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Review paper

Histone deacetylase inhibitors in cancer treatment

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Histone deacetylase (HDAC) inhibitors are emerging as an exciting new class of potential anticancer agents for the treatment of solid and hematological malignancies. In recent years, an increasing number of structurally diverse HDAC inhibitors have been identified that inhibit proliferation and induce differentiation and/or apoptosis of tumor cells in culture and in animal models. HDAC inhibition causes acetylated nuclear histones to accumulate in both tumor and normal tissues, providing a surrogate marker for the biological activity of HDAC inhibitors *in vivo*. The effects of HDAC inhibitors on gene expression are highly selective, leading to transcriptional activation of certain genes such as the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ but repression of others. HDAC inhibition not only results in acetylation of histones but also transcription factors such as p53, GATA-1 and estrogen receptor-α. The functional significance of acetylation of non-histone proteins and the precise mechanisms whereby HDAC inhibitors induce tumor cell growth arrest, differentiation and/or apoptosis are currently the focus of intensive research. Several HDAC inhibitors have shown impressive antitumor activity *in vivo* with remarkably little toxicity in preclinical studies and are currently in phase I clinical trial. The focus of this review is the development and clinical application of HDAC inhibitors for the treatment of cancer. [© 2002 Lippincott Williams & Wilkins.]

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Chromatin topology

Acetylation and deacetylation of nucleosome core histones play important roles in the modulation of chromatin structure and the regulation of gene transcription. Transcriptionally active genes are associated with highly acetylated core histones, whereas transcriptional repression is associated with low levels of histone acetylation.[1–3] The acetylation status of histones is controlled by the activities of two families of enzymes, the histone acetyltransferases (HATs) and histone deacetylases (HDACs).[4,5] The substrates for HATs and HDACs include ϵ-amino groups of lysine residues in the *N*-terminal regions of histones. In the nucleosome, positively charged hypoacetylated histones bind tightly to the phosphate backbone of DNA and maintain chromatin in a transcriptionally silent state by inhibiting access of transcription factors, transcriptional regulatory complexes and RNA polymerases to DNA. Acetylation neutralizes the positive charge on histones to allow a more open conformation of chromatin, thereby enhancing access of transcription factors and the transcription apparatus to promoter regions of DNA. Conversely, histone deacetylation restores a positive charge to lysine residues, condensing the chromatin into a tightly supercoiled conformation.[6–8]

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HATs and HDACs

Nuclear receptor co-activators have intrinsic HAT activity, whereas co-repressor proteins exist in large complexes with HDAC enzymes.[5,9,10] There are at least four groups of proteins with HAT activity and three classes of HDAC enzymes that have been identified in mammalian cells to date. Co-activator proteins with intrinsic HAT activity include GCN5, which is closely related to the yeast HAT GCN5;[11] the cyclic adenosine monophosphate response element (CREB)-binding protein CBP/p300 and p300/CBP-associated factor (P/CAF);[12] TAFII p250, a component of the basic transcription complex TFIIE;[13] and SRC-1 and ACTR, which are co-activators for ligand-dependent nuclear receptors.[14] Mammalian HDAC enzymes can be divided into three classes based on structural and functional homology with yeast counterparts as described in Table 1. The first class

Table 1. The human HDAC family

Class	Enzyme	Protein length (amino acids)	Catalytic domains	Mechanism of deacetylase activity	Subcellular localization	Tissue expression
I (RPD3-like)	HDAC1	482	one	Zn^{2+} dependent	nuclear	ubiquitous
	HDAC2	488	one	Zn^{2+} dependent	nuclear	ubiquitous
	HDAC3	428	one	Zn^{2+} dependent	nucleocytoplasmic shuttling	ubiquitous
	HDAC8	377	one	Zn^{2+} dependent	nuclear	ubiquitous
II (HDA1-like)	HDAC4	1084	one	Zn^{2+} dependent	nucleocytoplasmic shuttling	tissue specific
	HDAC5	1122	one	Zn^{2+} dependent	nucleocytoplasmic shuttling	tissue specific
	HDAC6	1215	two	Zn^{2+} dependent	nucleocytoplasmic shuttling	tissue specific
	HDAC7	unknown	one	Zn^{2+} dependent	nucleocytoplasmic shuttling?	tissue specific?
III (SIR2-like)	SIRT1	747	one	NAD^{+} dependent	Unknown	unknown
	SIRT2	373	one	NAD^{+} dependent	Unknown	unknown
	SIRT3	399	one	NAD^{+} dependent	Unknown	unknown
	SIRT4	314	one	NAD^{+} dependent	Unknown	unknown
	SIRT5	310	one	NAD^{+} dependent	Unknown	unknown
	SIRT6	355	one	NAD^{+} dependent	Unknown	unknown
	SIRT7	400	one	NAD^{+} dependent	Unknown	unknown

of deacetylases comprises HDAC1, HDAC2, HDAC3 and HDAC8, which are homologs of the yeast transcriptional regulator RPD3.[15–18] HDAC4, HDAC5, HDAC6 and HDAC7 are homologs of yeast HDA1, and form a second class of deacetylases that are specifically involved in cell differentiation.[19–24] The third class of HDACs is the sirtuins which are homologous to the yeast SIR2 family of proteins. There are seven human sirtuins (SIRT1–7) which require nicotinamide adenine dinucleotide (NAD) for deacetylase activity in contrast to the zinc-catalyzed mechanism used by class I and II HDACs.[25,26]

