REVIEW ARTICLE

Histone deacetylases (HDACs): characterization of the classical HDAC family

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Transcriptional regulation in eukaryotes occurs within a chromatin setting, and is strongly influenced by the post-translational modification of histones, the building blocks of chromatin, such as methylation, phosphorylation and acetylation. Acetylation is probably the best understood of these modifications: hyperacetylation leads to an increase in the expression of particular genes, and hypoacetylation has the opposite effect. Many studies have identified several large, multisubunit enzyme complexes that are responsible for the targeted deacetylation of histones. The aim of this review is to give a comprehensive overview of the structure, function and tissue distribution of members of the classical histone deacetylase (HDAC) family, in order to gain insight into the regulation of gene expression through HDAC activity. SAGE (serial analysis of gene expression) data show that HDACs are generally expressed in almost all tissues investigated. Surprisingly, no major differences were observed between the expression pattern in normal and malignant tissues. However, significant variation in HDAC expression was observed within tissue types. HDAC inhibitors have been shown to induce specific changes in gene expression and to influence a variety of other processes, including growth arrest, differentiation, cytotoxicity and induction of apoptosis. This challenging field has generated many fascinating results which will ultimately lead to a better understanding of the mechanism of gene transcription as a whole.

Key words: characterization, inhibitor, co-repressor, gene expression, histone deacetylase, tissue distribution.

INTRODUCTION

Transcription in eukaryotic cells is influenced by the manner in which DNA is packaged [1]. In resting cells, DNA is tightly compacted to prevent accessibility of transcription factors. DNA is packaged into chromatin, a highly organized and dynamic protein–DNA complex. The fundamental subunit of chromatin, the nucleosome, is composed of an octamer of four core histones, i.e. an H3/H4 tetramer and two H2A/H2B dimers, surrounded by 146 bp of DNA (Figure 1) [2,3]. Local chromatin architecture is now generally recognized as an important factor in the regulation of gene expression. During activation of gene transcription, this compact, inaccessible DNA is made available to DNA binding proteins via modification of the nucleosome [2]. This architecture of chromatin is strongly influenced by post-translational modifications of the histones. Compared with methylation and phosphorylation, the acetylation of core histones is probably the best understood type of modification. Histone acetylation occurs at the ϵ amino groups of evolutionarily conserved lysine residues located at the N-termini. All core histones are acetylated *in vivo*; modifications of histones H3 and H4 are, however, much more extensively characterized than those of H2A and H2B [1]. Important positions for acetylation are Lys^{9} and Lys^{14} on histone H3, and Lys^{5}, Lys^{8}, Lys^{12} and Lys^{16} on histone H4 (Figure 1) [4]. Steady-state levels of acetylation of the core histones result from the balance between the opposing activities of histone acetyltransferases and histone deacetylases (HDACs) [1]. In general, increased levels of histone acetylation (hyperacetylation) are associated with increased transcriptional activity, whereas decreased levels of acetylation (hypoacetylation) are associated with repression of gene expression (Figure 1) [1,2,5].

The fact that acetylation is a key component in the regulation of gene expression has stimulated the study of HDACs in relation to the aberrant gene expression often observed in cancer. Although no direct alteration in the expression of HDACs has yet been demonstrated in human oncogenesis, it is now known that HDACs associate with a number of well characterized cellular oncogenes and tumour-suppressor genes [e.g. Mad and retinoblastoma protein (Rb)], leading to an aberrant recruitment of HDAC activity, which in turn results in changes in gene expression [6,7]. In acute promyelocytic leukaemia, for example, the oncoprotein produced by the fusion of the *PML* (promyelocytic leukaemia) gene and the retinoic acid receptor α gene appears to suppress the transcription of specific genes through the recruitment of HDACs. Thus the cancer cell is unable to undergo differentiation, leading to excessive proliferation [8–10]. Similar phenomena have been described for retinoic acid receptor α–PLZF (promyelocytic leukaemia zinc finger protein) fusion, AML1 (acute myelocytic leukaemia pro-

Abbreviations used: CaMK, Ca^{2+}/calmodulin-dependent kinase; CtBP, C-terminal binding protein; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor(s); HDRP, HDAC-related protein; MEF2, myocyte enhancer factor 2; N-CoR, nuclear receptor co-repressor; NES, nuclear export signal; NLS, nuclear localization signal; NuRD, nucleosome remodelling histone deacetylase; Rb, retinoblastoma protein; RbAp46/48, Rb-associated protein 46/48; SAGE, serial analysis of gene expression; SMRT, silencing mediator for retinoic acid and thyroid hormone receptors; TSA, trichostatin A; YY1, Yin and Yang 1.

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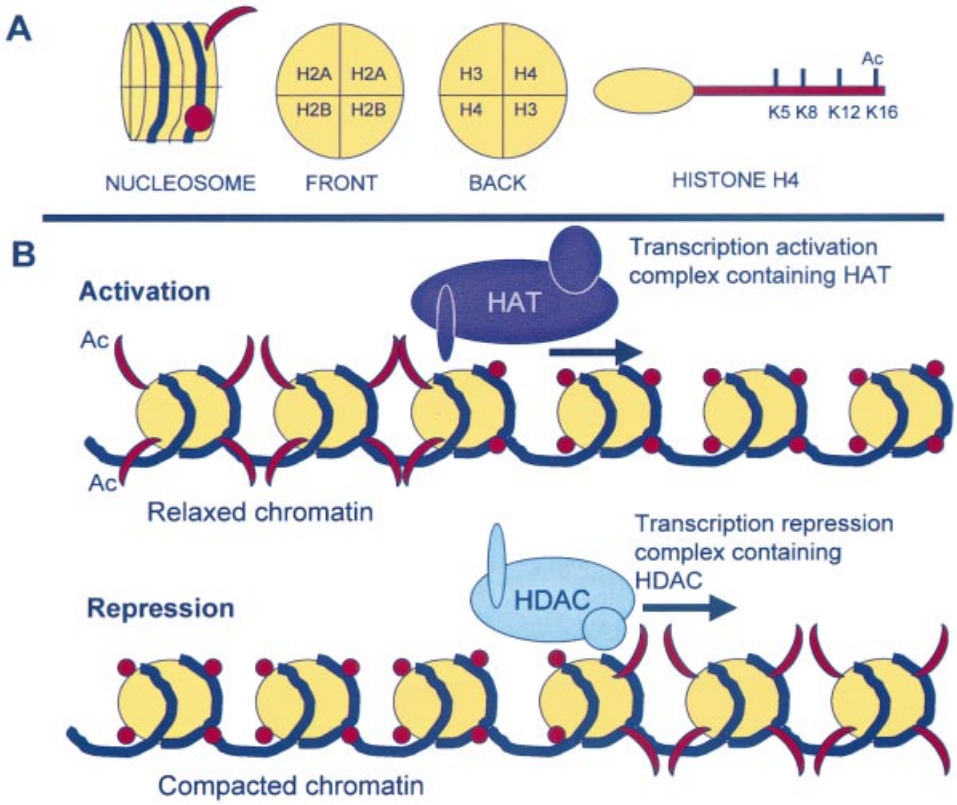


Figure 1 Various aspects of the transcription process and its regulation by histone modification

(**A**) Schematic representation of a nucleosome. Yellow represents the histones. Dark red depicts the histone tail that can be modified to loosen DNA (purple) winding. The dark red circle represents a tail without an acetyl (Ac) group. The dark red 'banana shape' represents a histone tail with an acetyl group, relieving the tight packaging of the DNA. (**B**) Transcriptional repression and activation in chromatin. Yellow circles represent core histone octamers; in the upper panel, acetylated histone tails (dark red) are depicted emerging from the octamer. DNA is purple, and the solid black arrow represents complex movement. Both histone acetyltransferase (HAT; activation) and HDAC (repression) require several cofactors (for DNA binding, for recruitment of the complex, for remodelling of the DNA helix to reduce the accessibility of transcription factors) for their activity [1,3,50].

tein 1)–ETO fusion, and also in the Myc/Mad/Max signalling pathway involved in solid malignancies [11–14].

