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REVIEW

HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics

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Histone deacetylases (HDACs) and histone acetyl transferases (HATs) are two counteracting enzyme families whose enzymatic activity controls the acetylation state of protein lysine residues, notably those contained in the N-terminal extensions of the core histones. Acetylation of histones affects gene expression through its influence on chromatin conformation. In addition, several non-histone proteins are regulated in their stability or biological function by the acetylation state of specific lysine residues. HDACs intervene in a multitude of biological processes and are part of a multiprotein family in which each member has its specialized functions. In addition, HDAC activity is tightly controlled through targeted recruitment, protein-protein interactions and post-translational modifications. Control of cell cycle progression, cell survival and differentiation are among the most important roles of these enzymes. Since these processes are affected by malignant transformation, HDAC inhibitors were developed as antineoplastic drugs and are showing encouraging efficacy in cancer patients.

Keywords: histone deacetylase, histone, post-translational modification, transcription, histone deacetylase inhibitors, protein acetylation

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Introduction

Core histones have long N-terminal extensions that have been known for decades to undergo extensive post-translational modifications such as acetylation, methylation and phosphorylation, as well as ubiquitination, sumoylation and ADP-ribosylation [1-5]. Interestingly, Vincent Alfrey *et al*. [6] proposed more than 40 years ago that modifications such as acetylation could have functional roles by modulating transcription efficiency, but it took about 30 years for the scientific community to follow-up on these observations and to establish a fundamental role for chromatin and its post-translational modification states in the epigenetic regulation of many processes impinging on DNA, such as transcription. The present review will focus on the histone deacetylase (HDAC) family of chromatin-modifying enzymes. Aberrant regulation of gene expression is at the basis of many human diseases and notably of many forms of cancer. Yet, attempts to pharmacologically target transcription complexes have been frustrated by the difficulty to disrupt protein-protein interactions with small molecules. The discovery that inhibition of chromatin-modifying enzymes allows to modulate transcription in eukaryotic cells paved the way for the development of novel pharmacologic agents. Recently, the HDAC inhibitor (HDACi) vorinostat was approved by the US FDA for the treatment of the cutaneous manifestations in patients with advanced, refractory cutaneous T-cell lymphoma demonstrating the therapeutic potential of HDAC inhibition. Our understanding of the biology of individual HDAC family members is rapidly increasing, showing that there is a considerable division of labor between the different HDAC subtypes that often have unique functions and that are promising targets for therapeutic intervention. The aim of this review is to highlight the diversity within the HDAC family, the

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mechanisms by which HDAC activity is regulated and the emerging insights into the structural biology of deacetylases. This knowledge will be fundamental for fully exploiting the therapeutic applications of HDAC inhibition. The last part of the review will therefore specifically focus on our ability to influence HDAC activity *in vivo* with small molecule inhibitors and the exciting possibility to modulate transcription (as well as non-transcriptional processes) with HDACi's to treat human disease.

The HDAC family

Acetylation occurs at the ε-amino groups of lysine residues present within the N-terminal extensions of the core histones. In all documented cases, the acetylation status of target lysines is tightly controlled by the balance of two counteracting enzymatic activities: those of histone acetyl transferases (HATs) and HDACs [7, 8]. Histone deacetylation represses transcription by different mechanisms. On the one hand, this process increases the charge density on the N-termini of the core histones thereby strengthening histone tail-DNA interactions and blocking access of the transcriptional machinery to the DNA template. In addition, histone modifications are specifically recognized by chromatin-interacting proteins. Bromodomains are found in several chromatin-associated proteins, such as some HAT family members, and are used for the recognition of acetylated lysine residues [9]. Deacetylation of a given lysine residue may allow its further modification by histone methyl-transferases [10]. Methylated lysine residues can in turn be recognized by proteins harboring chromodomains, Tudor domains or WD40 repeats [10, 11]. HP1 is a chromodomain protein that specifically recognizes trimethylated lysine 9 of histone H3, thereby favoring the assembly of heterochromatin [12-14]. Further heterochromatinization occurs by recruitment of DNA methyl transferases that will specifically methylate cytosine in the 5 position [15, 16]. Together with these post-translational modifications, there is a progressive decrease in the accessibility of DNA to the transcription machinery and hence an increasing transcriptional silencing (Figure 1).

In addition to histones, an increasing number of non-histone proteins are shown to undergo lysine acetylation: many of these proteins are transcription factors, but heat-shock proteins and structural proteins were also shown to be acetylated. Generally, acetylation has profound influences either on metabolic stability or on the biological function of the modified protein, and can be regarded akin to phosphorylation in terms of being a widely used post-translational mechanism of controlling protein function.

HATs utilize acetyl-CoA as a cofactor in the acetylation reaction. About 30 different HATs were identified that are

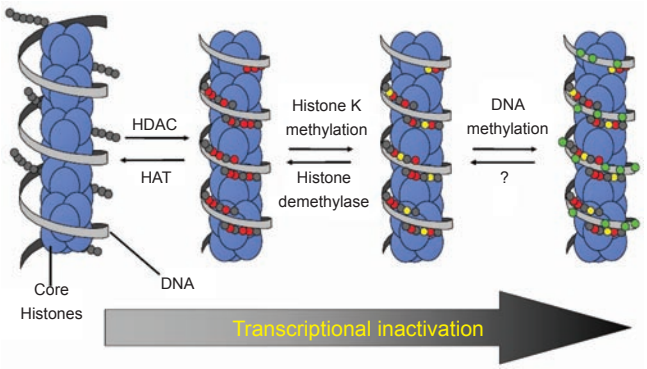

Figure 1 Chromatin modifications that induce progressively increasing transcriptional silencing. Note that modification by acetylation is fully reversible. Only recently enzymes that are able to remove methyl groups from histones (histone demethylases) have been identified. The occurrence of DNA demethylation in adult organisms is still debated.

grouped into five different families [17]. In the eukaryotic cell, HATs may reside either in the nucleus or in the cytosol. HDACs catalyze the inverse reaction by removing the acetyl group from the acetyl lysine residue. Higher organisms have evolved a considerable complexity in the HDAC family that was divided into three classes according to phylogenetic analyses and sequence homologies with the yeast proteins Rpd3, Hos1 and Hos2 (class I), HDA1 and Hos3 (class II) and the sirtuins (class III) [18-20]. Class I and class II proteins are evolutionarily related and share a common enzymatic mechanism, the Zn-catalyzed hydrolysis of the acetyl-lysine amide bond. Higher eukaryotes also express an additional Zn-dependent HDAC (HDAC11 in mammals) that is phylogenetically different from both class I and class II enzymes and is therefore regarded as a separate class (class IV). Class III proteins are evolutionarily unrelated to class I, II or IV and catalyze the transfer of the acetyl group onto the sugar moiety of NAD [20]. This latter reaction is strictly dependent on NAD as a cofactor, and class III HDACs are thought to link transcriptional regulation to energy metabolism that regulates NAD levels. In addition, class III enzymes have been suggested to also play a role in life span regulation by caloric restriction. This review will not deal in detail with class III enzymes but primarily focus on the Zn-dependent HDACs.