Regulation of gene expression

HATs are involved in the activation of gene transcription. Conversely, transcriptional co-repressors inhibit gene expression at least in part by recruitment of HDAC complexes to gene promoters. Early studies indicated that HDAC1 and HDAC2 are components of two large multisubunit protein complexes termed the mSin3 and Mi2 complexes. The co-repressors N-CoR (Nuclear Co-Repressor) and SMRT (Silencing Mediator for Retinoid and Thyroid receptors) interact with HDAC1 and HDAC2 through the mSin3 complex.[27–30] This HDAC–mSin3 complex can then be recruited to a variety of transcription factors including unliganded nuclear receptors,[27,29] MeCP2 (Methyl-CPG-binding protein 2)[31–33] and p53.[34] Other proteins interact with HDAC1 and HDAC2 through the Mi2 or NuRD (Nucleosome-Remodeling Histone Deacetylase) complex which is implicated in transcriptional repression by DNA methylation.[35] In addition to deacetylation of core histones, HDACs can down-regulate gene expression by deacetylating transcription factors such as p53,[36] GATA-1,[37] TFIIE and TFIIF,[38] and estrogen receptor (ER)-α.[39,40] HDAC1 and HDAC2 also participate in cell cycle-specific transcriptional regulation by binding the E2F transcription factor to repress transcription through an association with retinoblastoma (Rb) protein.[41,42]

The complex interactions of other members of the HDAC family are only beginning to be understood. For example, the nuclear target of HDAC4 is the human myocyte enhancer factor 2 (MEF2). MEF2 plays a role in differentiation of smooth and skeletal muscle, and MEF2-dependent transcription is silenced by deacetylase activity of HDAC4.[43] In contrast to the class I HDACs which are exclusively located in the nucleus, the class II enzymes HDAC4 and HDAC6 are predominantly cytoplasmic but shuttle between cytoplasm and nucleus in response to cellular differentiation signals.[22,43]

HDACs and cancer

Direct alterations in the HDAC genes have not been demonstrated in human cancers, but HDACs associate with several well-characterized oncogenes and tumor suppressor genes.[35] In proliferating cells, Myc/Max heterodimers activate the transcription of genes that stimulate cellular proliferation such as E2F which are regulated by E-box elements in their promoters.[44] However, in differentiating cells Mad replaces Myc and the Mad/Max heterodimer re-

presses growth stimulatory genes through an association with the HDAC–mSin3 complex.[45] The most common alteration in this pathway in human tumors is overexpression of Myc which prevents formation of Mad/Max heterodimers, thereby blocking Mad-mediated transcriptional repression.[46] c-Ski is a component of the HDAC–NCoR–mSin3 complex and its oncogenic form, v-Ski, is able to transform cells by blocking Mad-mediated repression in the absence of Myc overexpression.[47] This suggests that disruption of the HDAC–mSin3 complex is an important factor in oncogenesis.

Although Myc overexpression or abnormalities in the Myc/Mad pathway are common in human cancers, they do not occur in all tumors. However, the Rb/E2F cell cycle regulatory pathway is disrupted in almost every human tumor.[48] Rb interacts with class I HDACs through a complex variety of associations and there are many genetic alterations that disrupt this interaction in cancer. For example, deletions or mutations in the Rb gene are common in many solid tumors,[35] cervical cancer is often associated with human papilloma viruses expressing E7 oncoprotein which binds Rb and prevents its interaction with E2F and HDAC,[41] and there are genetic abnormalities that indirectly result in constitutive Rb phosphorylation and inactivation.[49] For both Mad and Rb, transcriptional repression is central to arrest of cell proliferation and the capacity to repress transcription is dependent on association of these proteins with HDAC.