It is clear that HDAC enzymes seldom operate alone. Many proteins, with various functions such as recruitment, co-repression or chromatin remodelling, are involved in forming a complex that results in the repressor complex shown in Figure 1. The most important signal involved in the initiation process of repression is situated in the DNA itself. Methyl groups bound to the cytosine residues situated 5′ to guanosines in DNA, in so-called CpG islands, are directly responsible for the recruitment of the HDAC complex via proteins such as methylated-CpG-binding proteins and methyl-CpG-binding-domain-containing proteins, or via the enzymes that methylate the CpG islands, the DNA methyltransferases. The methyl groups provide the basis for epigenetic gene silencing, such as imprinting and X chromosome inactivation, since a high proportion of methylated CpG will result in a loss of expression of the gene encoded by this stretch of DNA. Although it seems that HDAC could be solely responsible for the repression of gene transcription via recruitment to methylated CpG, this is not the case. When HDAC activity is inhibited, the transcription of the gene under study is not always (completely) restored [15–21].

In addition to deacetylation of histones, other proteins can also be deacetylated by HDACs, including p53, E2F, α-tubulin and MyoD, illustrating the complex function of HDACs in many processes in the cell [22,23].

Over the years, many different types of HDAC inhibitors (HDACi) have been developed, ranging from complicated structures of bacterial or fungal origin [trichostatin A (TSA), trapoxin] to the very simple butyrate. HDACi are capable of inhibiting HDACs with varying efficiency (at nanomolar to millimolar concentrations). Inhibition of HDACs can result in a general hyperacetylation of histones, which is followed by the transcriptional activation of certain genes through relaxation of the DNA conformation [24]. Generally, HDACi are known to be able to induce growth arrest, differentiation or apoptosis of cancer cells *in vitro* and *in vivo* [2,10,24–33]. DNA micro-arrays using malignant cell lines cultured in the presence of a HDACi indicated that a specific small number of genes (1–7%) showed altered expression [10,27,34,35]. Thus the effect of HDACi on gene expression is believed not to be a general one, but rather involves alteration of the transcription of a specific subset of genes [18,20,21].

The aim of this review is to give a comprehensive overview of the structure, function and tissue distribution of the classical HDAC family members, in order to gain insight into the regulation of gene expression via HDAC activity. Knowledge about the regulation of HDAC activity, as well as its level of expression and tissue distribution, is crucial in order to achieve a better understanding of changes in gene expression, e.g. during development or in malignancy. In this review we assess the tissue distribution of HDACs by using SAGE (serial analysis of gene

expression) databases on the Internet. In addition, a brief overview is given of HDACi and of the genes currently known to respond to HDACi treatment.

GENERAL OVERVIEW

Classification

There are two protein families with HDAC activity: the recently discovered SIR2 family of NAD^+-dependent HDACs (this family will not be subject of discussion in this review), and the classical HDAC family. Members of the classical HDAC family fall into two different phylogenetic classes, namely class I and class II (Figure 2) [4,36]. The class I HDACs (HDAC1, 2, 3 and 8) are most closely related to the yeast (*Saccharomyces cerevisiae*) transcriptional regulator RPD3. Class II HDACs (HDAC4, 5, 6, 7, 9 and 10) share domains with similarity to HDA1, another deacetylase found in yeast [4]. Recently a new member of the HDAC family has been identified, HDAC11 [37]. This protein contains all the necessary features to be designated as a HDAC, and although it is tempting to conclude from Figures 2 and 3 that HDAC11 is most closely related to the class I HDACs, no classification of HDAC11 to class I/II or the SIR2 family could be made, since the overall sequence similarity is too low [37]. Currently it is thought that HDACs of class I are expressed in most cell types, whereas the expression pattern of class II HDACs is more restricted (see Table 3), suggesting that they might be involved in cellular differentiation and developmental processes [38,39].

Localization

To exert their function, HDACs need to be in the nucleus, where their predominant substrate is found. The nuclear localization of HDACs occurs via a nuclear localization signal (NLS) or via co-localization together with other proteins/HDACs. Most HDACs contain a NLS, but some can be cytosolic as well; this depends on other regulatory domains. Class I HDACs are found almost exclusively in the nucleus (Figure 4). The localization of HDAC1 and HDAC2 is exclusively nuclear, due to the lack of a nuclear export signal (NES) [19]. HDAC3, however, has both a nuclear import signal and a NES, suggesting that HDAC3 can also localize to the cytoplasm. Interestingly, HDAC3 is nearly always localized in the nucleus in studies described so far, which might be explained in part by the recruitment of the HDAC3 complex by HDACs 4, 5 and 7 when they are bound to the DNA via co-repressors (Table 1) [36,40,41]. This possibly indicates a cell-type-specific domination of one signal over the other [41]. On overexpression of HDAC8 (necessary because of its low abundance), it could be demonstrated that this protein was localized in the nucleus [42].

Class II HDACs are able to shuttle in and out of the nucleus in response to certain cellular signals (Figures 4B and 4C). Figure 4(B) shows that the predominant localization of HDAC6 is in the cytoplasm [22,43]. HDAC11 resides in the nucleus; however, in activity assays, HDAC11 co-precipitates with the cytoplasmically localized HDAC6 [37,43]. HDAC10 can be localized in both the nucleus and the cytoplasm, although the function of the localization in both compartments has not been clarified [44,45]. The subcellular localization of HDAC9 can be cytosolic as well as nuclear, depending on the splice variant [43,46].

Figure 4(C) shows that the localization of HDACs 4, 5 and 7 is a carefully regulated process. The shuttling of HDACs 4, 5 and 7 between the cytosol and the nucleus has been studied extensively in differentiating muscle cells, resulting in a clear model [36,40,47]. Due to a (pre-) differentiation signal, HDAC4 is phosphorylated by Ca^{2+}/calmodulin-dependent kinase (CaMK), resulting in the export of HDAC4 together with CRM1, a cellular export factor for proteins with a leucine-rich NES. 14-3-3 protein (a cytosolic anchor protein) binds the phosphorylated form of HDAC4 and thereby retains HDAC4 in the cytosol. After fusion of muscle cells, terminal differentiation (post-differentiation) occurs, and HDAC4 is released from 14-3-3 due to a decrease in its phosphorylation status, and will consequently shuttle back to the nucleus.