Yeast and *Drosophila* HDACs: division of labor

Genome-wide roles of *Saccharomyces cerevisiae* HDACs were studied by microarray profiling of yeast HDAC knockout strains [21]. In those studies a division

of labor among the different classes emerged, with HDA1 primarily regulating genes involved in carbon metabolism, Rpd3 controlling primarily cell cycle genes and Sir2 emerging as a player in the regulation of amino-acid biosynthesis. Transcriptional profiles of Sin3 mutants showed striking similarity to Rpd3 mutants, in line with the notion that transcriptional repression by Sin3 involves the recruitment of Rpd3. This same paradigm was also found by others in higher organisms (see below).

The use of acetylation-site-specific antibodies for chromatin immunoprecipitation (ChIP) in yeast HDAC KO strains to probe intergenic region microarrays (ChIP on CHIP) has generated a very detailed genome-wide HDAC activity map of *S. cerevisiae* [22]. The functional division of labor highlighted by gene expression profiling was further substantiated by these studies. Thus, HDA1 and Rpd3 deacetylate distinct intergenic regions, with HDA1 being preferentially recruited to subtelomeric (HAST) regions containing a high proportion of genes induced by nutritional stress. From these studies, clues to substrate specificity also emerged, providing evidence for the preference of Rpd3 for H4K12ac, H4K5ac and H4K18ac over H4K16ac. Interestingly, the yeast HOS2 deacetylase was shown to be preferentially recruited to highly transcribed genes indicating that, in contrast to other HDACs, HOS2 is required for gene activations [23]. More recently, ChIP on CHIP analysis was performed by using a novel crosslinking technique in conjunction with anti-Rpd3 antibodies, leading to an Rpd3 binding map in *S. cerevisiae* [24]. In one of these studies, a similar distribution of Rpd3 and Sin3 was noted [25], consistent with the gene expression profiling data.

We used RNAi in combination with microarray analysis in *Drosophila* S2 cells to define the role of each of the five individual Zn-dependent HDACs known in this organism [26]. Unexpectedly, deregulated transcription was observed only upon silencing of *Drosophila* class I HDACs 1 and 3, but not upon RNAi of other family members. This suggests that many HDACs may either have primarily non-transcriptional roles or participate in transcription regulation only as a result of certain specific environmental stimuli. Indeed, many class II enzymes seem to follow the latter paradigm (see below), which also appears to be evolutionarily conserved, taking into consideration the preferred recruitment of HDA1 into HAST regions of the yeast genome. The major "housekeeping" deacetylase in *Drosophila* seems to be HDAC1. This protein is involved in the regulation of very diverse sets of genes, most notably those involved in cell proliferation and mitochondrial energy metabolism. As observed in the yeast system, in *Drosophila* too there is a noticeable functional overlap between HDAC1 and the transcriptional co-repressor Sin3 [26, 27], suggesting that most of its repressor function is exerted through deacetylase recruitment, a feature that has been evolutionarily conserved.

No systematic "omic" approaches to unveil individual roles of HDACs have been reported so far in mammalian cells, possibly because of the greater complexity of the family that has 18 known members in these organisms. Figure 2 shows a comparison between Zn-dependent HDACs in *Drosophila* and in mammals. Still, many aspects of the biology of individual subtypes have emerged, highlighting how each subtype has very specialized and mostly non-redundant functions.

Mammalian deacetylases

Among mammalian class I deacetylases (subtypes 1, 2, 3 and 8), HDACs 1 and 2 are most closely related (82% sequence identity) and found in the ubiquitously expressed mSin3A, NURD/Mi2/NRD and CoREST corepressor complexes [28]. HDAC1 KO mice die during embryonic development and their ES cells show decreased growth and enhanced expression of the cyclin-dependent kinase inhibitors p21 and p27 [29]. Compensatory upregulation of the expression of other class I HDACs in HDAC1 KO cells is apparently insufficient to counterbalance the loss of this subtype, suggesting both its functional uniqueness and the existence of regulatory cross-talk [30]. Both HDACs 1 and 2 (in addition to HDAC3) seem to be involved in the regulation of key cell cycle genes such as p21 ([31-33] and MP *et al*., manuscript in preparation). Still, HDAC2 also has some specialized functions, e.g. in counterbalancing proapoptotic signals that ensue from aberrant Wnt pathway activation in colon cancer [34]. HDACs 1 and 2 are not exclusively HDACs but may deacetylate non-histone substrates as well [35].

HDAC3 associates to and is activated by SMRT and NCoR co-repressors that play an important role in the regulation of gene expression by nuclear hormone receptors [28], which, in their unliganded state, recruit these corepressors and utilize the deacetylase activity to silence transcription. Studies on the retinoic acid receptor suggest that a specific lysine (H4K5) is preferentially deacetylated by HDAC3 recruited to the unliganded receptor [36]. Intriguingly, the SMRT/N-CoR corepressors are themselves capable of interacting with histones *via* an SANT domain and this interaction is strengthened upon deacetylation of H4K5, suggesting the existence of a positive feedback loop in the silencing mechanism. HDAC3 was found to be upregulated in $CD34^+$ $CD133^+$ early hematopoietic progenitors [37] and to play a transcription-independent role in mitosis [38], possibly pointing to functions in cell cycle progression and stem cell self-renewal. In contrast to

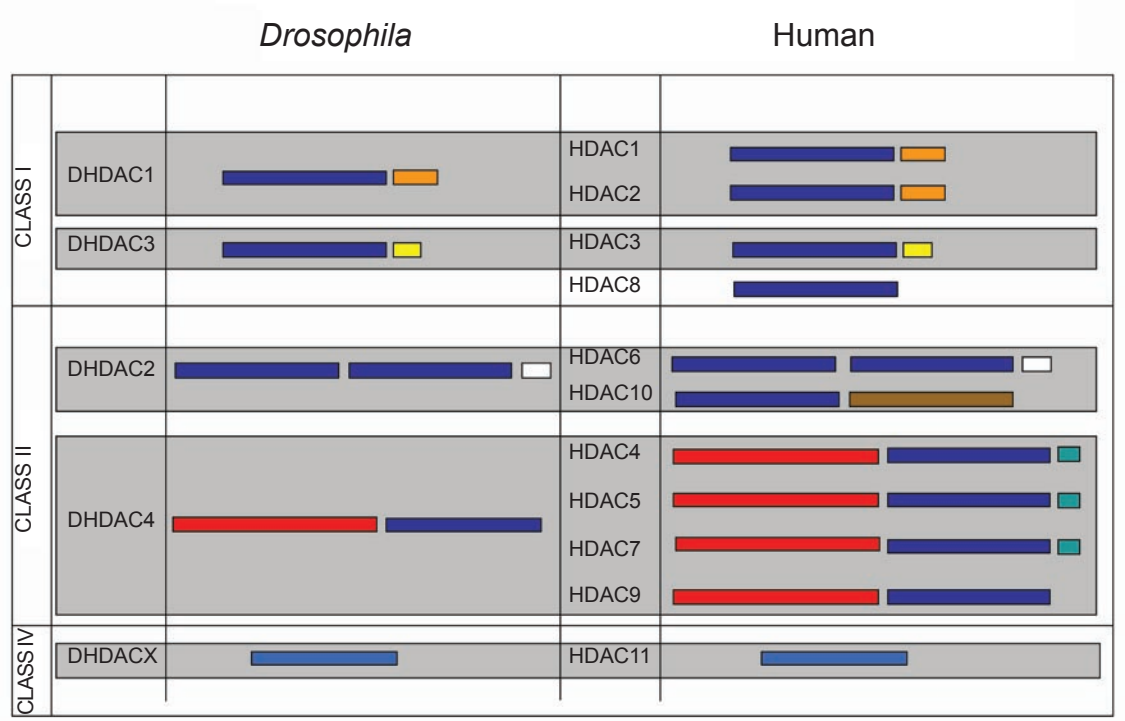

Figure 2 Zn-dependent histone deacetylases in *Drosophila* and mammals and their domain structure. Catalytic domains are shown in blue. Note that HDAC6 and DHDAC2 have two catalytic domains, whereas the second deacetylase domain (brown) is dysfunctional in HDAC10. C-terminal extensions are found in HDACs 1, 2 and 3 (class I) and in their *Drosophila* homologs (light brown and yellow). Also the N-terminal extensions found in class IIa HDACS (red) are conserved in DHDAC4, whereas the C-terminal extensions found in HDACs 4, 5 and 7 are not.

