggest that depudecin could be promising as an antiangiogenic agent and that its antiangiogenic action involves an inhibitory effect on vascular endothelial cell growth.

3.7 Psammaplins

Psammaplins, isolated from a marine sponge *Pseudoceratina purpurea*, inhibited HDAC and DNA methyltransferase activities (Pina et al., 2003). Psammaplin A (PsA) contains an α-oximatoamide functional group, which inhibits the HDAC activity at the catalytic site. The disulfide group is also an essential feature for HDAC inhibition. PsA showed a potent cytotoxicity against several cancer and endothelial cells (Jiang et al., 1995, 2004; Kim et al., 1999a, b; Nicolaou et al., 2001; Park et al., 2003; Pham et al., 2000; Shim et al., 2004). PsA-induced cytotoxicity may correlate with its inhibition on DNA replication (Jiang et al., 2004). Furthermore, PsA was found to inhibit mammalian aminopeptidase N (APN) that plays a key role in tumor cell invasion and angiogenesis (Shim et al., 2004). Interestingly, the antiproliferative effect of PsA was dependent on the cellular amount of APN expression. PsA suppressed the invasion and tube formation of endothelial cells stimulated by basic fibroblast growth factor. Several synthetic analogs of PsA are currently being developed as antiangiogenic and anticancer agents.

4 Mechanism of Actions of HDAC Inhibitors

HDAC inhibitors regulate several biological events including cell cycle, differentiation, and apoptosis in vitro and in vivo (Donadelli et al., 2003; Fandy et al., 2005; Fang, 2005; Fenic et al., 2004; Fronsdal and Saatcioglu, 2005; Henderson and Brancolini, 2003; Hu and Colburn, 2005; Imai et al., 2003; Mai et al., 2005; Marks et al., 2001a; Marks and Jiang, 2005; Nome et al., 2005; Sasakawa et al., 2003; Strait et al., 2005; Takimoto et al., 2005; Yoshida et al., 1990a, 2003). The mechanisms by which these inhibitors induce cell cycle arrest, differentiation, and apoptosis appear to involve multiple genes. In addition to inducing growth arrest and apoptosis, they also inhibit metastasis and angiogenesis (Deroanne et al., 2002; Kim et al., 2001b, 2004c; Sasakawa et al., 2003; Sawa et al., 2002; Williams, 2001; Zgouras et al., 2004). These biological processes are described in this section.

Inhibition of ErbB signaling pathway has been an attractive target for cancer therapy. Several studies have shown that HDAC inhibitors decreased expression of ErbB1 and ErbB2 in DU145 and ErbB2 in SKBr3 cancer cell lines (Chinnaiyan et al., 2005b). HDAC inhibitors also inhibited caveolin-1 and hypoxia-inducible factor 1α (HIF-α), and upregulated gelsolin, p19 (INK4D) and Nur77 expressions in DU145 cells (Chinnaiyan et al., 2005b). Synergistic effects of HDAC inhibitor and ErbB blockade have been shown on cell proliferative, apoptosis, and signaling pathways in cancer cells. Thus, anti-ErbB agents and HDAC inhibitors may offer a promising strategy of dual-targeted therapy. The beneficial effects of these agents may not derive solely from modulation of ErbB expression, but may result from effects on other oncogenic processes including angiogenesis, invasion, and cell cycle kinetics.

The ability of HDAC inhibitors to deactivate Akt through the reorganization of PP1 complexes not only provides a unique mode of Akt regulation, but also represent first example of modulating specific PP1-protein interactions by small-molecule agents. HDAC inhibitors have been reported to lower the apoptotic threshold of several molecularly targeted agents in cancer therapy. This therapeutic strategy is illustrated by the synergistic combination of HDAC inhibitors with other therapeutic agents, Hsp-90 antagonist 17-AGG (George et al., 2005; Rahmani et al., 2005), including the Bcr-Abl kinase inhibitor imatinib (Kim et al., 2004b; Nimmanapalli et al., 2003), the purine analog flutarabine (Maggio et al., 2004), the HER2 antibody trastuzumab (Fuino et al., 2003), the receptor tyrosine kinase FLT-3 inhibitor PKC412 (Bali et al., 2004), the proteosome inhibitor Bortezomib (Yu et al., 2003), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Shankar et al., 2005b; Singh et al., 2005). These chemosensitization effects may be mediated through both histone acetylation-dependent and acetylation-independent effects of HDAC inhibitors, of which the underlying mechanism warrants investigation.

MS-275 upregulates TGFβ signaling pathway via transcriptional activation of the TGFβ type II receptors (TβRII) (Lee et al., 2001), as a result of PCF recruitment to the NF-Y complex on the type II receptor promoter and selective

hyperacetylation of histones associated with the TβRII promoter (Park et al., 2002). Thus, MS-275induces TβRII promoter activity by the recruitment of the PCAF protein to the NF-Y complex, interacting with the inverted CCAAT box in the TβRII promoter. TβRII is often inactivated by mutation or transcriptionally repressed in many cancers, and is therefore a potential candidate for reactivation by HDAC inhibitor treatment.