Gene silencing by HDAC complexes is an important mechanism in the development of certain types of leukemia, most notably acute promyelocytic leukemia (APL).[50] APL is most commonly associated with a chromosomal translocation t(15;17) that results in fusion between the genes encoding the retinoic acid receptor (RAR)-α and the transcription factor PML. The resultant PML–RAR-α fusion protein is an oncoprotein that represses gene transcription by recruitment of HDAC to RAR-regulated genes, causing a maturational block in the myeloid line.[51,52] RAR-α binds to DNA as a heterodimer with the retinoid X-receptor (RXR). In the absence of retinoic acid, the RAR-α/RXR heterodimer binds a transcriptional co-repressor NCoR–mSin3–HDAC complex. In the presence of retinoic acid, the co-repressor complex is displaced and replaced by a co-activator complex with HAT activity.[51] However, in the PML–RAR-α fusion protein, RAR-α is no longer responsive to physiological concentrations of retinoic acid. In these circumstances RAR-α becomes a constitutive transcriptional repressor which blocks normal differentiation of promyelocytes, leading to APL. Pharmacological doses of all-*trans*-retinoic acid (ATRA) can overcome this block and induce differentiation of the malignant cell. However, APL eventually becomes resistant to further retinoid treatment. HDAC inhibition can overcome this resistance and restore sensitivity of APL cells to the differentiating effects of ATRA.[50,51,53] In a smaller subset of APL patients, a similar translocation t(11;17) results in a fusion between the same coding region of RAR-α to a different protein called PLZF (Promyelocytic Leukemia Zinc Finger). PLZF–RAR-α is completely insensitive to ATRA; this is thought to be due to an interaction domain within the PLZF protein that can bind the NCoR–mSin3–HDAC complex. The PLZF–RAR-α fusion protein therefore interacts with co-repressors through two domains, one of which is not sensitive to retinoic acid. However, HDAC inhibition can restore sensitivity of PLZF–RAR-α to retinoic acid and allow the leukemic cells to differentiate in response to ATRA.[50,51,53]

There is also evidence implicating aberrant HDAC-mediated transcriptional repression in lymphoma and some forms of acute myeloid leukemia (AML). For example, in non-Hodgkin's lymphoma the BCL6 transcriptional repressor is overexpressed, resulting in malignant transformation of lymphoid cells.[54] In the M2 subtype of AML, a t(8;21) translocation disrupts the AML1 gene, resulting in a fusion between the DNA-binding domain of AML1 and a second protein ETO.[55] Unlike the AML1 protein which normally functions in a complex to activate expression of genes required for myeloid differentiation,[56] AML1–ETO is a potent transcriptional repressor by recruitment of HDAC to the transcriptional repressor complex.[57] Treatment with an HDAC inhibitor can relieve ETO-mediated transcriptional repression and induce differentiation of AML1–ETO cells.[58] These observations underscore the importance of HDAC-mediated gene silencing in human oncogenesis and suggest the potential utility of HDAC inhibitors for the treatment of hematological malignancies.

HDAC inhibitors

A number of structurally dissimilar inhibitors of HDAC have been identified, many of which are natural products. Broadly speaking, these fall into the following categories based on the chemical structure of the compounds as depicted in Figure 1: (i) hydroxamic acids, e.g. trichostatin A

(TSA),[59,60] oxamflatin,[61] suberoylanilide hydroxamic acid (SAHA);[62] (ii) cyclic tetrapeptides containing the epoxyketone structure (2*S*,9*S*)-2-amino-8-oxo-9,10-epoxy-decanoyl (Aoe), e.g. trapoxin A and trapoxin B,[63] Cyl-1 and Cyl-2,[64] HC-toxin,[65] WF-3161,[66] chlamydocin;[67] (iii) cyclic peptides without Aoe, e.g. depsipeptide (FK228, formerly known as FR901228),[68] apicidin;[69] (iv) benzamides, e.g. MS-275 (previously called MS-27-275);[70] (v) short-chain and aromatic fatty acids, e.g. sodium butyrate,[71] sodium phenylbutyrate;[72] and (vi) miscellaneous compounds, e.g. depudecin.[73]

TSA and its glucopyranosyl derivative trichostatin C were first isolated from the culture broth of *Streptomyces hygroscopicus* as antifungal antibiotics active against *Trichophyton* species.[59,74] Several years later, the trichosatins were found to exhibit potent differentiating and antiproliferative properties at nanomolar concentrations in cultures of Friend erythroleukemia cells.[75] Although compounds such as dimethylsulfoxide and sodium butyrate were known to induce differentiation in this cell line, TSA was orders of magnitude more potent.[76] Stereoselective synthesis and analysis of the enantiomers of TSA indicated that the naturally occurring configuration is (*R*)-TSA and that (*S*)-TSA is biologically inactive.[77] The extremely potent biological activity and structural specificity of (*R*)-TSA pointed towards it binding to a specific molecular target. Subsequent studies showed that TSA was also active in various normal and tumor cell lines, arresting growth of rat fibroblast cells in both G_1 and G_2 phases of the cell cycle.[78] Nuclear histones from cells treated with TSA were found to be highly acetylated and pulse–chase analysis indicated that this was not due to increased acetylation but rather decreased deacetylation.[60] In experiments using partially purified mouse HDAC, TSA was a potent non-competitive inhibitor and the K_i of 3.4 nM was close to the effective antiproliferative concentration in cell lines. Furthermore, the K_i was 10-fold higher for HDAC purified from a mutant cell line resistant to TSA, confirming that HDAC was indeed the primary target of TSA.[60] Prior to the isolation of TSA, sodium butyrate was long known to be a non-competitive inhibitor of HDAC but lacked potency and specificity.[79,80] The effective concentration of butyrate for HDAC inhibition is in the millimolar range. In addition to HDAC inhibitory activity, this short-chain fatty acid also affects phosphorylation and methylation of histones and other nuclear proteins, DNA methylation, as well as having effects on the cytoskeleton and cell membrane.[71,80] The discovery of trichostatin A and characterization of its highly potent and specific HDAC inhibitory activity therefore proved to be a major advance.