HDACs 1 and 2 that are exclusively nuclear, HDAC3 may also be found in the cytoplasm and even associated with the plasma membrane, where it can be phosphorylated by Src [39]. Also HDAC3 is not an exclusive HDAC but may also deacetylate non-histone substrates, such as the RelA subunit of NF-κB, thereby affecting its stability and DNA-binding properties [40, 41]. The last member of class I, HDAC8, was recently found to be expressed in smooth muscle where it is required for muscle contractility [42]. This protein has also been linked to cancer since it is recruited by the leukemic INV [16] protein [43]; it regulates telomerase activity [44] and siRNAs targeting HDAC8 were shown to have antitumor effects in cell culture [45].

The class IIa HDACs (subtypes 4, 5, 7 and 9) are characterized by tissue-specific expression and stimulus-dependent nucleo-cytoplasmic shuttling [19]. They are target of several kinases, and some phosphorylated forms are confined to the cytosol by interaction with 14-3-3 proteins (see below). In the nucleus they associate with transcription factors, notably of the MEF and Runx families, and control differentiation and cellular hypertrophy in muscle and cartilage tissues [46, 47]. HDAC4 KO mice have a pronounced chondrocyte hypertrophy and die of aberrant ossification [46]. HDAC9 KO mice show cardiac hypertrophy [47] that is further exacerbated in the HDAC9+HDAC5 double KO animals [48]. HDAC7 has a specific role in the clonal expansion of T cells by suppressing Nur77-dependent apoptosis [49] and in vascular integrity through suppression of MMP10 [50]. Class IIb subtypes 6 and 10 have a duplication of their catalytic domains, but the second catalytic domain is thought to be dysfunctional in HDAC10. HDAC6 is the only deacetylase known to act on tubulin [51, 52]. Tubulin deacetylation is required for disposal of misfolded proteins in aggresomes [53]. HDAC6 also deacetylates Hsp90, pointing to a broader role of this subtype in protein folding [54-56]. Finally, very little is known about HDAC11, which cannot be clearly assigned to either class I or class II HDACs based on sequence motifs [57].

Cellular mechanisms controlling HDAC activity

Mammalian HDACs play global roles in the regulation of gene transcription, cell growth, survival and prolifera-

tion, and their aberrant expression or activity lead to cancer development. As expected for vital cellular regulators, the activities of HDACs are tightly controlled through a multitude of mechanisms, among which the recruitment into different co-repressor complexes and the modulation of deacetylase activity by protein-protein interactions or by post-translational modifications are particularly relevant ones.

With the exception of HDAC8, functional HDACs are never found as single monomeric polypeptides, rather they accumulate in high molecular weight multi-protein complexes in which different HDAC subtypes are often associated with specific co-regulators as well as with other chromatin-modifying enzymes [28].

HDAC biological activity can be separated into two distinct, not always interdependent areas, enzymatic activity (the ability to deacetylate histone or other non-histone protein substrates) and functional activity (the ability to regulate transcription and other biological processes). There are two orders of considerations that are worth mentioning to clarify the relevance of this distinction. First, not in all circumstances HDACs' biological functions are strictly dependent on their enzymatic activity. There is ample evidence that class IIa HDACs exert transcriptional repression, thanks to their ability to directly interact and inactivate specific target transcription factors (see [19 and 58] and references therein). In addition, they can recruit a number of distinct corepressors and/or protein-modifying enzymes, which in turn directly switch target transcription factors to their inactive form. Most of these multiple protein-protein interactions occur *via* class IIa HDACs' N-terminal regulatory domain, while the activity or even the physical presence of the C-terminal catalytic domain is often not strictly required. The existence of the MEF2-interacting transcription repressor (MITR; a naturally occurring HDAC9 splice variant) is paradigmatic, which retains transcriptional repressive functions despite lacking the C-terminal catalytic domain. Furthermore, the enzymatic activity of HDAC4, 5 and 7 was shown to be dependent on the association with the HDAC3/SMRT/N-CoR complex, thus suggesting that class IIa HDACs are not active deacetylases, but operate by recruiting preexisting enzymatically active HDAC3 protein complexes [19]. A second important consideration is that, with the exception of mammalian HDAC8, most purified recombinant HDACs are enzymatically inactive [59]. Any protein that associates with HDACs, therefore, has the potential to exert an enzymatic co-activating function. The deacetylase activity of HDAC3 strictly requires the interaction with its transcriptional corepressor protein partners SMRT or N-CoR, which contact and activate HDAC3 via a conserved deacetylase-activating domain (DAD) [60, 61]. Known HDAC1/2 complexes contain transcriptional corepressors mSin3A [62], MTA (metastasis-associated protein in NuRD complex) [63] and CoREST (corepressor of REST) [64]. MTA-2 is an essential component of an enzymatically active recombinant HDAC1/HDAC2 protein complex, while in CoREST the SANT domain is necessary for recruiting HDAC1 activity. SDS3 is a key component of the Sin3 corepressor complex that increases the enzymatic activity of HDAC1 in yeast and mammalian cells [65, 66]. Enzymatic activation by specific HDAC-cofactor interactions could therefore represent a general mechanism for spatially restraining HDAC activities to the specific promoter sites where corepressor complexes are targeted by sequence-specific DNA-binding proteins.

While the recruitment into different corepressor complexes and the modulation of deacetylase activity by protein-protein interactions mainly contribute to the functional diversity of different HDAC subtypes, post-translational modifications, such as phosphorylation and sumoylation, add additional complexity to the regulatory networks controlling these enzymes. All mammalian HDACs contain potential phosphorylation sites and the majority of them have been found to be phosphorylated *in vitro* and *in vivo* [59]. In the case of class IIa HDACs, phosphorylation regulates their subcellular localization and therefore their biological activities [19, 59]. The N-terminal regions of these HDACs contain a set of conserved serine residues that control their subcellular localization and confer signal responsiveness to downstream target genes [67, 68]. Phosphorylation of these residues creates binding sites for the 14-3-3 chaperone protein, which escorts phospho-HDACs from the nucleus to the cytoplasm, with consequent activation of HDAC target genes. Protein kinase D and various Ca^{2+}/calmodulin-dependent kinases transmit signals triggered by different extracellular stimuli to class IIa HDACs *via* their regulatory phosphorylation sites in a variety of cell types [69-76].