HDAC inhibitor may also enhance tumor-cell immunogenicity through transcriptional activation of MHC class I and II genes, costimulatory molecules (CD40, CD80, and CD86), intercellular adhesion molecule ICAM1, and type I and II interferons (Johnstone, 2002). These proteins play important roles in host defense mechanisms and cell signaling.

Nonepigenic mechanisms of HDAC inhibitors have recently been described. A number of tumor-associated proteins that mediate cell cycle, growth and/or apoptosis, including Ku70 (Cohen et al., 2004a, b; Subramanian et al., 2005), FOXO1 (Yang et al., 2005), p300 (Bouras et al., 2005), androgen receptor (Fu et al., 2003; Gaughan et al., 2002, 2005), Smad7 (Simonsson et al., 2005), Stat3 (O'Shea et al., 2005; Yuan et al., 2005), p53 (Juan et al., 2000; Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001), Hsp90 (Kovacs et al., 2005), NF-κB/RelA (Greene and Chen, 2004; Quivy and Van Lint, 2004; Yeung et al., 2004), and SRY (Thevenet et al., 2004) have been identified as substrates for various HDACs isoforms. Targeting the acetylation status of these signal mediators might underlie the antiproliferative activities of HDAC inhibitors in cancer cells. Furthermore, various HDACs have been shown to form complexes with cellular proteins including 14-3-3 proteins, α-tubulin, ubiquitin, and PP1(Brush et al., 2004; Canettieri et al., 2003; Grozinger and Schreiber, 2000; Hook et al., 2002; Kawaguchi et al., 2003; Yang and Gregoire, 2005). These protein–protein complexes may be responsible for altering the biological functions. HDACs 1 and 6 formed complexes with PP1 (Brush et al., 2004; Canettieri et al., 2003), of which the combined deacetylase/phosphatase activities underlie the ability of HDAC1 to modulate transcriptional activity of the cAMP-responsive element-binding protein (CREB) and that of HDAC6 to regulate microtubule dynamics. These studies provide new insight into the mechanism by which HDAC inhibitors elicited coordinate changes in cellular protein phosphorylation and acetylation and suggested that changes in these protein modifications at multiple subcellular sites may contribute to HDAC inhibitor's effects to suppress cell growth and transformation.

4.1 Cell Cycle Regulation by HDAC Inhibitors

During the cell-division cycle, chromosomal DNA must initially be precisely duplicated and then correctly segregated to daughter cells. Cell cycle control of transcription seems to be a universal feature of proliferating cells, although relatively little is known about its biological significance and conservation between organisms.

Given the key role of cell cycle integrity in tumor suppression and cancer therapy, a lot of attention has focused on the ability of HDAC inhibitors to alter the levels of cell cycle regulatory proteins. HDAC inhibitors induce growth arrest at both the G1 and G2/M phases of cell cycle and induce differentiation and/or apoptosis of various types of tumor cell lines (Acharya and Figg, 2004; Donadelli et al., 2003; Duan et al., 2005; Fandy et al., 2005; Fang, 2005; Lavelle et al., 2001; Marks and Jiang, 2005; Myzak et al., 2004; Nome et al., 2005; Rocchi et al., 2005; Rosato et al., 2003b; Sakajiri et al., 2005; Sato et al., 2004; Shankar et al., 2005b; Strait et al., 2005). HDAC inhibitors induced both $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ at protein levels, and caused hypophosphorylation of Rb (Fandy et al., 2005; Mitsiades et al., 2005; Nome et al., 2005; Shankar et al., 2005b). Other cell cycle inhibitors that participate in the proliferative arrest elicited by HDAC inhibitors are $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ (Hitomi et al., 2003; Yokota et al., 2004). Moreover, positive regulators of proliferation, such as cyclins D1 and D2, cMyc, or c-Src, are downregulated by HDAC inhibitors (Dehm and Bonham, 2004; Heruth et al., 1993; Lallemand et al., 1996; Souleimani and Asselin, 1993; Takai et al., 2004b). p53 is activated both by inhibitors of HDACs class I/II, as well as by inhibitors of the Sir2 family (Juan et al., 2000; Luo et al., 2000, 2001; Vaziri et al., 2001). Transcription factor Sp1 regulates $p21^{WAF1/CIP1}$ expression in a p53-independent fashion (Han et al., 2001; Sasakawa et al., 2002; Savickiene et al., 2004; Varshochi et al., 2005). Furthermore, $p21^{WAF1/CIP1}$ expression is also transcriptionally regulated by p53 (Parker et al., 1995).