Trapoxins A and B are cyclic tetrapeptides containing two L-phenylalanine residues and the epoxide group Aoe.[63] Trapoxin is a natural metabolite of the fungus *Helioma ambiens* and was first isolated as a result of its capacity to induce morphological reversion of a v-*sis*-transformed fibroblast cell line in culture.[81] At low nanomolar concentrations, trapoxin irreversibly inhibited HDAC activity in crude cell lysates and induced accumulation of hyperacetylated core histones in a number of mammalian cell lines.[63] Structural analysis indicated that the epoxide group is the only chemically reactive moiety in trapoxin. Reduction of the epoxide led to complete loss of biological activity, suggesting that trapoxin irreversibly inactivates HDAC by covalent interaction between its epoxide group and a nucleophilic active site residue in the enzyme.[63] Although HDAC activity had first been identified in crude nuclear extracts over 30 years previously, prior to the isolation of trapoxin, attempts at molecular characterization of HDAC had proved unsuccessful. However, the total syntheses of trapoxin B and K-trap, in which one of the phenylalanine residues of trapoxin is replaced by a protected lysine residue, led to the purification by affinity chromatography of two nuclear proteins whose peptide sequence was homologous to that of the yeast transcriptional regulator RPD3. Full-length cDNA encoding the catalytic subunit of human HDAC1 was then cloned, and pure recombinant HDAC1 was expressed and characterized for the first time.[15]

As a consequence of the early work on sodium butyrate, TSA and trapoxin, a number of other HDAC inhibitors have been isolated from natural sources. These include apicidin, a cyclic tetrapeptide fungal metabolite of *Fusarium* species first discovered because of its antiprotozoal activity.[69] Unlike trapoxin and other natural cyclopeptides like HC-toxin and chlamydocin, apicidin lacks the α-keto-epoxide (Aoe) moiety and has potent but reversible HDAC inhibitory activity at low nanomolar concentrations.[82] Depsipeptide (also known as FK228), isolated from *Chromobacterium violaceum*, is another cyclic peptide without Aoe that reversibly inhibits HDAC activity at micromolar concentrations.[68] Depudecin is a fungal metabolite of *Alternaria brassicicola* with an unusual chemical structure containing two epoxides and six chiral centers in an 11-carbon chain.[83,84] Depudecin has irreversible HDAC inhibitory activity at micromolar concentrations.[73]

A number of synthetic compounds and analogs of natural compounds have been developed that inhibit