CK2 is a key modulator of class I HDAC activity

With the exception of HDAC8, shown to be phosphorylated and thus inactivated by protein kinase A (PKA) [77], casein kinase 2 (CK2) emerged as a key enzyme for all other class I HDAC isotypes [78-82]. CK2 is a multifunctional protein kinase, ubiquitously distributed in both the cytoplasm and the nucleus of eukaryotic cells and most prominently involved in the regulation of cell growth, survival and proliferation [83-85], and transcription-related chromatin remodeling [86-88]. Its activity has been consistently found elevated in many human cancers and experimental tumors, and its deregulated expression was shown to contribute to tumorigenesis and impart oncogenic potential [85]. In this regard, it is relevant that class I HDACs play similar global

roles in homeostasis, signal transduction, cell cycle control and cancer development [89, 90]. HDAC1 and HDAC2 C-terminal regions contain at least two serine residues at equivalent positions (S421/S422 and S423/S424, respectively) that are phosphorylated by CK2 [78, 80]. Based on site-directed mutagenesis studies, HDAC3 S424 was more recently found to be phosphorylated in cells and to be a CK2 phosphoacceptor site *in vitro* [82]. Mass spectrometry analysis [91] reinforced this evidence directly identifying S424 as a unique HDAC3 phosphoacceptor site *in vivo*. This residue resides in a canonical CK2 consensus sequence and is highly conserved among HDAC3 homologs from different species. However, the C-terminal sequence of HDAC3 diverges significantly from the corresponding regions of HDAC1 and HDAC2, and by sequence alignment comparisons S424 has no equivalent in the other HDAC class I members. These studies raise a number of yet to be reconciled contradictory conclusions, mainly concerning the regulatory effects exerted by phosphorylation on the enzymatic activity, transcriptional repression potential and functional protein-protein interactions [78-81, 91, 92]. Interestingly, HDAC1 and HDAC2 phosphoserine mutations, shown to be deleterious for the enzymatic activity, also disrupt interactions with endogenous associated proteins [78-80], including MTA2 and CoREST, essential members of HDAC1/2 enzymatically proficient complexes [63, 64]. Phosphorylation might therefore increase the affinity of HDACs for key interacting proteins, which in turn enhance their enzymatic activity. Phospho-serine mutagenesis approaches, however, include the caveat that the mutations themselves could alter the HDACs conformation inducing an enzymatically less active form and/or decreasing protein-binding affinity. Published evidence has shown that HDAC1 and HDAC2 immunocomplexes contain functional CK2 [79, 81]. More recently, we have identified active CK2 in the HDAC3 immunocomplex capable of efficiently phosphorylating both β-casein and the associated HDAC3 and of autophosphorylating its own regulatory β subunit [91]. In the same work, phosphorylation of HDAC3 did not affect either the deacetylase activity of the protein or its ability to stably interact with N-CoR. Our findings are therefore not in agreement with recently published evidence showing a three- to four-fold reduction of HDAC3 enzymatic activity upon mutation of S424 [82]. Under our experimental conditions, N-CoR and CK2 co-elute with HDAC3 in sub-stoichiometric amounts and only a fraction of ectopically expressed HDAC3 is phosphorylated on S424. Collectively, these findings support a model in which phosphorylation and association with N-CoR could represent two distinct and mutually exclusive mechanisms regulating HDAC3 enzymatic activity, the latter being dominant under our experimental conditions. Concurrent interactions with both N-CoR and CK2 could be incompatible so that only the cofactor-free HDAC3 fraction would be a potential phosphorylation substrate. Alternatively, since S424 is located at the extreme HDAC3 C-terminus previously found to critically contribute to the interaction with SMRT/N-CoR DAD [60, 93], the stable association with the cofactor might interfere with the accessibility of the CK2 phosphoacceptor site to the kinase. Future studies aimed to map the HDAC3 region responsible for CK2 interaction would help clarifying these points. In a number of circumstances, HDAC3 is capable of establishing direct protein-protein interactions and displays biological activity independent of its recruitment in N-CoR/SMRT complexes, suggesting alternative mechanism(s) through which it can exert its transcriptional co-repression function in normal and tumor cells [94-101]. It would be tempting to speculate that HDAC3 phosphorylation might assume a critical role in activating the enzyme in N-CoR/SMRT-independent contexts.

Non-catalytic functions of class I HDACs

In addition to the pleiotropic biological roles accomplished through their deacetylase activities, in some circumstances both class I and class II HDACs have been recently shown to regulate key cellular processes by interacting with and targeting different enzymes to their specific substrates. For instance, HDAC1 and HDAC6 can influence the phosphorylation-dephosphorylation state of CREB and Akt, respectively, by forming reversible HDAC-PP1 complexes, thus regulating the delivery of PP1 to these substrates [102, 103]. In this respect, the interaction of class I HDACs with CK2 could lead to two different regulatory mechanisms, one related with direct phosphorylation of the HDAC within the complex, and the other relying on delivering the protein kinase to specific targets. Interestingly, two of the better-characterized HDAC3-interacting proteins, i.e. SMRT and NF-κB RelA/p65 subunit, are also CK2 substrates [104, 105]. Little is known about how CK2 activity is regulated *in vivo*. The association with key regulators such as HDACs could represent one mechanism for restraining CK2 activity in different cellular compartments and on different protein targets.

The role of phosphatases in the regulation of HDAC activity

While most studies have focused on HDAC phosphorylation, an increasing number of published data suggest the involvement of protein phosphatases being equally relevant [82, 102, 103, 106]. Although PP1 was shown to associate specifically with HDAC1, 6 and 10, it is not

known whether these HDAC isotypes are direct substrates of this phosphatase. In the case of HDAC3, however, a distinct serine/threonine protein phosphatase, PP4, has been recently identified that functionally interacts with and dephosphorylates HDAC3, thus negatively modulating its deacetylase activity [82]. Interestingly, the HDAC3 N-terminal region involved in PP4 interaction was also shown to be essential for the association with the SMRT DAD [60], thus reinforcing the hypothesis that HDAC3 can be subjected to two independent regulatory mechanisms, one mediated by its activating cofactor and the other by phosphorylation/dephosphorylation.

Sumoylation

In addition to phosphorylation, another post-translational modification that has been shown to regulate HDAC activity and function is the conjugation of small ubiquitin-related modifier (SUMO-1) [59]. Two independent reports [107, 108] identified HDAC1 as a substrate for SUMO-1 modification *in vitro* and *in vivo* at K444 and K476. However, as for HDACs phosphorylation studies, these studies also lead to opposite conclusions regarding the functional relevance of sumoylation on HDAC1 activity. Also some class II HDAC members (HDAC4, HDAC6 and MITR) have been found to be modified by SUMO-1 [109]. In particular, HDAC4 sumoylation was shown to positively modulate its repressive and deacetylase activities and to be linked to nuclear import regulatory mechanisms. It is worth noting that the reduced repressor activity of sumoylation-deficient HDAC1 and HDAC4 was found to be independent of their ability to associate with known binding proteins including mSin3A and N-CoR. However, several recent studies have determined that sumoylation of some transcriptional regulators, such as p300, Dnmt3a and ELK-1, regulates their association with HDACs and, consequently, their capacity to repress transcription [110, 111]. More recently, a number of independent publications have shown that class IIa HDACs, in particular HDAC4, can stimulate MEF2 sumoylation, and thus MEF2-dependent transcriptional repression [113-115].