HDAC5 resides in the nucleus during the proliferation of muscle cells (pre-differentiation) and is triggered to relocalize from the nucleus to the cytoplasm during differentiation. A mediator believed to be involved in this compartmental change

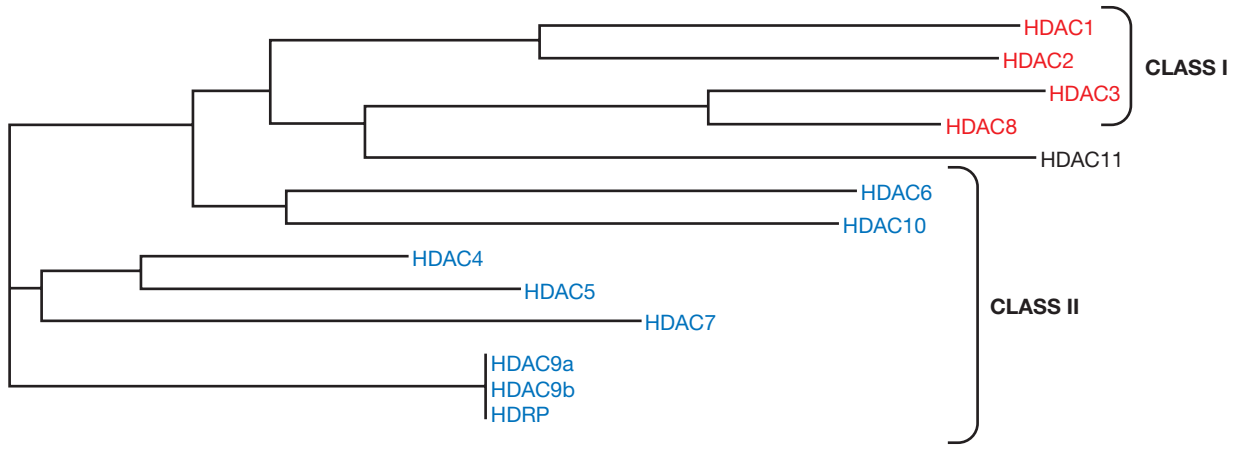


Figure 2 Evolutionary relationship between the HDACs

The actual distances may be greater than shown. The class I HDACs (red) are related to yeast (*Saccharomyces cerevisiae*) RPD3, and the class II HDACs (blue) are related to the yeast HDA1 enzyme. RPD3 is most related to HDAC1 and HDAC2; HDA1 is most closely related to HDAC6 [4,42,69]. From the phylogenetic tree, it can be concluded that HDAC9a, HDAC9b and HDAC9c/HDRP form a distinct group within class II that seem to be less related to other members of class II. HDAC11 (black) does not show enough identity with class I or class II HDACs to be placed in either class.

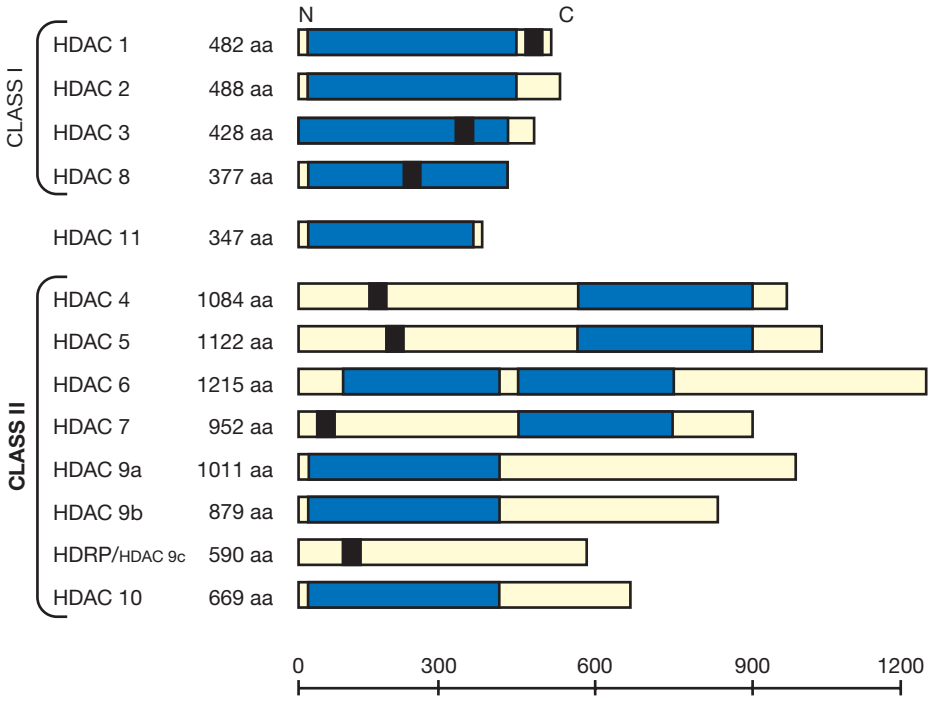


Figure 3 Schematic depiction of the different isoforms of HDAC

Bars depict the length of the protein. The catalytic domain is shown in blue. Note that HDRP does not possess any deacetylase activity. Black depicts a NLS. N, N-terminus, C, C-terminus. For further details, see [1,38,40,41,43,46,48–50,54,60,67,72,73,76,125].

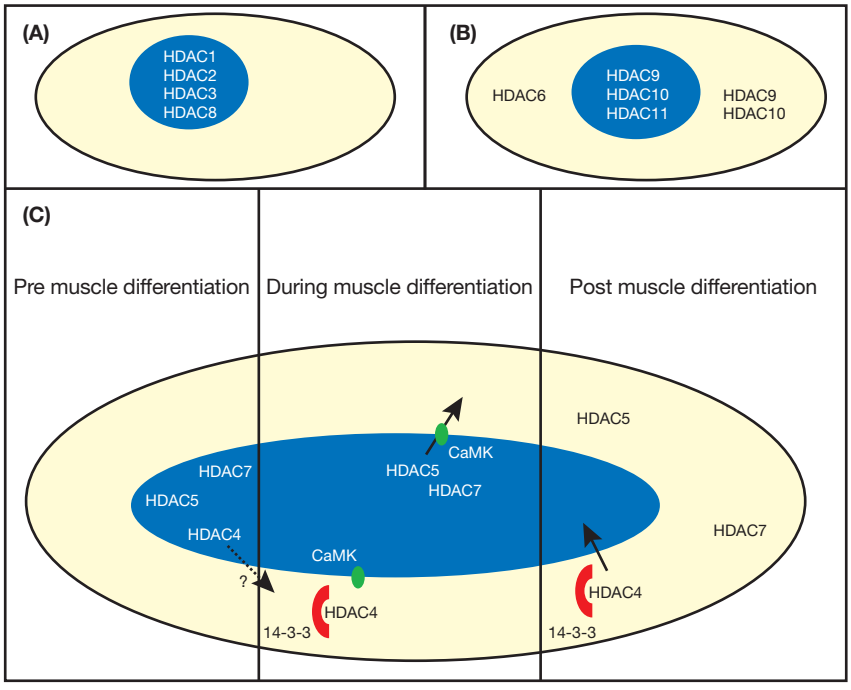


Figure 4 Localization of HDACs

The nucleus is depicted in blue. 14-3-3 protein (red) can retain HDAC4/5 or HDAC7 in the cytoplasm when they are phosphorylated. CaMK (green) is involved in nuclear export via the calcium/calmodulin signalling pathway. Subcellular localization of (**A**) class I HDACs and (**B**) class II HDACs is shown. (**C**) Shuttling of HDAC4, HDACs5 and HDACs7 during muscle differentiation.