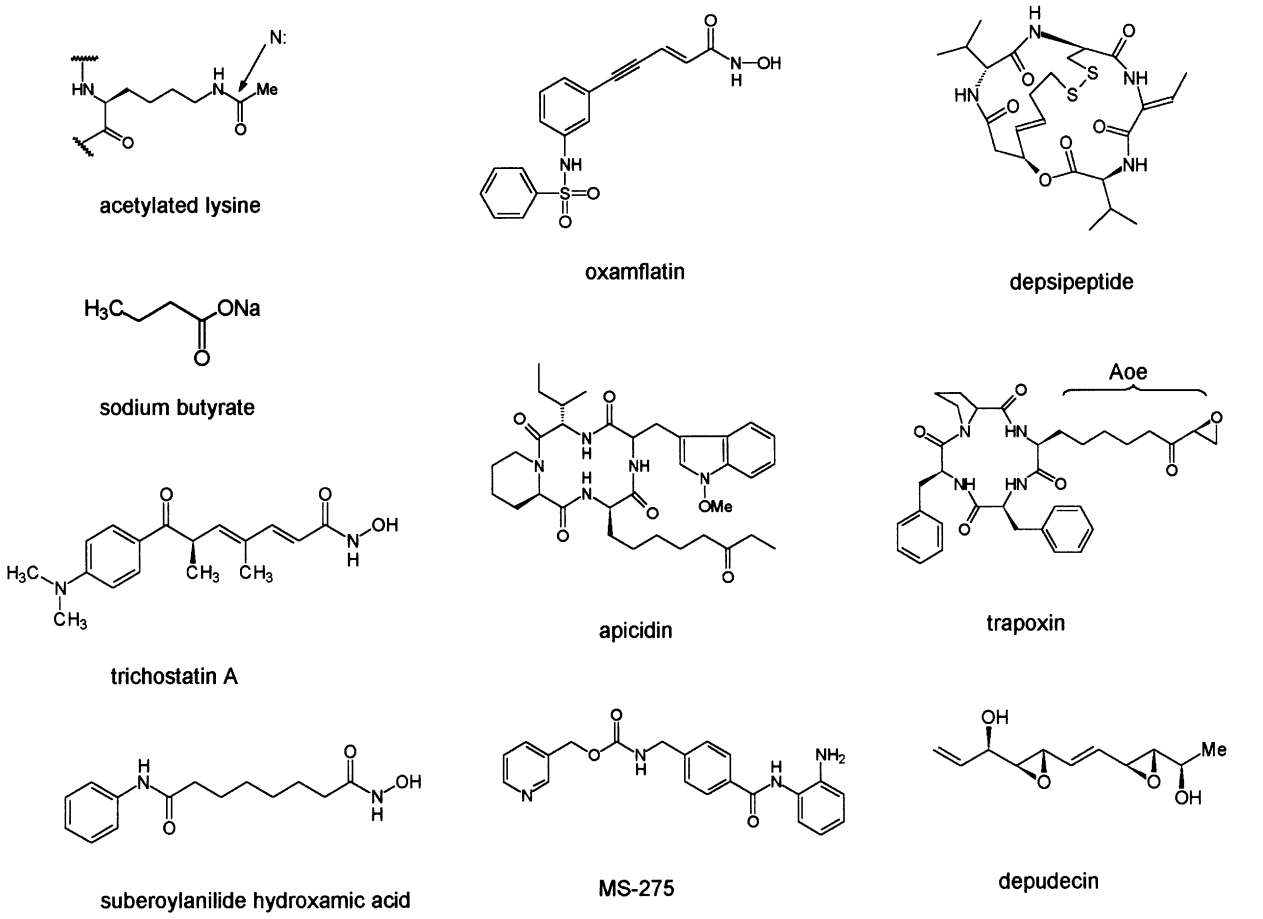

Figure 1. Chemical structures of acetylated lysine and the HDAC inhibitors discussed in the text. The hydroxamic acids TSA, SAHA and oxamflatin are reversible HDAC inhibitors. The aliphatic chain and hydroxamic acid group of each inhibitor are analogous to the lysine side chain and acetyl group of ϵ-*N*-acetylated lysine residues in nuclear histones. Chelation of a zinc atom in the catalytic pocket by the hydroxamic acid moiety is the main mechanism of enzyme inhibition. Trapoxin is a cyclic tetrapeptide inhibitor containing the functionally important amino acid Aoe. The Aoe side chain is isosteric to that of acetylated lysine and acts as a substrate mimic, alkylating active site residues in the HDAC enzyme and irreversibly inhibiting its activity.

HDAC activity. MS-275 is one of the most potent of a series of benzamide derivatives that has micromolar HDAC inhibitory activity.[70] Oxamflatin is an aromatic sulfonamide derivative with a hydroxamic acid moiety and inhibits HDAC at micromolar concentrations.[61] SAHA is the prototype in a series of synthetic hydroxamic acid-based hybrid polar compounds, also with micromolar HDAC inhibitory potency.[62] Structure–activity studies have identified the importance of the hydroxamic acid for activity in these hybrid polar compounds since substitution/modification at this site abolishes activity.[85]

The X-ray crystal structure of the HDAC catalytic core has been solved to atomic resolution by crystallization of the HDAC-like protein, HDLP.[86] HDLP is an HDAC homolog from the thermophilic bacterium *Aquifex aerolicus* that shares a 375-amino-acid region of sequence homology with human HDAC1, has deacetylase activity for histones and is inhibited by TSA. Co-crystallization studies of HDLP with TSA or SAHA have enabled the mechanism of HDAC inhibition by hydroxamic acid-based inhibitors to be characterized. The active catalytic site is formed by a tubular pocket, a zinc-binding site and two asparagine–histidine charge-relay systems. TSA and SAHA are substrate mimics; the aliphatic chain and hydroxamic acid group of each inhibitor is analogous to the lysine side chain and acetyl group of the HDAC substrate. These inhibitors bind inside the pocket by inserting the aliphatic chain into the tube and make contact with residues at the rim, walls and bottom of the pocket. Chelation of a zinc atom near the bottom of the catalytic pocket by the hydroxamic acid moiety is the main mechanism of enzyme inhibition. The

dimethylaminophenyl group of TSA serves as a cap which is required to pack the inhibitor at the rim of the tubular active site pocket. The amino acid sequences of the loops that form the active site pocket in HDLP are conserved across the HDAC family. This suggests that the deacetylase catalytic reaction and mechanism of inhibition by TSA and SAHA are the same in HDLP and in class I and class II mammalian HDAC enzymes.[86]

TSA competes with trapoxin for binding to HDAC1, suggesting that the side chain of Aoe also acts as a substrate mimic, presumably alkylating one of the conserved charge-relay histidine residues in the active site pocket of the enzyme.[15] Support for this model also derives from recent work in which a novel trapoxin analog containing a hydroxamic acid instead of the epoxyketone has been synthesized.[87] This cyclic hydroxamic acid-containing peptide (CHAP)1, a hybrid of TSA and trapoxin, reversibly inhibited HDAC1 at low nanomolar concentrations in contrast to the irreversible inhibition seen with trapoxin. Structure–activity studies indicate that the cyclic tetrapeptide portion of CHAP-based HDAC inhibitors affects potency. Whilst the short-chain fatty acid sodium butyrate is an HDAC inhibitor at millimolar concentrations, an analog of trapoxin B with a carboxylic side chain rather than the epoxyketone inhibited HDAC1 activity at 100 nM concentration. Thus the inhibitory activity of a short-chain fatty acid can be potentiated by a cyclic tetrapeptide structure, consistent with it acting as a cap which packs the inhibitor at the rim of the active site pocket.[86]