Knowledge gained from understanding the many different aspects of HDAC regulation is expected not only to broaden our comprehension of fundamental cellular events but also to be translated into the design of a new generation of mechanism-based therapeutic agents urgently needed for the treatment of major human diseases, such as cancer.

Structure and function of HDACs

The understanding of the mechanism of action and the rational design of HDACi's are greatly facilitated by the availability of structural information on the molecular architecture of HDAC enzymes. The first structural and mechanistic information on HDACs was derived from the crystal structure of an HDAC-like protein (HDLP) with unknown function, from the hyperthermophilic bacterium *Aquifex aeolicus* [116]. The structure revealed a compact domain belonging to the α/β fold with a central eight-stranded parallel β-sheet flanked on each side by four α-helices. Seven additional α-helices are grouped close to the C-terminal end of the β-sheet. Portion of these helices together with loops connecting the secondary structure elements form, along one face of the structure, a narrow apolar channel with a depth of 11 Å at the bottom of which the polar catalytic core containing one zinc ion is found. In the X-ray structures of the HDACi's, Trichostatin A (TSA) and vorinostat (suberoylanilide hydroxamic acid, SAHA) in complex with HDLP [116], the aromatic end of the inhibitors (cap moiety) points away from the protein, while the long carbon chain (spacer) fits into the narrow hydrophobic pocket and ends with the hydroxamic acid establishing polar interactions with protein residues and the zinc at the bottom (zinc-binding, enzyme-inhibiting group). For 5 years, the HDLP crystal structure was the only model available for structure-activity relationship studies of HDACi's. In 2004, two independent groups published X-ray structures of inhibitor-bound human HDAC8 (approximately 30% sequence identity with HDLP; [117, 45]). The structure of HDAC8 forms a single compact α/β domain composed of a central eight-stranded parallel β-sheet and eleven α-helices (Figure 3A), similar to HDLP [116] and to the dimanganese enzyme arginase [118]. Striking differences between HDAC8 and HDLP are observed for most of the loops emerging from the core of the protein, for the distal helices and for a long extended loop, which connects the last two helices at the C-terminus. This region in HDAC8 is completely different from HDLP, including the loss of two helices (Figure 3A). Overall, these variations give rise to a more accessible active site in HDAC8 with a slightly deeper and larger pocket (Figure 3A).

Recently, the structure of the bacterial FB188 HDAH (HDAC-like amidohydrolase from *Bordetella/Alcaligenes* strain FB188) was also determined [119]. This protein has about 30% sequence identity with the two catalytic domains of HDAC6, and 20% and 21% sequence identity with HDLP and HDAC8, respectively. The overall structure of FB188 HDAH reveals an α/β domain similar to HDLP and HDAC8 (Figure 3A). It consists of a seven-stranded parallel β-sheet flanked on one side by three α-helices and on the other side by four α-helices with additional seven α-helices shielding the top of the β-sheet. The differences with HDLP and HDAC8 are predominantly restricted to the loops surrounding the entrance of the active site, especially