Table 1 Co-repressors of HDACs

The co-repressors required for the functioning of HDACs are grouped into different functional classes. Some of the co-repressors appear in more than one class due to their dual function. Several co-repressors are not always necessary, depending on tissue type and transcriptional requirements. Some HDACs are not listed, since little is known about their co-repressor requirements. Nuclear hormone receptors [e.g. oestrogen receptor (ER), glucocorticoid receptor (GR), thyroid hormone receptor (TR) and retinoic acid receptor (RAR)] function as transcriptional activators, binding the respective responsive element directly, when ligand is present. In the case of SMRT/N-CoR binding and function, no ligand is bound to the receptors, and thus they function as transcriptional repressors. Recruiters bind DNA, are sequence-specific, are methylation-specific, or are intermediates between DNA and the HDAC complex. From the group of recruiters, only one is usually present; all the other groups represent necessary co-repressors of HDAC activity. Abbreviations: RBP, Rb binding protein; BRCA, breast cancer-associated susceptibility protein; NF-κB, nuclear factor-κB; MeCP, methylated CpG binding protein; MTA, metastasis-associated protein; SAP, Sin3-associated protein; TBL, transducin β-like protein; MBD3, methyl-CpG-binding-domain-containing protein 3; PML, promyelocytic leukaemia; DNMT, DNA methyltransferase; ERK, extracellular-signal-regulated kinase; CtBP, C-terminal binding protein.

	HDAC1 and HDAC2 [16–19,39,47,56–59,95,114,115]			HDAC3 [36,41,50,55,65–67]	HDAC4 [32,36,46,50,66,73]	HDAC5 [32,36,46,50,66,73]	HDAC7 [32,40,48–50,54]	HDAC9 [43,46,67]
	Sin3 complex	NuRD complex	Co-REST complex					
Histone binding	RbAp48	RbAp48		RbAp48				
Recruiters	Ikaros	MBD3		HDAC4, HDAC5, HDAC7, HDRP, YY1, Rb,	MEF2	MEF2, GATA-2	MEF2	MEF2
	YY1, RBP-1, Sp1, BRCA1, Rb, HDRP, heterochromatin protein-1, NF-κB, MeCP2, HDAC10			GATA-2, HDAC9, HDRP, HDAC10				
Nuclear hormone receptor binding	mSin3A, N-CoR, SMRT*			SMRT, N-CoR	SMRT, N-CoR	SMRT, N-CoR	SMRT, N-CoR	SMRT, N-CoR
Remodelling (helicase/ATPase family members)		Mi-2						
Necessary/modulates HDAC activity		MTA2, p70, p32,	Co-REST	SMRT/N-CoR	SMRT/N-CoR, HDAC3	SMRT/N-CoR, HDAC3	SMRT/N-CoR, HDAC3	
Unknown	RbAp46, SAP18, SAP30	RbAp46		TBL1				
Association as intermediate	Mad/Max, Mxi/Max, MeCP-1, ER, GR, TR, RPX homeodomain proteins, c-Ski, Sno, Aiolos, p53, NY-F, REST, Su(9)H1, PML-RARα, DNMT			CRM1, HDAC3	ERK1/2, CtBP, HDAC10	CtBP, HDAC10	CRM1, CtBP, HDAC10	HDAC1, HDAC3, SMRT, N-CoR

* SMRT does not interact directly with HDAC1.

of HDAC5 is CaMK (the 14-3-3 consensus binding domain is present), although, since HDAC5 also has a NES domain, it could not be confirmed that CaMK is solely responsible for the transport of HDAC5 out of the nucleus. Thus the shuttling of HDAC5 takes place in the opposite direction and on a different time scale when compared with that of HDAC4. Both HDAC4 and HDAC5 reside initially in the same compartment, but end up in the cytosol and the nucleus respectively.

HDAC7 has a very high degree of sequence similarity with HDAC5, except that HDAC7 does not have a NES domain. Like HDAC5, HDAC7 is able to shuttle from the nucleus to the cytoplasm during muscle cell differentiation, indicating that calcium signalling (14-3-3 protein binding) might again be involved. In cell types other than muscle cells, the regulation of the localization of HDAC5 and HDAC7 is less clear. In general, the localization of HDAC7 seems to depend on the presence of CaMK and 14-3-3 (other unknown proteins might also be required). When CaMK and 14-3-3 are present, HDAC7 resides in the cytoplasm; however, if both CaMK and 14-3-3 are not expressed, HDAC7 is found in the nucleus. Even though there is a large degree of similarity in function and localization between HDAC5 and HDAC7, both enzymes are found in the nucleus in some cell lines (CV-1, MCF7), whereas in other cell lines (HepG2) HDAC5 is located in the nucleus and HDAC7 is localized in the cytosol. The presence of the NES domain on HDAC5 might have a dominant role in determining the localization of HDAC5 in these cell lines, but clearly more research is needed to clarify these contradictory findings [43,48,49].

Mechanism of action

The mechanism of action of the HDAC enzymes involves removing the acetyl group from the histones comprising the nucleosome. Hypoacetylation results in a decrease in the space between the nucleosome and the DNA that is wrapped around it (Figure 1). Tighter wrapping of the DNA diminishes accessibility for transcription factors, leading to transcriptional repression (Figure 1) [1,3,50]. The catalytic domain of HDAC is formed by a stretch of $\sim$ 390 amino acids consisting of a set of conserved amino acids. The active site consists of a gently curved tubular pocket with a wider bottom [51]. Removal of an acetyl group occurs via a charge-relay system consisting of two adjacent histidine residues, two aspartic residues (located approx. 30 amino acids from the histidines and separated by approx. 6 amino acids), and one tyrosine residue (located approx. 123 amino acids downstream from the aspartic residues) [38,51]. An essential component of the charge-relay system is the presence of a Zn^{2+} ion. This atom is bound to the zinc binding site on the bottom of the pocket. However, other cofactors are required for HDAC activity (Table 1): most recombinantly expressed enzymes are found to be inactive. HDACi function by displacing the zinc ion and thereby rendering the charge-relay system dysfunctional. TSA, with its hydroxamic acid group and its five-carbon atom linker to the phenyl group, has the optimal conformation to fit into the active site [51]. TSA is the most potent reversible HDACi currently known, with an IC_{50} in low nanomolar range [52]. All HDACs are thought to be approximately equally sensitive to inhibition by TSA [19,32,50,53].