Despite the large number of HDAC inhibitors that have been described, little is known about their target enzyme specificity. There is increasing evidence to support a distinct biological role for each of the HDAC enzymes and it is likely that inhibition of specific members of the HDAC family will have specific functional consequences such as on gene expression, cell cycle regulation, proliferation, differentiation and apoptosis. To date, such isoform-specific inhibitors have not yet been developed. Studies characterizing the activities of inhibitors for HDAC1, HDAC4 and HDAC6 indicate that TSA is a relatively non-selective inhibitor of all three enzymes.[87] TSA is a simple analog of acetyl-lysine with a small cap group and is likely to inhibit both class I and class II HDACs. However, cyclic tetrapeptides such as trapoxin make extensive contact with the rim of the active site pocket and in the shallow grooves around the rim. The cyclic tetrapeptide structure may therefore mimic the substrate structure around the acetylated lysine and confer specificity. Indeed, recent studies show that HDAC6 is resistant to trapoxin and CHAP1 as well as to CHAP counterparts of other natural cyclic tetrapeptides containing Aoe. HDAC6 is unique in having two deacetylase domains.[21,88] In common with other class II HDACs, HDAC6 is located in the cytoplasm and a fraction of the protein translocates into the nucleus in response to differentiation stimuli, suggesting that its substrates include non-histone proteins.[22] Interestingly, HDAC6 inhibition by high concentrations of trapoxin is reversible, indicating that it does not alkylate the enzyme which may account for the resistance of HDAC6 to cyclic tetrapeptides containing Aoe.[87]

More recently, the X-ray crystal structure of the catalytic core of SIRT2, an NAD-dependent HDAC, has been reported.[26] In *Saccharomyces cerevisiae* and *Caenorhabiditis elegans*, the homolog Sir2 is a transcriptional repressor at telomeres and ribosomal RNA gene clusters that plays an important role in determining the life span of these organisms. The unique structure of the Sir2 class of HDACs, their requirement for NAD rather than zinc for deacetylation and insensitivity to inhibition by HDAC inhibitors such as TSA suggest that the human sirtuins may have very different biological functions to those of the class I and class II HDACs.[26]

Cellular activities of HDAC inhibitors

The biological effects of HDAC inhibitors in cultured mammalian cells include reversion of the transformed phenotype, inhibition of proliferation, cell cycle arrest in G_1 phase, and induction of differentiation and/or apoptosis in tumor cell lines. The precise mechanisms underlying these cellular responses have yet to be characterized.

Malignant transformation often results in a marked alteration in the morphology and cytoskeletal architecture of cells.[89] One of the most characteristic changes is the loss of actin stress fibers.[90] HDAC inhibitors have the capacity to re-organize the actin-containing microfilament system, revert the morphological changes following transformation of cells in culture by oncogenes such as v-*sis*, v-*src* and v-*ras*, and induce morphological and biochemical differentiation of a number of tumor cell lines.[63,91] These include murine erythroleukemia cells, human neuroblastoma, teratocarcinoma, breast and colorectal adenocarcinomas, and bladder carcinoma cell lines.[76] Detransforming activity is suppressed by actinomycin D and cycloheximide, suggesting that

both mRNA and *de novo* protein synthesis are required.[84] Gelsolin is a calcium and phosphoinositide-regulated actin-binding protein with an important role in maintaining the actin cytoskeleton. Gelsolin protein levels are reduced in many transformed cell lines and tumor tissues.[92] Levels of expression of gelsolin mRNA and protein increase in response to HDAC inhibition, and correlate with reversion of transformed morphology and a differentiated phenotype. Induction of gelsolin expression by HDAC inhibitors is likely to result from derepression of the gelsolin gene.[91,93] The relevance of gelsolin to the observed morphological changes is supported by the observation that the morphological effects of HDAC inhibitors are suppressed after microinjection of anti-gelsolin antibodies.[73]