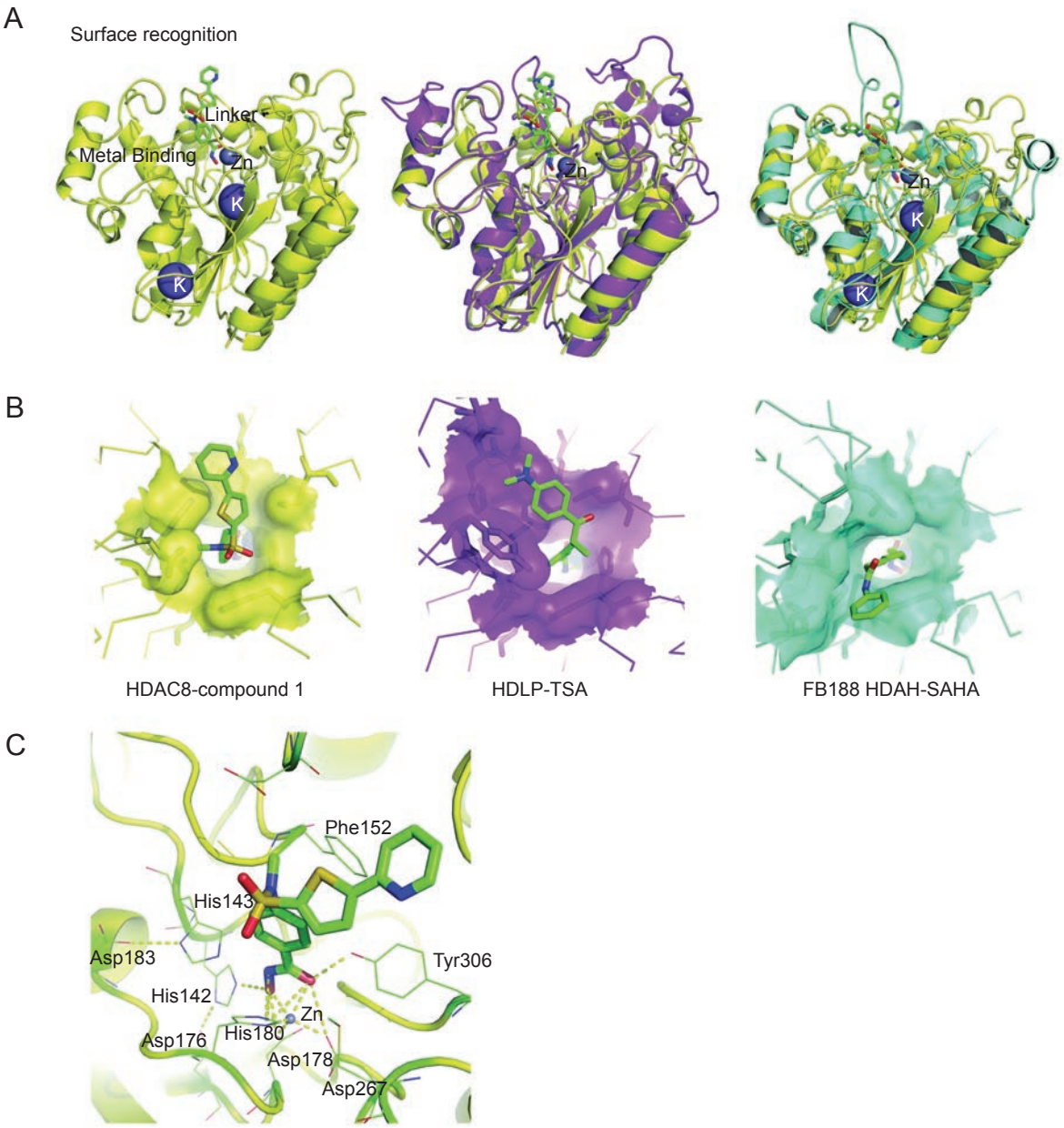

Figure 3 Crystal structure of inhibitor-bound HDAC8 and comparison with inhibitor-bound HDAC-like enzymes. **(A)** Left, Ribbon diagram of human HDAC8 monomer in complex with the hydroxamic acid inhibitor, *N*-hydroxy-4-{methyl [(5-pyridin-2-yl-2-thienyl) sulfonyl] amino} benzamide, compound 1, pdb code 1w22. The protein is shown as a yellow ribbon and the inhibitor is drawn in stick representation (oxygen, nitrogen, sulfur and carbon are colored red, blue, yellow and green, respectively). Zn^{2+} and K^{+} ions are indicated and drawn as violet spheres. The HDACi pharmacophore consists of a metal binding moiety at the zinc site, a linker moiety in the channel and a surface recognition moiety on the rim of the channel. Center, Superposition of human HDAC8 (yellow) and HDLP (magenta) in complex with TSA, pdb code 1c3r. Compound 1 and TSA are drawn in stick representation and colored as in **(A)** left. In the HDLP structure, only the Zn^{2+} ion is present and indicated as a violet sphere. Right, Superposition of human HDAC8 (yellow) and FB188 HDAH (cyan) in complex with SAHA (116), pdb code 1zz1. Compound 1 and SAHA are drawn in stick representation and colored as in **(A)** left. In the FB188 HDAH structure, both Zn^{2+} and K^{+} ions are present and indicated as violet spheres. **(B)** Molecular surface representation at the entrance of the active site channel for HDAC8 (yellow), HDLP (magenta) and FB188 HDAH (cyan). Compound 1, TSA and SAHA are drawn in stick representation and colored as in **(A)**. **(C)** Expanded view showing HDAC8 active site with the bound inhibitor, compound 1. Residues relevant for catalysis and involved in inhibitor binding are indicated and drawn in line representation. The inhibitor is drawn in stick representation. The Zn^{2+} ion is indicated as a violet sphere. Polar interactions are shown as dashed yellow lines. Red, oxygen; blue, nitrogen; yellow, sulphur; and green, carbon.

the long N-terminal loop connecting α-helices 1 and 3 and the C-terminal loop regions (Figure 3A). The structural variability in the loop regions at the entrance of the active site channel results in different molecular surfaces for HDLP, HDAC8 and HDAH (Figure 3B). It is reasonable to assume that this structural variability will be found across the entire family of HDACs and points to a critical role of these regions in conferring different substrate specificities and different protein-protein interactions.

Like HDLP, one zinc ion is bound penta-coordinated to Asp, His, Asp and the hydroxamate moiety of several inhibitors [117, 45] (Figure 3C). This zinc ion binds to a site corresponding to the higher affinity catalytic Mn^{2+} site of arginase: Zn^{2+} ligands Asp168/Asp178, His170/His180 and Asp258/Asp267 of HDLP/HDAC8 correspond to Mn^{2+} (B) ligands Asp124, His126 and Asp234 of arginase [118]. In addition, also the number and the spacing between the zinc ligands are typical of zinc enzymes where this metal is catalytically active: three protein side chains bind to the catalytic zinc ion, the first two ligands are separated by a short spacer of two amino acids and these ligands are separated from the third ligand by a long spacer of 87 amino acids [120]. An exact conservation of the catalytic center is found also in HDAH [119], where the zinc ion is penta-coordinated by Asp180, His182 and Asp268 and the hydroxamate moiety of the inhibitor.

The structure of HDAC8 revealed another important difference with HDLP, which is the presence of two potassium-binding sites (Figure 3A, [44]): site 1 located near (7.0 Å) the zinc-binding site and directly connected to it by coordinating Asp178 and His180 (both involved in zinc chelation); site 2 located 15 Å away from site 1. The two potassium ions seem to anchor helix α7 and its loops to the central β-sheet, and CD spectroscopy data suggest that these ions are required for the structural stability of the enzyme, while sodium ions that have the same external configuration but a smaller ionic radius, decrease HDAC8 stability [45, 118]. Purified HDAC8 in the presence of NaCl has sodium instead of potassium in their structures. The two potassium-binding sites are also found in the HDAH crystal structures [120], and the high degree of conservation of ligands in the entire HDAC family suggests that bound potassium is a feature of all HDAC proteins [45].

The development of HDACi's as anticancer agents

Imbalance of histone and non-histone protein acetylation is common in human cancer. HDACs are overexpressed, aberrantly recruited by oncoproteins or mutated in malignant cells, and several mutations affecting HATs were discovered in tumors [121]. TSA, a natural product, was among the first HDACi's to be discovered [122]. This molecule shows pronounced anticancer activity and has generated much interest in the discovery of novel HDACi chemotypes. Only a minor fraction of genes is deregulated by HDACi and among these are genes involved in cell cycle progression and apoptosis [123, 124]. In addition to transcriptional effects, HDACi also affect progression through mitosis by altering chromatin acetylation, and may alter expression of HSP90 client proteins, among which there are many players in oncogenesis, by affecting HSP90 acetylation. Detailed descriptions on the role of HDACs in cancer and the mechanisms by which HDACi's interfere with tumor cell growth and survival can be found in reviews elsewhere [121, 125, 126]. Major hurdles in the discovery of new HDACi have been recently solved. First of all, several groups have been able to express and purify functional HDACs from human, insect and bacterial cells. The technique is mostly based on tagging the COOH terminus of the target HDAC with a short amino-acidic sequence like FLAG, Myc or $His_{[6x]}$ [127-132] that allows a straightforward purification from transfected/transformed cells. Usually these affinity purified proteins are over 90% pure and often allow co-purification of many of the cofactors described in the HDAC complexes, indicating that these protein complexes are representative of the natural ones. Several groups have made use of stable clones and large-scale transient transfection mostly in HeLa and HEK293 cells, which allows purification of large amounts of recombinant HDACs. Availability of highly purified HDAC preparations is an important achievement that allows for a classic biochemical approach to the discovery of subtype-specific HDACi.

The capability to produce recombinant, tagged versions of all known HDACs needs to integrate with relevant cell-based functional assays. Up to now, very few cell-based assays have been described. Among these, a more limited subset was suitable for high throughput screening; in all these cases, HDACi upregulated the expression of a reporter gene, usually luciferase, under the control of portions of the natural $p21^{WAF1/Cip1}$ and 5-lipoxygenase gene promoters or of a minimal SV40 promoter containing multiple SMAD-binding motifs. Despite these efforts, all cell-based assays described require several manipulation steps, and as is the case with an end point assay, do not permit continuous monitoring of the enzymatic reaction. To solve the problem, we have taken advantage of the β-lactamase (Bla) reporter gene, and selected a stable HeLa cell clone expressing the Bla reporter under the control of a $p21^{WAF1/Cip1}$ minimal promoter (–183 through +25 bp) (Pallaoro *et al*., manuscript in preparation). A relevant advantage of the Bla reporter system versus all other commercial reporter genes is its superior sensitivity [133]. Assay sensitivity derives from the combination of

two features, an enzymatic reaction and its FRET detection system. The combined features allow detection of the activity of as little as 50 Bla molecules per cell compared to the 10^5 of GFP or 10^4 of luciferase molecules. Clearly, such sensitivity is very demanding in terms of fine tuning of reporter gene expression and requires no or extremely low basal expression levels and high inducibility. By combining the indicated minimal promoter lacking the p53 response elements and the HeLa cell line (which has a p53 null background) with the sensitivity features described earlier, we assured robustness, a straightforward readout format and a low probability of identifying false positives that would activate p21 *via* HDAC-inhibition unrelated mechanisms. A combination of ChIP, siRNA and HDAC overexpression approaches showed that this assay selectively measures the inhibition of class I HDACs in cells.