CLASS I HDACs

HDAC1 and HDAC2

HDAC1 and HDAC2 are highly similar enzymes, with an overall sequence identity of approx. 82% (Figure 2). The catalytic domain on the N-terminus forms the major part of the protein

Table 2 Chromosomal localization of HDACs and reliable tags used to determine expression

Data were obtained from the NCBI SAGE database (http://www.ncbi.nlm.nih.gov/UniGene/) in combination with the Human Transcriptome Map (http://www.amc.uva.nl/), freely available on the Internet. The numbers shown in Table 3 were generated on the basis of the tags. Tags shown are unique for the gene and are considered reliable. UTR, untranslated region; EST, expressed sequence tag.

Enzyme	UniGene cluster # (Gi#)	Chromosomal location	SAGE tags
HDAC1	Hs.88556 (12653070)	1p34	TCCAAAGTAA
HDAC2	Hs.3352 (4557640)	6q21	CTTTATGTGA
HDAC3	Hs.279789 (13128861)	5q31*	ACAATGACAA
HDAC4	Hs.91400 (13259519) UTR?	2q37	CAACCTCCAG CACGCCTGGG
HDAC5	Hs.9028 (13259520)	17q21	GAGCAGGAGC
HDAC6	Hs.6764 (15079316)	Xp11	GCAAGGTTGC TGTCCTCCCA
HDAC7	Hs.275438 (13259521)	12q13, a and b isoforms	7a: TTTTTGTAAA (7b is unclear)
HDAC8	Hs.112272 (EST) (8923768)	Xq13	TGGTCTAGTT
HDAC9	Hs.116753 [9a, 17158038; 9b, 17158040; 9c, 7662279 (= HDRP, 3882208)]	7p15–p21; three isoforms	TGGCTGAGCA
HDAC10	Hs.26593 (16903565)	22q13; two splice variants	CAACCCACGC
HDAC11	Hs.? (10438543)	3p25	

* From Blast searching and looking at UniGene clusters, it seems that HDAC3 has high identity with part of chromosome 11, which is possibly another HDAC-like gene.

(Figure 3) [1,6,54,55]. HDAC1 and HDAC2 are inactive when produced by recombinant techniques, implying that cofactors are necessary for HDAC activity to occur. *In vivo*, HDAC1 and HDAC2 only display activity within a complex of proteins. These complexes consist of proteins necessary for modulating their deacetylase activity and for binding DNA, together with proteins that mediate the recruitment of HDACs to the promoters of genes [56]. Three protein complexes have been characterized that contain both HDAC1 and HDAC2: Sin3, NuRD (nucleosome remodelling and deacetylating) and Co-REST (Table 1). Both the Sin3 complex (named after its characteristic element mSin3A) and the NuRD complex consist of a core complex containing HDAC1, HDAC2, Rb-associated protein 48 (RbAp48, which binds histone H4 directly) and RbAp46. The core complex alone does not possess maximal HDAC activity, and additional cofactors are needed (Table 1) [39,56–59]. In addition to functioning through these complexes, HDAC1 and HDAC2 can also bind directly to DNA binding proteins such as YY1 (Yin and Yang 1, a cellular nuclear matrix regulatory protein), Rb binding protein-1 and Sp1 [2,50,54,55,60–64].

In addition to the regulation of HDAC1 and HDAC2 activity by the availability of co-repressors, a second means of regulating activity is via post-translational modifications. Both activity and complex-formation are regulated by phosphorylation. HDAC1 and HDAC2 are phosphorylated at a low steady-state level in resting cells. Hyperphosphorylation of HDAC1 and HDAC2 leads to a slight but significant increase in deacetylase activity, and at the same time to disruption of complex-formation between HDAC1 and HDAC2 and between HDAC1 and mSin3A/YY1. When hypophosphorylation of HDAC1 and HDAC2 occurs, the activity of HDAC1 and HDAC2 decreases, but complex-formation is increased. The apparently contradictory consequences of phosphorylation maintain HDAC activity at a certain optimal level. Mutational analysis of HDAC1 shows that Ser^{421} and Ser^{423} are crucial phosphorylation sites; when they are mutated, complex-formation is hampered and HDAC activity is decreased [39,47].

HDAC3

HDAC3 is evolutionarily most closely related to HDAC8, with 34 % overall sequence identity, and HDAC3 has the same domain structure as all class I HDACs (Figures 2 and 3). In HDAC1 and HDAC2, the regions that correspond to amino acids 181–333 of HDAC3 are very similar to each other (93 % identity). However, the corresponding region in HDAC3 has only 68 % identity with HDAC1 and HDAC2. Surprisingly, the non-conserved C-terminal region of HDAC3 is required for both deacetylase activity and transcriptional repression. In addition to the NLS that other class I HDACs possess, a NES is also present in HDAC3 (amino acids 180–313). The balance between these two signals is probably dependent on cell type and on environmental conditions [41]. HDAC3 shares structural and functional features with other class I HDACs, but it exists in multisubunit complexes that are different from other known HDAC complexes. This could imply that individual HDACs have distinct functions due to their complex specificity.

SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) and N-CoR (nuclear receptor co-repressor) are necessary factors for HDAC3 activity (Table 1). (N-CoR and SMRT are two distinct, but highly related, proteins that share similar domain structure and function. Both act as co-repressors. See [43,54] for more details.) Both SMRT and N-CoR have a conserved deacetylase-activating domain for HDAC3 activation [65]. HDAC3 is able to form oligomers *in vitro* and *in vivo* with other HDACs [41]. Using overexpression coupled to immunoprecipitation, it was shown that HDAC3 can co-precipitate with HDACs 4, 5, and 7 through complex-formation with SMRT and N-CoR [40,41]. Endogenous HDAC3 mostly associates with itself, and only a small fraction of HDAC3 interacts with HDAC4. HDAC3 can also be found in a complex with HDAC-related protein (HDRP) (see HDAC9 below) [36,55,66,67]. HDAC3 shares the ability of HDAC1 to mediate the Rb–RbAp48 interaction, suggesting a mediating role of these HDACs in the cell cycle process. HDAC3, however, does not interfere with Rb–E2F activity, in contrast with HDAC1 and HDAC2 (Table 1) [57,68].

HDAC8

Of all the class I HDACs, HDAC8 is most similar to HDAC3 (34 % identity; Figure 2). HDAC8 consists largely of the catalytic domain with an NLS in the centre (Figure 3) [32,38,43,50]. Due to its very recent discovery, it is not yet known whether HDAC8 function is regulated by a co-repressor complex of proteins. Two different transcripts of 2.0 kb and 2.4 kb have been found.

Discrepancies with respect to chromosomal localization existed, as the gene has been mapped to Xq21.2–Xq21.3 and to Xq13, although the latter is now considered to be correct (Table 2). Northern analysis, after lengthy incubations, revealed a varying degree of HDAC8 expression in several tissue types, suggesting a very low abundance of HDAC8 mRNA [38,69].