4.2 Apoptotic Induction by HDAC Inhibitors

HDAC inhibitors induce apoptosis in several types of cancers including breast, prostate, lung and thyroid carcinoma, leukemia, and multiple myeloma (Amin et al., 2001; Chen et al., 2005; de Ruijter et al., 2003; Donadelli et al., 2003; Fandy et al., 2005; Fandy and Srivastava, 2006; Kim et al., 2003; Mitsiades et al., 2005; Mori et al., 2004; Papeleu et al., 2005; Rosato et al., 2003a; Sakajiri et al., 2005; Singh et al., 2005; Vigushin and Coombes, 2002; Zhang et al., 2004d). In addition to TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors, the regulation of Bcl-2 family members is also important for inducing sensitivity by HDAC inhibitors. We and others have shown that HDAC inhibitors selectively induce proapoptotic members such as Bax, Bak, Noxa, Bim and Puma and inhibit antiapoptotic Mcl-1, Bcl-X_L and Bcl-2 expression (Fandy et al., 2005; Fandy and Srivastava, 2006; Khan et al., 2004; Mitsiades et al., 2003; Neuzil et al., 2004; Shankar et al., 2005b; Singh et al., 2005; Zhang et al., 2003, 2004b). Bcl-2 family members mainly exert their apoptotic effects by acting at the level of mitochondria and play a crucial role in cancer development (Green and Reed, 1998). HDAC inhibitors cleave poly(ADP-ribose) polymerase (PARP) and caspase-8, caspase-9, caspase-3, caspase-7, and caspase-2. Transfection of Bcl-2 cDNA partially suppressed SAHA-induced cell death. HDAC inhibitors can also induce TRAIL, suggesting the activation of

death receptor pathway without the requirement of exogenous TRAIL. Thus, HDAC inhibitors can induce apoptosis by linking both death receptor and mitochondrial pathways of apoptosis.

Dysregulation in apoptosis has been associated with the development of cancer (Johnstone et al., 2002). Recent studies have shown the involvement of mitochondria in many apoptotic signaling pathways (Kandasamy et al., 2003; Wei et al., 2000). Members of the Bcl-2 family of proteins that regulate apoptotic signaling through mitochondria are key regulators of apoptosis in mammalian development, and their deregulation is associated with disease, particularly cancer (Grimm et al., 1996; Gross et al., 1999). There are three classes of Bcl-2 family members: apoptosis promoters (e.g., Bax and Bak); apoptosis inhibitors (e.g., Bcl-2, Bcl-X_L, and adenoviral E1B 19K); and the BH3-only Bcl-2 family members (e.g., Bid, Puma, Noxa, Bad, and Nbk/Bik) (Gross, 2001). BH-3 only proteins may function as death sensors that mediate activation of the mitochondrial apoptosis pathway in response to oncogenic stress signals or DNA damage. Noxa and PUMA are transcriptionally induced by p53 and mediate apoptosis induced by p53. These proapoptotic activities of certain BH3-only proteins essentially depend on the presence of Bax and Bak. Inactivation of both Bax and Bak was required for tumor growth and was selected for in vivo tumorigenesis (Degenhardt et al., 2002a, b). $Bax^{-/-}$ and $Bak^{-/-}$ double knockout mouse embryo fibroblasts (DKO MEFs) were resistant to death signaling pathway, indicating that they are the required downstream components of mitochondrial signaling pathways (Kandasamy et al., 2003). Bim has been implicated in modulating lymphocyte homeostasis in immune cells. $Bim^{-/-}$ mice succumb to autoimmune kidney disease, accumulation of lymphoid and myeloid cells, and perturbed T-cell development (Bouillet et al., 2002; Bouillet and Strasser, 2002). Therefore, the regulation of Bcl-2 family members by HDAC inhibitor may play important roles on apoptosis by inducing a death activity or by antagonizing a survival activity. Furthermore, HDAC inhibitors can disrupt cellular redox state (e.g., ROS), and damage mitochondria in cells undergoing apoptosis.

Direct inhibitor of apoptosis protein (IAP)-binding protein with low pI/second mitochondrial activator of caspases, HtrA2/Omi and GstPT/eRF3 are mammalian proteins that bind via N-terminal IAP-binding motifs (IBMs) to the baculoviral IAP repeat (BIR) domains of IAPs. These interactions can prevent IAPs from inhibiting caspases, or displace active caspases, thereby promoting cell death (Deveraux and Reed, 1999). IAPs (cIAP-1, cIAP-2, NIAP, Livin/ML-IAP, survivin, and XIAP) protect cells against apoptosis by acting as caspase inhibitors (Deveraux and Reed, 1999). IAPs bind to and directly inhibit caspase-3, caspase-7, and caspase-9 (Deveraux and Reed, 1999; Deveraux et al., 1999). IAP proteins are regulated by interactions with the mitochondrial proteins (e.g., Smac/DIABLO), which may be released into the cytosol upon apoptotic stimulation and through IAP sequestration results in elevated caspase activity (Du et al., 2000; Verhagen et al., 2000). Some IAP proteins are also regulated by proteolysis via the ubiquitin-proteasome pathway and caspase-dependent cleavage of XIAP in cells undergoing apoptosis. The inhibition of XIAP, cIAP1, and cIAP2 expressions by HDAC inhibitors may contribute in sensitization of cells to TRAIL. In this context, we have shown that

TRAIL inhibits the expression of IAPs in breast and prostate cancer cells (Shankar et al., 2005b; Singh et al., 2005). The combination of HDAC inhibitors and TRAIL may further inhibit the expression of some of the IAPs and contribute to the synergistic induction of apoptosis by these agents.