Classes of HDACi's and their anticancer properties

To date, there are five distinct classes of HDACi as illustrated in Figure 4: short-chain fatty acids like butyric acid (BA), hydroxamic acids such as vorinostat, electrophilic ketones, aminobenzamides exemplified by MS-275, and natural cyclic peptides like romidepsin and apicidin. Various HDACi from several of these chemical classes have already advanced into clinical trials, and recently Zolinza (vorinostat) was approved by the US FDA for the treatment of the cutaneous manifestations in patients with advanced, refractory cutaneous T-cell lymphoma following two or more prior systemic therapies.

Among the first HDACi to be identified were the short chain fatty acids like BA and valproic acid [134-137]. These agents are the least potent of the various classes of HDACi with IC_{50}s in the millimolar range. Indeed in 1975, BA was shown to induce differentiation in Friend Leukemia cells, and then 3 years later to cause a dramatic and reversible increase in histone acetylation [136]. These findings together with growth arrest seen in many cell lines caused a child with acute myelogenous leukemia to be treated with 500 mpk i.v. infusion of BA daily, resulting in reduction and then disappearance of the myeloblasts. However, more extensive clinical trials failed to reproduce these effects, possibly due to the short half-life ($t_{1/2}$ = 6 min) and low C_{max} (50 μM) achievable. In an effort to obtain higher plasma

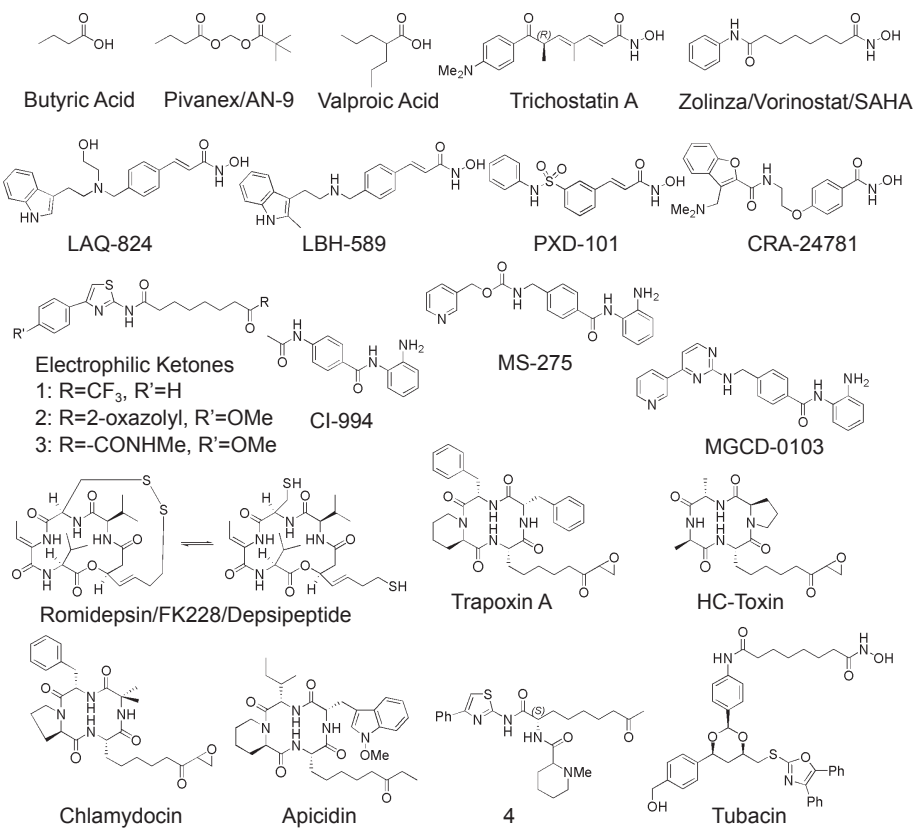

Figure 4 Representative structures of HDACi's with anticancer activity.

levels, several prodrugs of BA have been developed. One such prodrug, Pivanex (AN9), is highly lipophilic and is able to cross cell membranes more efficiently and has been shown to affect the growth, differentiation and apoptosis of various cancer cell lines faster and at lower concentrations than BA [137].

TSA, a fungistatic antibiotic, was the first of a series of hydroxamic acid HDACi to be identified and has become one of the main research tools for probing the function of histone acetylation [122, 138]. Like BA, interest in this natural product arose due to the demonstration that it was able to induce Friend leukemia cell differentiation, as well as G_1 and G_2/M cell cycle arrest. In 1990, TSA was identified to be a potent HDACi, giving rise to a marked accumulation of acetylated histones *in vivo* and strongly inhibiting HDAC, with a K_i of 3.4 nM [122]. More recently, an X-ray crystal structure of this compound bound to HDAC8 has revealed that the hydroxamic acid group tightly coordinates the active site zinc ion [117]. Poor pharmacokinetics preclude any *in vivo* work with this HDACi; nevertheless, these findings stimulated the development of structurally related HDACi.

Zolinza, formerly known as SAHA, or generically known as vorinostat, is the most clinically advanced HDACi [139-141] and was approved by the FDA for the treatment of cutaneous manifestations of advanced, refractory cutaneous T-cell lymphoma. In a phase IIb study, ~30% of the patients responded to the daily dose of 400 mg orally [139]. Vorinostat was identified in the mid-1990s as an agent capable of inducing differentiation of murine erythroleukemia cells and subsequently has been shown to induce differentiation, cell growth arrest and/or apoptosis in a wide variety of cell lines at low micromolar concentrations [140, 142]. Vorinostat is able to inhibit both class I and II HDACs at nanomolar concentrations, and like TSA has been shown to bind at the active site of the enzyme. Vorinostat has been demonstrated to have antiproliferative effects against a range of cultured transformed cells and these effects in part are due to altered gene expression where vorinostat causes both increased and decreased expression of about 2-10% of expressed genes. *In vivo* vorinostat inhibits tumor growth with little toxicity in a wide range of animal models of solid tumors (including breast, prostate, lung and gastric cancers) and hematological malignancies. Clinical studies have been initiated against diverse tumor types, and meaningful clinical responses have been observed in patients with T-cell and B-cell lymphomas, acute myeloid leukemia, mesothelioma, and laryngeal or thyroid cancers. Stable disease has also been achieved in patients with renal-cell carcinoma, urothelial cancer or Hodgkin's lymphoma. Vorinostat has also been shown to have synergistic or additive effects with a wide range of chemotherapeutics in preclinical studies, and clinical combination studies are underway. Additional examples of hydroxamic acid HDACi include LAQ824 and LBH589, PXD-101, and CRA-24781 [143-148].

In an attempt to move away from the hydroxamic acid zinc-binding group, which causes these HDACi to display short half-lives, a series of electrophilic ketones have been developed [149-151]. Electrophilic ketones had previously been shown to be readily hydrated and be able to inhibit zinc-dependent enzymes. Various activated ketones were tested including trifluoromethyl ketones such as 1 in Figure 4, which was shown to be a modest HDACi (IC_{50} = 310 nM), with weak antiproliferative activity (IC_{50} = 4.2 μM) against the mouse HT1080 tumor cell line. The corresponding methyl ketone was inactive, demonstrating the requirement for the electrophilic carbonyl group. Unfortunately, these compounds are readily reduced to the inactive alcohol, displaying poor PK parameters, short $t_{1/2}$ and low exposure upon i.v. dosing. A related series of heterocyclic ketones suffered a similar fate; and despite oxazoles 2 (Figure 4) being a potent HDACi (IC_{50} = 30 nM), only modest cellular effects were observed. Related α-ketoamides displayed the same liability of rapid reduction, yet efficacy *in vivo* was achieved with 3 (Figure 4), a potent non-hydroxamic acid HDACi (IC_{50} = 9 nM) showing antiproliferative activity on HT1080 cells. Indeed, in a mouse HT1080 xenograft model 3 gave rise to tumor growth inhibition when dosed every other day at 30 or 100 mpk i.p., although some toxicity was seen at the highest dose.