CLASS II HDACs

HDAC4, HDAC5 and HDAC7

HDAC4, HDAC5 and HDAC7 are found in the same region of the phylogenetic tree, and represent a subgroup within the class II HDACs. HDAC4 and HDAC5 are most similar to each other (overall similarity 70%), but HDAC7 is also closely related ($\sim$ 58 and $\sim$ 57% overall similarity respectively) (Figure 2). All three HDACs have their catalytic domain on the C-terminal half of the protein, and the NLS is situated close to the N-terminus (Figure 3). Binding domains for C-terminal binding protein (CtBP), myocyte enhancer factor 2 (MEF2) and 14-3-3 are conserved in all three HDACs on the N-terminus [1,43]. Furthermore, HDAC5 has a NES within the catalytic domain, suggesting nuclear–cytoplasmic trafficking. HDAC4, HDAC5 and HDAC7 are able to interact with SMRT/N-CoR, and the co-repressors BCoR (Bcl-6-interacting co-repressor) and CtBP (Table 1).

The N-termini of HDAC4, HDAC5 and HDAC7 interact specifically with and repress the myogenic transcription factor MEF2. MEF2 plays an essential role, as a DNA binding transcription factor, in muscle differentiation [70]. When MEF2 is associated with HDAC4, 5 or 7, the function of MEF2 as a transcription factor is inhibited, thus blocking muscle cell differentiation. CaMK activity overcomes this inhibition by dissociating the MEF2–HDAC complex due to phosphorylation of HDAC4/5/7. Subsequently, transport (along with the cellular export factor CRM1) of the HDAC out of the nucleus can occur, illustrating another type of regulation of HDAC activity [65,71,72]. Interestingly, HDAC4, 5 and 7 associate with HDAC3 *in vivo* via co-recruitment to the SMRT/N-CoR factors in the nucleus (Table 1), with an absence of HDAC3 leading to inactivity. This suggests that HDAC4, 5, and 7 function as a link between DNA-binding recruiters and the HDAC3-containing HDAC complex [36,40,41].

The subcellular localization of HDAC5 and HDAC7 differs from that of HDAC4 at the different stages of muscle cell differentiation (Figure 4). These HDACs might complement each other in order to control the differential regulation of gene expression during the various stages of differentiation in muscle cells. These three HDACs are able to 'fine tune' the repression of gene expression due to the need for co-repressors for their activity, and even more intriguingly via their ability to change localization in response to a specific signal, thus providing a carefully regulated sequence of changes in gene expression during differentiation [32,36,40,43,46,48–50,54,63,72–74].

HDAC6

The phylogenetic tree shows that HDAC6 is evolutionarily most closely related to HDAC10 (Figure 2). In general, though, the identity of HDAC6 with other human HDACs is low, with some resemblance to yeast HDA1 (*Saccharomyces cerevisiae*) indicating an early separation from the other HDACs in evolution. HDAC6 is a rather unique enzyme within the classical family of HDACs, because it contains two catalytic domains arranged in tandem (Figure 3) [32,50]. Another unique feature of HDAC6 is the presence of a HUB (HDAC6-, USP3-, and Brap2-related zinc finger motif) domain on the C-terminus. This domain is a signal for ubiquitination, suggesting that this HDAC is particularly prone to degradation [43]. The catalytic domains of HDAC6 are most similar to the catalytic domain of HDAC9. HDAC6 functions as a tubulin deacetylase, regulating microtubule-dependent cell motility [22]. Although it resides predominantly in the cytoplasm (having a NES) to exert its function, HDAC6 is also found in the nucleus in a complex together with HDAC11. The function of HDAC6 has been little studied in comparison with other HDACs, but it seems to have many special and interesting features [32,42,43,50,75].

HDAC9

The phylogenetic tree in Figure 3 shows that HDAC9 splice variants are clustered as a separate group related to HDAC4/5/7 within class II of the classical HDAC family. The HDAC9 catalytic domain is located on the N-terminus, as for the class I HDACs. There are three known splice variants, HDAC9a, HDAC9b and HDRP/HDAC9c, but more variants are suspected (Figures 2 and 3) [46]. HDAC9c/HDRP lacks the catalytic domain and is 50% similar to the N-terminus of HDAC4 and HDAC5. By analogy with HDAC4/5/7, HDRP is able to recruit HDAC3, thus circumventing the lack of a catalytic domain. In addition, HDAC9 is also able to interact with MEF2 (CaMK/14-3-3), indicating that HDAC9 may have an important function in muscle differentiation. The alternative splicing might also represent another way of fine-tuning HDAC activity. Certain cell types might express one isoform, while others express the other [43,46,67].

HDAC10

HDAC10 is the most recently discovered member of the class II HDACs. Two mRNA species, with a slight difference in length, have been found, suggesting the existence of two splice variants of HDAC10 [44]. Analysis of protein sequence identity shows that HDAC10 is most closely related (37% overall similarity) to HDAC6 (Figure 2) [75]. HDAC10 has a catalytic domain on its N-terminus, and a NES and a putative second catalytic domain on the C-terminus. Also, two putative Rb binding domains have been found on HDAC10, suggesting a role in regulation of the cell cycle. Furthermore, HDAC10 is found to interact with HDACs 1, 2 and 3 (and/or SMRT) and HDACs 4, 5 and 7, but not with HDAC6, although some contradictory results are presented in the literature. The fact that HDAC10 is able to associate with many other HDACs indicates that it might function as a recruiter rather than as a deacetylase. However, when expressed by recombination, HDAC10 alone does show deacetylating activity [44,45,75,76].

HDAC11

From the phylogenetic analysis, it appears that HDAC11 is most closely related to HDAC3 and HDAC8, suggesting that it might be more closely related to the class I HDACs than to the class II HDACs (Figure 2). The classification of HDAC11, however, has not yet been determined, since its overall sequence identity with the other HDACs is limited. HDAC11 contains a catalytic domain situated at the N-terminus (Figure 3), with proven HDAC activity that can be inhibited by trapoxin (a TSA analogue). HDAC11 was found not to reside in any of the known HDAC complexes (Sin3, N-CoR/SMRT), possibly indicating a biochemically distinct function of HDAC11 [37].

TISSUE DISTRIBUTION OF HDACs

We used SAGE data from the AMC Human Transcriptome Map, available on the Internet, to generate a first indication of the tissue distribution of HDACs in normal and tumour tissue [77]. A general overview of HDAC expression specified by tissue type is given in Table 3. The numbers indicate the amounts of mRNA molecules that were found, standardized to 100000 tags. To enhance the reliability of the data, it will be necessary to verify these expression profiles by Northern analysis. HDAC11 expression was not included, since no UniGene cluster number or SAGE tag (Table 2) is available as yet.