HDAC inhibitors activate the p53 molecule through acetylation of 320 and 373 lysine residues, upregulate PIG3 and NOXA, and induce apoptosis in cancer cells expressing wild and pseudo-wild-type *p53* genes (Terui et al., 2003). SAHA induced polyploidy in human colon cancer cell line HCT116 and human breast cancer cell lines, MCF-7, MDA-MB-231, and MBA-MD-468, but not in normal human embryonic fibroblast SW-38 and normal MEFs (Xu et al., 2005a). The polyploid cells lost the capacity for proliferation and committed to senescence. The induction of polyploidy was enhanced in HCT116 $p21^{WAF1-/-}$ or HCT116 $p^{53-/-}$ cells than in wild-type HCT116. The development of senescence of SAHA-induced polyploidy cells was similar in all colon cell lines (Xu et al., 2005b). The present findings indicate that the HDAC inhibitor could exert antitumor effects by inducing polyploidy, and this effect is more marked in transformed cells with nonfunctioning $p21^{WAF1/CIP1}$ or *p53* genes.

In chronic myelocytic leukemia (CML) the activity of the Bcr-Abl tyrosine kinase is known to activate a number of molecular mechanisms, which inhibit apoptosis (Nimmanapalli et al., 2003; Xu et al., 2005b). SAHA markedly decreases protein expression levels of Bcr-Abl, c-Myc, and HDAC3 in CML, suggesting that SAHA exerts its biological activity by inhibiting survival pathway (Xu et al., 2005b). Differential expression of HDAC has been reported in various cancers. To explore the mechanisms of disease-specific HDAC activity in AML, the expression of HDAC in primary AML blasts and in four control cell types (namely CD34+ progenitors from umbilical cord, quiescent or cycling (postculture) cells, cycling CD34+ progenitors from GCSF-stimulated adult donors, and peripheral blood mononuclear cells) was characterized. Only Sirt1 was consistently overexpressed in AML samples compared with all controls, while HDAC6 was overexpressed relative to adult, but not neonatal cells (Bradbury et al., 2005). HDAC5 and SIRT4 were consistently underexpressed. HDAC inhibitors (valproate, butyrate, TSA, and SAHA) caused hyperacetylation of histones in AML blasts and cell lines (Bradbury et al., 2005). Such treatment also modulated the pattern of HDAC expression, with strong induction of HDAC11 in all myeloid cells tested, and lesser, more selective, induction of HDAC9 and SIRT4. The distinct pattern of HDAC expression in AML and its response to HDAC inhibitors is of relevance to the development of HDAC inhibitor-based therapeutic strategies and may contribute to observed patterns of clinical response and development of drug resistance.

4.3 Antiangiogenic Properties of HDAC Inhibitors

Tumor growth requires the development of new vessels that sprout from preexisting normal vessels in a process known as “angiogenesis” (Folkman, 2002). These new vessels arise from local capillaries, arteries, and veins in response to the release of

soluble growth factors from the tumor mass, enabling these tumors to grow beyond the diffusion-limited size of approximately 2 mm diameter. Tumor growth and metastasis depend upon the development of a neovasculature in and around the tumor (Folkman, 2002, 2003a, b, d; Folkman and Kalluri, 2004; Liotta et al., 1991). Angiogenesis is regulated by the balance between stimulatory (e.g., bFGF, IL-8, MMP-2, MMP-9, TGFβ1, and vascular endothelial growth factor [VEGF]) and inhibitory (e.g., angiostatin, IL-10, and interferon) factors released by the tumor and its environment (Folkman, 2003b, c). For example, overexpression of bFGF (Allen and Maher, 1993; Ravery et al., 1992) and VEGF (Brown et al., 1993a, b; O'Brien et al., 1995) has been found in the tissue, serum, and urine of patients with bladder cancer and has been associated with cancer progression, suggesting a direct involvement of these proteins in angiogenesis.

HDAC inhibitors also modulate angiogenesis in a potentially therapeutic manner. HDAC1 downregulates expression of p53 and the von Hippel–Lindau tumor suppressor gene and stimulates angiogenesis of human endothelial cells. HDAC inhibitors prevent endothelial cell proliferation and angiogenesis by downregulating angiogenesis-related gene expression (Bapna et al., 2004; Caponigro et al., 2005; Chinnaiyan et al., 2005b; Deroanne et al., 2002; He et al., 2005; Kim et al., 2001b, 2004c; Kwon et al., 2002a; Liu et al., 2003; Michaelis et al., 2004, 2005; Mie Lee et al., 2003; Momparler, 2003; Murakami et al., 2004; Nam and Parang, 2003; Pili et al., 2001; Qian et al., 2004; Rossig et al., 2002; Sasakawa et al., 2003; Sawa et al., 2002; Takimoto et al., 2005; Wang et al., 2003; Wiedmann and Caca, 2005; Williams, 2001; Zgouras et al., 2004). Phenyl butyrate, LBH589, LAQ824, and TSA have antiangiogenic activity both in vitro and in vivo (Pili et al., 2001; Qian et al., 2004, 2006; Williams, 2001). Other HDAC inhibitors such as SAHA, FK228, VPA, and apicidin also have antiangiogenic acitivity (Kim et al., 2001b; Kwon et al., 2002a; Michaelis et al., 2004). Angiogenesis inhibition induced by HDAC inhibitors was associated with modulation of angiogenesis-related genes both in cancer cells (e.g., inhibition of HIF-1α and VEGF) and in endothelial cells (inhibition of Tie-2 and survivin), and inhibition of endothelial cell migration and proliferation (Kim et al., 2001b; Kwon et al., 2002a; Michaelis et al., 2004; Williams, 2001). Furthermore, LBH589 inhibited endothelial tube formation and matrigel invasion (Qian et al., 2006). These data suggest that the effects of HDAC inhibitors on angiogenesis can be further enhanced in the presence of TRAIL.