Transcriptional repression by recruitment of HDAC activity is central to the control of cell proliferation by the Myc/Mad pathway and Rb/E2F cell cycle regulatory pathway. Inhibition of HDAC activity might therefore be predicted to prevent cell cycle arrest. Paradoxically, HDAC inhibitors do not promote cell proliferation but rather induce cell cycle arrest. The most likely explanation is that HDAC inhibitors also act upstream of Myc and E2F in the G_1 phase of the cell cycle.[35] The putative target is the tumor suppressor protein $p21^{WAF/CIP1}$, a cyclin-dependent kinase (CDK) inhibitor which binds CDKs and inhibits their activity, leading to hypophosphorylation of the pocket proteins Rb, p107 and p130, and suppression of cell proliferation.[94] Normally, $p21^{WAF/CIP1}$ is induced by p53 in response to DNA damage, hence the terminology WAF or CIP1 (Wild-type p53-Activated Factor or CDK-Inhibitor Protein-1).[95] HDAC inhibitors markedly activate transcription of $p21^{WAF/CIP1}$ which is associated with hyperacetylated histones in the chromatin of the p21 gene. Although the precise mechanism of activation is unclear, it is certainly p53-independent[96] and requires Sp-1 binding sites within the p21 gene promoter.[97,98] The favorable association between a diet high in fiber and a reduced incidence of colon cancers is thought to result from induction of $p21^{WAF/CIP1}$ mRNA expression within the colon by short-chain fatty acid products of fiber fermentation.[35,94] Studies in the human colorectal carcinoma cell line HCT116 showed that TSA was a potent inhibitor of proliferation of the parental line but a p21-deleted HCT116 cell line was relatively resistant. However, proliferation of the $p21^{-/-}$ HCT116 cells was still inhibited by TSA in a dose-dependent manner, indicating that p21 is not the sole determinant of growth inhibition by TSA, at least in this cell line.[99] Thus $p21^{WAF/CIP1}$ is thought to play an important if not determinant role in the arrest of cell growth by HDAC inhibitors.

HDAC inhibition induces accumulation of hyperacetylated histones in most regions of chromatin but only affects the expression of a small subset of genes. In differential display experiments comparing gene expression in TSA-treated and untreated human lymphoid cell lines, TSA caused a 2-fold or greater change in levels of expression of only eight out of 340 genes examined.[100] The basis for this selectivity is not known. Hyperacetylation of histones correlates with increased gene transcription but does not necessarily account for maximal activation and an increasing number of non-histone transcription factors have been identified that are also targets for acetylation. The functional effects of acetylation of non-histone proteins vary. For example, acetylation enhances activity of the tumor suppressor p53,[101,102] the Kruppel-like factor EKLF[103] and the erythroid differentiation factor GATA-1,[37] but represses transcriptional activity of T cell factor and direct acetylation of the co-activator ACTR contributes to inhibition of ligand-induced nuclear receptor signaling.[104] Recent studies in our laboratory indicate that ER-α is directly hyperacetylated in response to TSA treatment of an MCF7 breast adenocarcinoma cell line,[39] whilst another group has reported that acetylation of ER-α lysine residues in the hinge/ligand binding domain suppresses ligand sensitivity and regulates transcriptional activation by HDAC inhibitors,[40] Furthermore, conservation of the acetylated ER-α motif in other nuclear receptors suggests that direct acetylation may play an important role in the regulation of diverse nuclear receptor signaling functions.[40]

Induction of apoptosis by HDAC inhibitors has been reported in several human cancer cell lines but the mechanism underlying this effect is not well understood.[105–110] One possibility is that histone acetylation relaxes chromatin and enhances accessibility of DNA to apoptotic endonucleases.[105,108] In support of this hypothesis, cycloheximide, an inhibitor of protein synthesis, can prevent the formation of apoptotic bodies and nuclear fragmentation in Jurkat cells treated with sodium butyrate or TSA,[107] and has also been shown to inhibit apoptosis in human neuroblastoma cells treated with the HDAC inhibitor *m*-carboxycinnamic acid bishydroxamic acid.[110] Other studies suggest that HDAC inhibitor-induced apoptosis is related to effects on gene expression including p21, c-*myc* and gelsolin.[91,98,111–113] Interestingly, apoptosis of human lung cancer cells induced by TSA or depsipeptide is greatly augmented in the presence of the DNA

methyltransferase inhibitor 5-aza-2′-deoxycytidine (DAC).[114] DNA methylation status is an important determinant of chromatin structure and function.[31,32,115,116] For example, in mammals the inactive X chromosome is both hypoacetylated and hypomethylated.[117] Methylated DNA is transcriptionally repressed, and the methyl-CpG-binding protein MeCP2 forms a complex with HDAC that affects chromatin architecture and gene regulation.[31,32] DNA methylation status also influences the level of local histone acetylation.[118] DNA methylation induces hypoacetylation of chromatin and the methytransferase Dnmt1 interacts directly with HDAC to form a transcriptionally inactive chromatin structure.[119–121] Recent studies indicate that concurrent treatment of cells with TSA and DAC can restore mRNA expression of methylated tumor suppressor genes.[122] However, in ER-α breast cancer cell lines, TSA treatment alone is sufficient to re-activate transcription of the methylated ER gene.[123]

In vivo studies of HDAC inhibitors

The short-chain fatty acid sodium butyrate, aromatic fatty acids sodium phenylbutyrate and its metabolic precursor sodium phenylacetate, and the lipophilic butyric acid analog, pivaloyloxymethylbutyrate have all been tested in animal tumor models.[71,124] They are weak HDAC inhibitors active at millimolar concentrations and have limited efficacy *in vivo* against solid tumors and leukemias.[71,125]