The SAGE data for HDACs 1, 2, 3, 5, 6, 7 and 10 suggest that they are more or less generally expressed in all tissues examined. HDAC8 and HDAC9 seem to be expressed more in tumour tissues than in normal tissues, and this feature is even more striking for HDAC4. The lack of HDAC4 expression in normal somatic tissue could suggest that HDAC4 is not essential. The role of HDAC4 in muscle differentiation may, however, imply that HDAC4 is expressed only in embryonic muscle tissue, which is lacking from the databases. Unlike HDAC4, HDAC5 is expressed in heart tissue, together with HDAC7 and HDAC9, which is in agreement with their proposed function in muscle tissue. Surprisingly, HDAC expression in tumour tissue was only slightly higher than in normal tissue (increased in 37, decreased in 13 and the same in seven). Using Northern analysis, nearly all HDAC mRNAs have been detected in many other tissue types (for references, see individual sections). Northern analysis is a much more sensitive technique than SAGE, indicating that the expression of HDACs is generally rather low. Rare transcripts have a slight chance of not being present in a SAGE database due to a limited amount of tags sequenced. Thus the SAGE data show that HDAC expression is low and rather uniform, and that HDACs are almost equally well expressed in normal tissue and tumour tissue. A comparison between nine cell lines/primary tumour SAGE databases of neuroblastoma, however, showed a large variation in HDAC expression within this tumour category, ranging from no detectable HDAC expression to 31 tags/100000 for HDAC2 (results not shown).

It has also been reported that HDAC expression can change under the influence of HDACi and cell density [78,79]. Furthermore, since HDAC6 and HDAC8 are located on chromosome X, a chromosome known for its ability to undergo X-inactivation via deacetylation, it might be interesting to find out whether these HDACs are subject to or directly involved in X-inactivation [80]. Studies using overexpression and deletion of RPD3 and HDA1 (representatives of class I and class II HDACs) in yeast showed differences in specificity with regard to the deacetylation of lysine residues in histones H3 and H4. This might indicate that, due to differences in expression of the HDACs (classes I and II) in various cell types, different acetylation patterns could occur, resulting in different transcriptional regulation [81,82].

HDACi

It is currently thought that many HDACi function by blocking access to the active site (reversible or irreversible) of HDAC. There are many inhibitors known, but the most potent discovered so far is TSA. TSA is a fermentation product of *Streptomyces*. Originally TSA was used as an anti-fungal agent, but later it was discovered to have potent proliferation-inhibitory properties with cancer cells. TSA belongs to the group of hydroxamic acids, and is effective at nanomolar concentrations *in vitro* (Table 4). The ability of TSA to inhibit HDAC was reported for the first time by Yoshida et al. in 1990 [52]. Because the production of TSA is costly and highly inefficient (20 steps, resulting in a 2%

Table 3 General HDAC expression per 100000 tags by tissue type

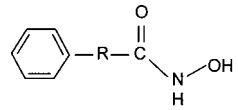
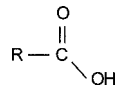
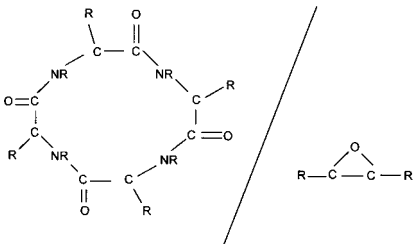
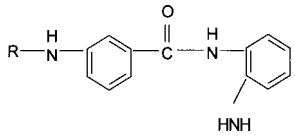
HDAC expression in various normal and cancerous cells is shown. The values are calculated averages. These expression data are based on the SAGE tags given in Table 2. 'All normal' and 'all tumour' represent the overall averages of all normal and tumour tissues respectively found in the Human Transcriptome Map (http://www.amc.uva.nl). ND, no data available.

	Expression (per 100000 tags)									
	Class I				Class II					
Tissue	HDAC1	HDAC2	HDAC3	HDAC8	HDAC4*	HDAC5*	HDAC6	HDAC7	HDAC9	HDAC10
Normal tissue										
All normal	1.4	3.0	1.85	0.2	0.01	0.42	4.5	2.2	0.55	1.15
Brain	0.3	1.6	0.8	–	–	7.2	4.5	0.8	0.8	2.38
Breast	–	–	4.3	–	–	4.0	8.6	2.9	–	3.3
Colon	7	5.0	–	–	–	4.6	4.0	–	–	2.0
Kidney	1.8	11.9	2.7	0.9	–	–	–	–	–	4.6
Ovary	4.1	3.1	5.2	–	–	–	4.1	9.3	2.1	2.9
Pancreas	0.7	1.5	3.0	0.7	–	–	2.2	1.5	–	–
Prostate	1.3	3.3	3.3	–	–	3.3	5.3	2.7	–	5.2
Heart*	–	2.3	–	–	–	9.5	2.3	2.3	1.1	–
Tumour tissue										
All tumour	1.75	4.1	1.95	0.55	0.19	3.4	5.5	2.15	1.0	1.3
Brain	1.9	3.8	2.5	0.9	6.1	6.1	7.2	1.5	1.7	3.1
Breast	0.2	3.0	1.1	–	–	–	8.9	3.2	–	4.3
Colon	1.8	2.3	1.5	0.6	1.6	3.7	2.0	1.5	2.6	2.5
Neuroblastoma	0.5	9.1	2.0	0.5	–	8.5	1.5	1.0	1.5	ND
Ovary	2.6	6.1	2.6	1.0	3.1	–	4.2	2.9	1.0	2.6
Pancreas	2.3	2.3	3.0	1.5	2.9	7.95	3.0	1.5	0.8	2.8
Prostate	2.1	3.2	1.9	0.2	2.4	3.8	2.6	1.6	–	2.95
Heart	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

* From NCBI UniGene; no hits in Human Transcriptome Map.

Table 4 HDACi

Shown are the features of four groups of HDACi, including *in vitro* efficiency of the members, and general structure. Oxamflatin, apicidine and depsipeptide (FK228) are cyclic hydroxamic acid-containing tetrapeptides, i.e. hybrids between hydroxamic acids and cyclic tetrapeptides. They are grouped in accordance with their major feature [83]. The cyclic tetrapeptides/epoxides form a heterogeneous group of compounds with a high degree of overlap, indicating that many of the members have both features.

Group and structure	Compounds	*In vitro* IC_{50} range
Hydroxamic acids 	TSA [8,24,26,27,32,35,64,79,85,86,101,102,107,108,112,116]	nM
	Suberoyl anilide bishydroxamide (SAHA) [8,10,32,85,86,112]	μM
	M-carboxycinnamic acid bishydroxamide (CBHA) [32,103,105]	μM
	Scriptaid [32,85,86]	μM
	Pyroxamide [32,98]	μM
	Oxamflatin [32,84–86,112]	nM
Short-chain fatty acids 	Butyrate [27,30,32,35,79,85,86,102,107,112,116]	mM
	Phenylbutyrate [8,9,25,85,88–90,117]	mM
	Valproic acid [26,32]	mM
Cyclic tetrapeptides/epoxides 	Trapoxin [32,33,85,86,112]	nM
	HC-toxin [116]	nM
	Chlamydocin [118]	nM
	Depudesin [32,85,86,111,112]	μM
	Apicidine [30,32,85,86,100,112]	nM–μM
	Depsipeptide (FK228) [31,32,85,86,92,112,122,123]	nM
Benzamides 	*N*-acetyldinaline (CI-994) [32,85–87,112,121]	μM
	MS-275 [32,85,86,110,112]	μM

yield), the search for alternative HDACi is ongoing and of high importance [83,84].