HDAC inhibitors upregulate p53 and von Hippel–Lindau expression (Kim et al., 2001b). The combination of adenoviral vector carrying wild-type *p53* (*Ad-p53*) gene therapy with sodium butyrate resulted in a complete regression of xenografted human gastric tumor (KATO-III) cells in nude mice (Takimoto et al., 2005). Tumors treated with the combination showed higher numbers of TUNEL-positive cells and lower CD34 staining than those treated with a single modality (Takimoto et al., 2005). This was further supported by the finding that the brain-specific angiogenesis inihibitor-1 (BAI-1), an inhibitor of vascularization, was induced by sodium butyrate treatment in cells transfected with Ad-p53 (Takimoto et al., 2005). These data suggest that HDAC inhibitors can be combined with *p53* gene therapy for the treatment of cancer. The HDAC inhibitors have shown the

dual function of targeting both tumor cells and proliferating endothelial cells and to inhibit tumor angiogenesis by gene modulation. Rational clinical testing of these agents either alone or in combination with angiogenesis inhibitors is warranted.

5 Combination of HDAC Inhibitors with Trail/Apo-2L

HDAC inhibitor either alone or in combination with TRAIL can be used in cancer therapy. We and others have shown that several HDAC inhibitors can enhance the apoptosis-inducing potential of TRAIL in TRAIL-sensitive cells and sensitize TRAIL-resistant breast, prostate, and lung cancer cells, and malignant mesothelioma, leukemia, and myeloma cells (Facchetti et al., 2004; Fandy et al., 2005; Goldsmith and Hogarty, 2005; Inoue et al., 2004; Nebbioso et al., 2005; Rosato et al., 2003a; Shetty et al., 2005; Singh et al., 2005; Vanoosten et al., 2005). The sensitization of TRAIL-resistant cells appears to be due to downregulation of the antiapoptotic protein Bcl-2, Bcl-X_L, and Mcl-1, and upregulation of proapoptotic genes *Bax*, *Bak*, *TRAIL*, *Fas*, *FasL*, *DR4*, and *DR5*, and activation of caspases. HDAC inhibitors upregulate proapoptotic genes in cancer cells but not in normal cells (Insinga et al., 2005a, b). Sodium butyrate and TSA enhanced TRAIL-mediated apoptosis to a greater extent than depsipeptide, MS-275, and oxamflatin (Vanoosten et al., 2005). Both sodium butyrate and TSA treatment also increased mRNA and surface expression of TRAIL-R2/DR5 that was dependent on the transcription factor Sp1, thus providing a possible mechanism behind the increased sensitivity to TRAIL. These results show that sensitivity to HDAC inhibitors in cancer cells is a property of the fully transformed phenotype and depends on activation of a specific death pathway. Since HDAC inhibitors sensitize TRAIL-resistant cancer cells to undergo apoptosis by TRAIL, they appear to be promising candidates for combination chemotherapy.

Several studies have demonstrated the engagement of mitochondria during activation of death receptor pathway (Debatin and Krammer, 2004; Sartorius et al., 2001; Shankar et al., 2005b; Suliman et al., 2001). Cross talk between the death-receptor (extrinsic) and mitochondrial (intrinsic) pathways requires caspase-8/caspase-10-dependent cleavage of Bid (Fandy et al., 2005; Shankar et al., 2005b; Singh et al., 2005; Suliman et al., 2001). tBid activates Bax and Bak to release cytochrome c and other mitochondrial proteins (Luo et al., 1998; Wei et al., 2000). Since HDAC inhibitors induced cleavage of Bid, the truncated Bid may trigger activation of mitochondria in the absence of ligand TRAIL. We have shown that the pan-caspase inhibitor z-VAD-fmk completely inhibited TRAIL-induced apoptosis in the presence of HDAC inhibitor (Fandy et al., 2005; Shankar et al., 2005b; Singh et al., 2005). The caspase-8 inhibitor z-IETD and DN-FADD completely inhibited the synergistic interaction between HDAC inhibitor and TRAIL. Furthermore, in the presence of HDAC inhibitors, TRAIL induced caspase-3 and caspase-9 activation and caused cleavage of their substrate poly(ADP-ribose)

polymerase (PARP). Antiapoptotic proteins Bcl-2 and Bcl-X_L inhibit HDAC inhibitors and/or TRAIL-induced apoptosis by blocking cytochrome c release. The phosphorylation deficient mutant of Bcl-2 and Bcl-X_L also blocked HDAC inhibitors and/or TRAIL-induced apoptosis. In cell-intrinsic pathway of apoptosis, mitochondria amplify the apoptotic signals leading to activation of caspase-9 (Kandasamy et al., 2003). Caspase-9 in turn activates downstream caspases and the cleavage of apoptotic substrates that finally kill cells. The synergistic effects of HDAC inhibitors and TRAIL on apoptosis occur through activation of downstream caspase-3, which can be activated by both extrinsic and intrinsic pathways (Fandy et al., 2005; Shankar et al., 2005b; Singh et al., 2005).