A fourth class of HDACi is the aminobenzamides exemplified by CI-994, MS-275 and MGCD0103 [152-156]. CI-994 was the first member of this class to be identified and arose from the serendipitous observation that dinaline, a potential anticonvulsant and the des-acetyl derivative of CI-994, caused bone marrow suppression. Investigation into the potential antitumor activity revealed efficacy *in vivo* in preclinical models [152, 153]. It was identified that dinaline is rapidly acetylated *in vivo* and CI-994 is the active agent. More recently, the mechanism of action has been determined to be due to HDAC inhibition. The most advanced aminobenzamide currently in clinical trials is MS-275, which is in development for melanoma [154, 155]. Recently disclosed clinical data showed that this compound administered orally at either 3 mg biweekly or 7 mg weekly was well tolerated and 7/28 patients had stable disease for 8 to more than 48 weeks. The infrequent dosing regime is due to this compound's extended half-life in man, $t_{1/2}$ = 45-100 h. Preclinically MS-275 was shown to inhibit HDACs with IC_{50} = 2.0 μM and was demonstrated to cause hyperacetylation of histone H4, induce $p21^{WAF1/CIP}$ and cause G_1 cell cycle arrest. Antiproliferative activities ranging from 42 nM to 4.7 μM were seen against a wide panel of cancer cell lines, and *in vivo* studies with this agent in several xe-

nograft models showed that tumor growth inhibition was observed in seven out of eight models at non-toxic doses when administered orally. The exact manner by which this and related compounds inhibit HDACs is not understood, although the 2′-amino group on the benzanilide has been shown to be crucial for activity, as its removal or isomerization to the related 3′-amino position resulted in loss of HDACi activity. A related aminobenzamide, MGCD0103 [156], is undergoing phase I/II trials in solid tumors and hematological cancers. This compound is a selective inhibitor of HDACs 1, 2, 3 and 11 with IC_{50}s = 0.1, 0.2, 2 and 2 μM respectively, and in contrast to hydroxamic acid HDACi, it does not inhibit the other isoforms.

Romidepsin (also known as FK228 or depsipeptide) is a natural product produced by *Chromobacterium violaceum* [157, 158]. The compound was demonstrated to show potent antitumor activity both *in vitro* and *in vivo* although several years passed before the mechanism of action of this agent was elucidated. Although romidepsin apparently contains no functional group able to interact with the active site zinc ion, the compound was shown to have appreciable HDACi activity, and have effects on cell cycle, chromatin structure and cause accumulation of acetylated histones. Further work revealed that it is a natural pro-drug, activated by cellular reduction of the disulfide bond to the corresponding thiols [158]. Molecular modeling suggests that these groups are capable of acting as zinc-binding groups. In the presence of the reductant DTT, the potency of romidepsin increases 36-fold to 1 nM; furthermore, alkylation of these thiol groups almost abolishes the HDAC inhibitory activity. Romidepsin has been shown to be a selective class I HDACi, with IC_{50}s of 36, 47, 510 and 14 000 nM respectively on HDACs 1, 2, 4 and 6. Clinical studies with this agent are under way and interim phase II data in patients with advanced CTCL has been reported. Following a 4-h i.v. infusion on days 1, 8 and 15 of a 28-day cycle, the compound was reported to be well tolerated with manageable toxicities, including nausea, vomiting and fatigue [158].

A final class of HDACi is a series of cyclic peptides exemplified among others by Trapoxin A, H-C toxin, chlamydocin and apicidin [159-163]. All these structures contain a proline or pipecolinic acid residue and a non-proteinogenic long aliphatic amino acid (L-Aoe or L-Aoda) that may act as a substrate analog. Most of these natural products contain the epoxide group that was believed to be essential for biological activity and this was supported by the fact that trapoxin was shown to potently and irreversibly inhibit HDACs [163]. More recently, apicidin was isolated and demonstrated to have potent, broad spectrum antiprotozoal activity and was shown to be a potent HDACi [162]. It displays antiproliferation activity against a wide panel of cancer cell lines. Apicidin is unique in that it lacks the epoxide whose presence is integral for biological activity in closely related cyclic tetrapeptide.

We have used apicidin as a starting point for a drug discovery program and were to evolve a series of potent, low molecular weight, non-hydroxamic acid HDACi's, exemplified by 4 (Figure 4), which is selective for HDACs 1, 2, 3 and 6, and displays only weak or no activity on HDACs 4, 5, 7 and 8 [164]. Furthermore, 4 showed submicromolar activity against several human tumor cell lines.

In a search for selective deacetylase inhibitors, Schreiber designed a library of 7 392 members capable of inhibiting deacetylase activity and used a multi-dimensional, high throughput, cell-based assay to screen for induction of α-tubulin or histone acetylation[165]. Follow-up of the hits enabled a selective, reversible inhibitor of α-tubulin deacetylation to be identified – Tubacin. Tubacin was shown to induce a three-fold increase in α-tubulin acetylation at the 10 μM concentration, with EC_{50} = 2.5 μM. At these concentrations no alterations in gene expression were seen nor did it affect cell cycle progression, demonstrating the selectivity of this compound. Further studies identified that HDAC 6 was the target of Tubacin and that this isoform plays a role in microtubule stability and cell mobility. Subsequent deconvolution of the library also identified a selective inducer of histone acetylation, Histacin [166].

Summary and perspectives

The possibility of using lysine acetylation in a highly diversified manner in different biological processes seems to be the evolutionary driver behind the complexity of the HDAC family in mammals. Not only has lysine acetylation emerged as a common post-translational mechanism that has many different roles that go beyond chromatin conformation but also distributing the task of controlling lysine acetylation levels on different players has largely expanded the possibility to fine tune this reaction and to incorporate it in many different physiological contexts. In line with this notion, the tight regulation of deacetylase activity at different levels and through different mechanisms is a common theme for probably all HDAC family members. Interfering with the expression or function of individual HDACs was shown to elicit unique biological responses. Since HDACs are involved in many pathological processes, their inhibition could provide clinical benefits. This has been extensively proven for cancer but there is hope that the present compounds may prove useful in several other human diseases. Even though many aspects of the functions of individual HDAC subtypes in mammals were elucidated, more comprehensive *in vivo* data are needed to extrapolate potential therapeutic benefits. Con-

ditional knockout animals or catalytically-dead knock-ins would be tremendously important in this assessment. The medicinal chemistry efforts have shown that it is possible to extensively modulate the subtype specificity of HDACi. Highly selective compounds may turn out to be formidable tools for improving our understanding of the biology of this fascinating class of enzymes and they may also open new avenues for selective targeting of pathology-associated HDAC subtypes in a therapeutic setting.

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