Today TSA is used mainly as a reference substance in research for newly developed HDACi. Many related compounds belonging to the hydroxamic acid group have been developed, but oxamflatin is the only compound in this group with similar *in vitro* potency to TSA (Table 4).

Another well known group of HDACi are short-chain fatty acids such as butyrate, phenylbutyrate and valproic acid. These compounds are far less efficient in their HDAC-inhibiting capability than TSA (millimolar compared with nanomolar range). Cyclic tetrapeptide antibiotics are the third group of inhibitors, and these compounds are characterized by their complicated structure and their high HDAC-inhibitory potential. Most of these compounds are products of bacteria or fungi, but apicidine and depsipeptide are composed of a chemically engineered combination of hydroxamic acids and cyclic tetrapeptides, as is oxamflatin. Benzamides, including CI-994 (*N*-acetyldinaline) and MS-275, comprise the final group of established HDACi.

Although the effects of HDACi in inhibiting HDAC activity are considered to be the same, there are two exceptions with regard to the mechanism of inhibition. All HDACi inhibit HDAC in a reversible fashion, except for trapoxin and depudesin, which inhibit the enzyme irreversibly through a different mechanism, namely via covalent binding to the epoxyketone group [32,83,85]. It is thought that almost all HDACs are approximately equally sensitive to the different HDACi. Exceptions are HDAC6 and HDRP, because of their 'abnormal' amount of catalytic domains. It has been reported that HDAC6, comprised of a double catalytic domain, is sensitive to one inhibitor (TSA; reversible), but resistant to inhibition by another (trapoxin; irreversible) [83]. Furthermore, it was discovered that class II HDACs are five times less susceptible to inhibition by valproic acid than are class I HDACs, and HDAC4 has been reported to be less sensitive to inhibition by butyrate. The development of new HDACi targeted at a specific HDAC, together with elucidation of the tissue distributions of the subtypes, might make a tailored use of HDACi possible [26,43].

Some of the compounds mentioned above have been entered into clinical trials [butyrate, phenylbutyrate, depsipeptide, pyroxamide, suberoyl anilide bishydroxamide (SAHA), valproic acid, CI-994]. In many studies, an increase in histone acetylation

Table 5 HDAC-responsive genes

Listed are genes whose expression has been shown to be affected by HDACi. No discrimination is made between increased and decreased expression. Abbreviations: RAR, retinoic acid receptor; MMP, matrix metalloproteinase; PKC, protein kinase C; HSP, heat-shock protein; ICAM, intercellular cell-adhesion molecule; HES, hairy and enhancer of split; hTERT, human telomerase reverse transcriptase; IL, interleukin; VEGF, vascular endothelial growth factor; CPA3, carboxypeptidase A3.

Function	Genes
Cell cycle	Cyclin A [64], cyclin E [19], cyclin B1 [64], cyclins D1 and D3 [19,104], $p21^{WAF1/Cip1}$ [27,64,98,109,122,123], p53 [79,97]
Apoptosis	CD95/CD95 [99], gelsolin [35,100], Bax [100,109], Bcl-2 [19]
Transcription factors	GATA-2 [27], c-Myc [107], RARα and β [102]
Other	ErbB1 [97], erbB2 [97], Raf-1 [97], MMP-2 [30], tob-1 [27], p55 Cdc [27], PKCδ [27], HM89 [27], HSP70 [27], ICAM-1 [27], STRA13 (related to HES genes) [106], hTERT [24,108], IL-6 receptor [109], IL-2, -8 and -10 [19], VEGF [120], Notch [102], CPA3 [124]

has been observed; this resulted in a partial response in some patients, but in a few patients a complete response was achieved. The proposed mechanism consists of the re-expression of silenced genes and/or the silencing of downstream genes due to the regained access of their promoters to other modulatory factors [8,9,31,32,85–93]. The intriguing results achieved in these clinical trials might contribute greatly to enhancing our understanding of HDAC function, although little is known as yet about the specific functions of the individual HDACs and the specific consequences of using HDACi in normal cells. Therefore studies investigating the mechanism of action of all HDACs, plus the global effects of using HDACi, are warranted.

RESPONSIVE GENES

By using HDACi in *in vitro* model systems, effects on the expression of many genes have been documented. The general effect often seen in cell lines is cytotoxicity, differentiation, inhibition of proliferation and induction of apoptosis. The mechanism by which changes in gene expression are thought to occur following HDAC inhibition is increased acetylation, resulting in increased recruitment of DNA-binding transcription factors, which in turn leads to increases in the expression of particular genes and decreases in the expression of other (downstream) genes. The amounts of acetylated histones (H4 or H3) increase in the presence of HDACi, as can be seen for some other acetylated proteins (e.g. p53, YY1). For example, the increased expression of p21 and of the luteinizing hormone receptor is only partly due to a general increase in acetylation, Sp1 sites in the promoter seem to be obligatory for reaching full induction of expression after HDACi treatment. This is possibly because HDAC1/2 can bind directly to Sp1, but Sp1 sites are also able to protect against DNA methylation, resulting in a more transcription-ready state of the promoter [16,94,95]. On the other hand, the possibility of a direct response via other (unknown) DNA binding proteins that use HDACi as a ligand cannot be ruled out as yet (Table 5). The changes in expression (either increases or decreases) of the genes listed in Table 5 are the result of inhibition of HDACs. It is clear that, by studying the effects of HDACi on the expression of various genes and their regulatory pathways, a more detailed picture will emerge of how the inhibition of HDACs, combined with the HDAC expression profile of that cell, ultimately determines the fate of the cell. Since it is not always entirely clear which sequence of events has culminated in the remission of disease in one patient and not in others, the results of *in vitro* studies will be indispensable for developing a better understanding of the mechanisms involved [10,24–30,34,51,59,79,83,93,96–112].

CONCLUDING REMARKS

HDACs appear to be key enzymes in the regulation of gene expression. HDAC function seems to be regulated by its intrinsic features, abundance, cellular compartmentalization and association with cofactors. Each cell type requires a specific gene expression pattern, thus prescribing a certain requirement of HDAC expression. Surprisingly, cellular transformation and oncogenesis did not result in major changes in HDAC expression. In the tumour tissues we examined, at least one HDAC proved to be present. The intra-tissue variation in HDAC expression might underlie the great variation in responses noted so far in *in vitro* studies and in clinical trials with HDACi. A wide variety of processes are associated with the inhibition of HDACs, such as apoptosis, necrosis, differentiation, inhibition of proliferation and cytostasis. In addition, drug resistance can be overcome, and the restoration of expression of silenced genes is known to occur. Although few studies have been performed with normal healthy cells, animal experiments and clinical trials have reported few or no side effects of the tested HDACi within the therapeutic range [8,31,98,101–103]. Last but not least, the efficiency of HDACi is greatly dependent on their stability *in vivo*. Most HDACi are not very stable due to their innate structure, and can also be readily degraded by first-pass liver metabolism [50].

Currently, many efforts are being made to expand our knowledge of the HDACs and to develop potent and stable HDACi [113]. In the future, this might give rise to the tailored use of HDAC-specific HDACi in order to dissect the complex functions of HDACs in a cell-type-specific manor.

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