The sensitization of cancer cells to HDAC inhibitors appears to be p53 independent. We have recently shown that chemotherapeutic drugs (Singh et al., 2003) or irradiation (Shankar et al., 2004a, b) can sensitize breast and prostate cancer cells by upregulating death receptors DR4 and/or DR5 in cells harboring wild-type (MCF-7) and mutated (MDA-MB-231 and MDA-MB-468) p53. Recent studies have shown that HDAC inhibitors induce apoptosis in leukemia in a p53-independent manner but not in normal hematopoietic progenitors (Insinga et al., 2005b; Nebbioso et al., 2005). Other transcription factors such as NF-κB and SP1 have been shown to regulate the expression of death receptors (Chen et al., 2003; Keane et al., 1999; Nagane et al., 2000; Ravi et al., 2001).

Treatment of nude mice with HDAC inhibitors resulted in acetylation of histone H3 and H4, and downregulation of hypoxia-inducible factor 1-alpha and VEGF expression in tumor cells. Furthermore, control mice demonstrating increased rate of tumor growth had increased numbers of CD31-positive or von Willebrand Factor (vWF)-positive blood vessels, and increased circulating vascular VEGFR2-positive endothelial cells compared to HDAC inhibitor and/or TRAIL-treated mice. Sequential treatments of athymic nude mice with HDAC inhibitors followed by TRAIL cause a synergistic apoptotic response through activation of caspase-3 and caspase-7, which is accompanied by regression of tumor growth, inhibition of angiogenesis, and enhancement of survival of xenografted nude mice. Together with our previous studies showing that cancer chemotherapeutic drugs and irradiation upregulate DR4 and/or DR5 expression, thereby enhancing TRAIL-induced apoptosis in vivo (Chinnaiyan et al., 2000; Shankar et al., 2004b, 2005a; Singh et al., 2003), these studies demonstrate the antitumor interactions of HDAC inhibitors with the TRAIL death-receptor pathway. Similarly, several recent studies including ours have demonstrated the additive or synergistic effects of HDAC inhibitors and TRAIL on apoptosis in vitro (Facchetti et al., 2004; Fandy et al., 2005; Goldsmith and Hogarty, 2005; Inoue et al., 2004; Nebbioso et al., 2005; Neuzil et al., 2004; Rosato et al., 2003a; Shankar et al., 2005b; Shetty et al., 2005; Singh et al., 2005; Zhang et al., 2003). The ability of HDAC inhibitors to sensitize cancer cells to TRAIL suggests that HDAC inhibitors can reduce the minimal effective dose or side effects of TRAIL. Thus, these data provide the framework for clinical evaluation of HDAC inhibitors and TRAIL for the treatment of human cancer.

6 Combination of HDAC Inhibitors with Irradiation

HDAC inhibitors have been shown to radiosensitize prostate, breast, and glioma cell lines (Camphausen et al., 2004; Kim et al., 2004a; Nome et al., 2005). TSA has been shown to radiosensitize human glioblastoma U373MG and U87MG cell lines in a dose- and time-dependent manner (Kim et al., 2004a). VPA enhanced the radiosensitivity of brain tumor SF539 and U251 cell lines in vitro and U251 xenografts in vivo, which correlated with the induction of histone hyperacetylation (Camphausen et al., 2005). Similarly, MS-275 can enhance radiosensitivity of DU145 prostate carcinoma and U251 glioma cells suggesting that this effect may involve an inhibition of DNA repair (Camphausen et al., 2004). The combination of HDAC inhibitors with irradiation may be useful for the treatment of cancer and merit further investigation. Given the limited efficacy of standard treatments for patients with cancer, these data provide support for clinical trials integrating HDAC inhibitor with radiation therapy.

Caspase-2 and caspase-3 cleave HDAC4 in vitro, and caspase-3 is critical for HDAC4 cleavage in vivo during UV-induced apoptosis (Paroni et al., 2004). After UV irradiation, GFP-HDAC4 translocates into the nucleus coincidentally/immediately before the retraction response, but clearly before nuclear fragmentation. Together, these data indicate that caspases could specifically modulate gene repression and apoptosis through the proteolytic processing of HDAC4. Among molecular cell cycle-targeted drugs currently in the pipeline for testing in early-phase clinical trials, HDAC inhibitors may have therapeutic potential as radiosensitizers.

7 Combination of HDAC Inhibitors with Chemotherapeutic Drugs

Chemotherapeutic treatment with combinations of drugs is frontline therapy for many types of cancer. Combining drugs which target different signaling pathways often lessens adverse side effects while increasing the efficacy of treatment and reducing patient morbidity. It has recently been shown that HDAC inhibitors facilitate the cytotoxic effectiveness of the topoisomerase I inhibitor camptothecin in the killing of tumor cells (Bevins and Zimmer, 2005). SAHA has been shown to act as a chemopreventive agent in mammary tumors in the rat (Cohen et al., 1999) and inhibited the growth of established tumors (Butler et al., 2000; Chinnaiyan et al., 2005a; Cohen et al., 1999). SAHA and sodium butyrate interacted synergistically with camptothecin in inducing apoptosis of breast and lung cancer cell lines. Experiments have shown that cells arrested in G2-M by camptothecin were most sensitive to subsequent addition of HDAC inhibitor. In camptothecin-arrested cells, sodium butyrate decreased cyclin B levels, as well as the levels of the antiapoptotic proteins XIAP and survivin. Overall, these findings suggest that reducing the levels

of these critical antiapoptotic factors may increase the efficacy of camptothecin in the clinical setting if given in a sequence that does not prevent or inhibit tumor cell progression through the S phase.