A number of more potent HDAC inhibitors have *in vivo* activity with little or no toxicity in experimental models. Depsipeptide increases survival of mice bearing murine ascitic leukemias and melanoma, and inhibits growth of solid tumors including murine colon carcinoma, reticulum cell sarcoma and fibrosarcoma in normal mice, and human lung carcinoma and stomach adenocarcinomas xenografts implanted in nude mice, respectively.[126] Depsipeptide partly inhibits the growth of human APL in immunodeficient mice. The antitumor activity of depsipeptide and ATRA is syngergistic in this model, and combination treatment also enhances survival.[127] Oxamflatin has been shown to increase the survival of mice bearing transplanted murine melanoma tumors without observable toxicity.[61] The benzamide derivative MS-275 administered orally effectively inhibits growth of several human tumor xenografts implanted in nude mice, including leukemias, colorectal, gastric, ovarian and pancreatic carcinomas. At high doses of MS-275 there are signs of toxicity including weight loss and poor general appearance, but the compound still has excellent antitumor activity without toxicity at half the maximum tolerated dose.[70] TSA was reported to be inactive *in vivo* against a human melanoma xenograft nude mouse model,[128] but insolubility of TSA in the aqueous vehicle used for administration may account for the apparent lack of activity observed in this model. In contrast, our group has shown that TSA has potent antitumor activity without toxicity *in vivo* in the *N*-methyl-*N*-nitrosourea carcinogen-induced rat mammary cancer model and e*x vivo* histopathological studies indicate that induction of differentiation is the likely mechanism of antitumor activity.[39] Other investigators have reported that SAHA or related hydroxamic acid-based hybrid polar compounds have antitumor activity *in vivo* against human melanoma[128] and androgen-independent prostate tumor xenograft models without toxicity,[129] as well as preventive activity in carcinogen-induced rat mammary[130] and murine lung carcinoma models.[129] The cyclic hydroxamic acid-containing peptide CHAP31 is a potent inhibitor of transplanted murine melanoma growth in mice, and has antitumor activity against human breast, lung, melanoma and gastric tumor xenografts in nude mice. CHAP31 is considerably more stable in cell culture than either TSA or trapoxin, and effective plasma concentrations are maintained for several hours after i.v. administration in the rat. However, the therapeutic index of CHAP31 is low and significant weight loss occurs at effective antitumor doses, thus limiting its potential for clinical drug development.[131]

Despite their limitations of low potency and lack of selectivity, Phase I clinical and pharmacokinetic studies of short-chain and aromatic fatty acids have been performed in cancer patients. Following i.v. or oral administration, millimolar or high micromolar peak plasma concentrations near to those effective *in vitro* can be achieved without significant adverse effects.[132,133] Sodium butyrate has been used to induce partial remission of acute myeloid leukemia[134] and sodium phenylbutyrate in combination with ATRA has successfully induced remission in a patient with relapsed APL that had become refractory to retinoids alone.[72] Treatment with HDAC inhibitors can increase the accumulation of hyperacetylated nuclear histones in tumor tissue, and in normal tissues such as bone marrow and peripheral blood mononuclear cells. Detection of acetylated histones can therefore be a useful surrogate marker of HDAC inhibitor activity *in vivo.* Hyperacetylation of histones was detectable in bone marrow and peripheral blood mononuclear cells during treatment of the

patient with sodium phenylbutyrate.[72] However, the response in this patient was short-lived and four subsequent patients with APL failed to respond to concurrent treatment with sodium phenylbutyrate and ATRA. Depsipeptide,[68,135] MS-275, SAHA and a second hybrid polar hydroxamic acid-based HDAC inhibitor, pyroxamide,[136] are all currently in phase I clinical trials.

The impressive antitumor activity of HDAC inhibitors *in vivo* in animal models and lack of toxicity at doses that effectively inhibit tumor growth strongly support HDAC as a suitable molecular target for anticancer drug development. Many questions still remain unanswered regarding the optimal evaluation and utilization of HDAC inhibitors for cancer prevention and treatment. The maximum tolerated dose which is commonly used as an endpoint in phase I studies of cytotoxic agents may not be appropriate to evaluate the toxicity of HDAC inhibitors which are predominantly cytostatic. Furthermore, tumor regression may not be a suitable biological endpoint for clinical studies of antitumor efficacy.

Pharmacodynamic surrogates such as histone hyperacetylation in peripheral mononuclear cells and pharmacokinetic endpoints that relate to inhibition of the target in preclinical animal models may be a better guide to the clinical plasma levels required. Further work is also necessary to determine the clinical utility of combination therapy with an HDAC inhibitor and hypomethylating agent such as DAC, or with a cytotoxic chemotherapeutic drug. Nonetheless, it is clear that HDAC inhibitors are emerging as an exciting new class of relatively non-toxic drugs that are potentially useful for the treatment of solid and hematological malignancies.

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