MS-275 also synergistically interacted with fludarabine in inducing apoptosis of human lymphoid and myeloid leukemia cells (Maggio et al., 2004). Prior exposure of Jurkat lymphoblastic leukemia cells to MS-275 increased mitochondrial injury, caspase activation, and apoptosis in response to fludarabine, resulting in highly synergistic antileukemic interactions and loss of clonogenic survival. Simultaneous exposure to MS-275 and fludarabine also led to synergistic effects, but these were not as pronounced as observed with sequential treatment. Similar interactions were noted in the case of (a) other human leukemia cell lines (e.g., U937, CCRF-CEM); (b) other HDAC inhibitors (e.g., sodium butyrate); and (c) other nucleoside analogues (e.g., 1-beta-D-arabinofuranosylcytosine, gemcitabine). Potentiation of fludarabine-induced apoptosis by MS-275 was associated with acetylation of histones H3 and H4, downregulation of the antiapoptotic proteins XIAP and Mcl-1, enhanced cytosolic release of proapoptotic mitochondrial proteins (e.g., cytochrome c, Smac/DIABLO, and AIF), and caspase activation. These events were accompanied by the caspase-dependent downregulation of $p27^{/KIP1}$, cyclins A, E, and D1, and cleavage and diminished phosphorylation of retinoblastoma protein. Prior exposure to MS-275 attenuated fludarabine-mediated activation of MEK1/2, extracellular signal-regulated kinase, and Akt, and enhanced c-Jun NH(2)-terminal kinase phosphorylation; furthermore, inducible expression of constitutively active MEK1/2 or Akt significantly diminished MS-275/fludarabine-induced lethality. Combined exposure of cells to MS-275 and fludarabine was associated with a significant increase in generation of ROS; moreover, both the increase in ROS and apoptosis were largely attenuated by coadministration of the free radical scavenger L-*N*-acetylcysteine. Finally, prior administration of MS-275 markedly potentiated fludarabine-mediated generation of the proapoptotic lipid second messenger ceramide. Taken together, these findings indicate that MS-275 induces multiple perturbations in signal transduction, survival, and cell cycle regulatory pathways that lower the threshold for fludarabine-mediated mitochondrial injury and apoptosis in human leukemia cells.

A synergistic interaction of retinoic acid and CBHA was shown in a mouse model of neuroblastoma. DNA hypomethylating agents have been found to have synergistic effects with HDAC inhibitors. The combination of TSA with azacytidine caused a dramatic potentiation in the activation of silenced genes (Baylin and Bestor, 2002; Baylin et al., 2001; Chen et al., 1997). Depsipeptide and TSA induced apoptosis in human lung cancer cells. HDAC inhibitor-induced apoptosis was greatly enhanced in the presence of the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine, suggesting the DNA methylation status plays an important role on the effectiveness of HDAC inhibitors (Zhu et al., 2001). Furthermore, HDAC inhibitors enhanced paclitaxel-induced cell death in ovarian cancer cell lines independent of p53 status (Chobanian et al., 2004). Similarly, commonly used anticancer drugs doxorubicin and decitabine have been reported to have synergistic effects with HDAC inhibitors (Blagosklonny et al., 2000; Gozzini and Santini, 2005).

Thus, the combination of anticancer drugs with other epigenetic therapies provides potentially safer therapeutic options.

8 Chemoprevention by HDAC Inhibitors

In recent years, the use of naturally occurring chemopreventive agents have attracted many investigators because of their nontoxic effects. The preclinical data on selected chemopreventive agents have been very promising. Evidence indicates that a diet high in fresh fruits and vegetables decreases risk of certain cancers because they contain fiber, folate, and vitamins with antioxidant activity (Howe et al., 1992; Janne and Mayer, 2000). Studies have shown that the dietary fiber provides a protective effect against colon cancer (Howe et al., 1992; Trock et al., 1990). It appears that the fermentation of dietary fiber in the lumen of the colon produces the short chain fatty acid *n*-butyrate, which has anticarcinogenic activity on a variety of cellular functions, including differentiation, motility, invasion, adhesion, proliferation, and apoptosis. There is a positive correlationship between high fecal butyrate levels and decrease tumor incidence and tumor growth (Cassidy et al., 1994; Hylla et al., 1998; McIntyre et al., 1993). Butyrate is a physiological regulator of colonic epithelial cell proliferation, differentiation, and survival; and it induces histone hyperacetylation and inhibits methylation (de Haan et al., 1986; Riggs et al., 1977). Butyrate induces expression of $p^{21/WAF1/CIP1}$ through a process involving histone hyperacetylation and recruitment of Sp3 to the proximal p21 promoter (Sowa et al., 1999), and p21 is required for butyrate-mediated growth arrest in colon carcinoma cells (Archer et al., 1998). Although p21 is a p53 target gene, p21 induction by butyrate and other HDAC inhibitors is p53-independent (Xiao et al., 1997). Thus, HDAC inhibitors can induce p21-associated growth arrest in the absence of wild-type p53 function.

Sulforaphane (SFN), a compound found at high levels in broccoli and broccoli sprouts, is a potent inducer of phase 2 detoxification enzymes and inhibits tumorigenesis in animal models. SFN also has a marked effect on cell cycle checkpoint controls and cell survival and/or apoptosis in various cancer cells. SFN dose-dependently increased the activity of a β-catenin-responsive reporter, without altering β-catenin or HDAC protein levels (Myzak et al., 2004). SFN inhibits HDAC activity in colon and prostate cancer cells (Myzak et al., 2005). The inhibition of HDAC was accompanied by an increase in acetylated histones. SFN caused enhanced interaction of acetylated histone H4 with the promoter region of the $p^{21/WAF1/CIP1}$ gene and the bax gene. SFN induced cell cycle arrest and apoptosis through caspase activation. These findings provide new insight into the mechanisms of SFN action in benign prostate hyperplasia, and they suggest a novel approach to chemoprotection and chemotherapy of prostate cancer through the inhibition of HDAC.

In summary, several reports have described butyrate, diallyl disulfide, and SFN as HDAC inhibitors, and many other dietary agents likely will be discovered to attenuate HDAC activity. Dietary HDAC inhibitors, as weak ligands, regulate the

expression of genes involved in cell growth, differentiation, and apoptosis. An important question is the extent to which dietary HDAC inhibitors, and other dietary agents that affect gene expression via chromatin remodeling, modulate the expression of genes so that cells can respond most effectively to external stimuli and toxic insults.

9 Clinical Trials with HDAC Inhibitors

Phase I and II clinical trials indicate that HDAC inhibitors from several different structural classes are very well tolerated and exhibit clinical activity against a variety of human malignancies; however, the molecular basis for their anticancer selectivity remains largely unknown. Furthermore, HDAC inhibitors have also shown preclinical promise when combined with other therapeutic agents, and innovative drug delivery strategies, including liposome encapsulation, may further enhance their clinical development and anticancer potential. An improved understanding of the mechanistic role of specific HDACs in human tumorigenesis, as well as the identification of more specific HDAC inhibitors, will likely accelerate the clinical development and broaden the future scope and utility of HDAC inhibitors for cancer treatment.

Several HDAC inhibitors (SAHA, MS-275, CI-994, and depsipeptide) are currently undergoing clinical trials (Blanchard and Chipoy, 2005; Hess-Stumpp, 2005; Kelly et al., 2005). HDAC inhibitors represent a relatively new group of targeted anticancer compounds, which are showing significant promise as agents with activity against a broad spectrum of neoplasms, at doses that are well tolerated by cancer patients. SAHA is most advanced in development, currently in phase I and II clinical trials for patients with both hematologic and solid tumors (Kelly et al., 2005). Clinical trials on depsipeptide alone have shown low toxicity and evidence of antitumor activity (Sandor et al., 2002). Additionally, the compound has potential for synergism with radiotherapy, chemotherapy, and biologicals. Second-generation HDAC inhibitors, such as LAQ824 and PDX101, are currently under phase I clinical trials. Simultaneously, synthetic benzamide-containing HDAC inhibitors, CI-994 and MS-275, have reached phase I and II clinical trials, respectively.

10 Conclusions

Epigenetic modifications causing gene transcriptional repression have been associated with malignant transformation and are intriguing new targets in the treatment of cancer. In contrast to genetic deletions causing irreversible loss of gene function, epigenetic gene silencing mediated by DNA methylation and histone deacetylation can be reversed via pharmacologic inhibition of DNA methyltransferases and HDACs, respectively. When this occurs, normal patterns of gene expression, cell

differentiation, and apoptosis may be restored and disease response obtained. The HDAC has been considered an attractive target molecule for cancer therapy. The inhibition of HDAC activity by a specific inhibitor induces growth arrest, differentiation, and apoptosis of several cancer cells.

Our studies have shown that HDAC inhibitors upregulate proapoptotic members of Bcl-2 family and death receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5), and downregulate antiapoptotic genes of Bcl-2 family; thus it is possible that sensitization of cancer cells to chemotherapy, irradiation, or TRAIL by HDAC inhibitors may occur at various stages of apoptotic pathways. Furthermore, the ability of HDAC inhibitors to inhibit angiogenesis may further affect tumor growth by regulating angiogenesis-related signaling pathways. Preliminary studies in animal models have revealed a relatively high tumor selectivity of HDAC inhibitors, strengthening their promising potential in cancer chemotherapy. Some of these inhibitors are undergoing phase I and phase II clinical trials. Furthermore, the combination of HDAC inhibitors with commonly used anticancer drugs, irradiation, or TRAIL will be useful for cancer therapy. Since the HDAC inhibitors are frequently used in epigenetic studies and are considered to be promising anticancer drugs, these findings will have implications in both laboratory and clinical settings.

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