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Perspective

Histone Deacetylase Inhibitors: From Bench to Clinic

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1. Introduction

The interest in histone deacetylase (HDAC[a]) inhibitors began almost 30 years ago during some studies designed to understand why dimethyl sulfoxide (DMSO) caused terminal differentiation of murine erythroleukemia cells.[1] This early observation paved the way for the development of novel pharmacological agents in the field of chromatin remodeling. In October 2006 the FDA approved the first HDAC inhibitor, SAHA (Figure 1), to treat the rare cancer cutaneous T-cell lymphoma (CTCL), and in the meantime there has been an aggressive development effort to bring HDAC inhibitors to the market for every major tumor type, both solid and hematologic, either as a single therapy or in combination, and a number of molecules are currently undergoing clinical trials (Figure 1). These compounds owe their antitumor action to their ability to reverse some of the aberrant epigenetic states associated with cancer. In spite of this, the mechanisms that underlie these effects are far from being completely elucidated. The research in this domain is intense, and our understanding of the biology involved is rapidly increasing.

Beyond cancer, novel additional therapeutic applications, such as neurodegenerative diseases and inflammation, have been proposed for histone deacetylase inhibitors.

A number of reviews has appeared recently in literature on HDAC inhibitors, mainly on the pharmacology and on the patent issues.[2] In this review we specifically focus on the medicinal chemistry efforts carried out in the field since the Miller paper of 2003, with some additional considerations concerning the biology and the pharmacology of these molecules.[3]

2. HDACs Biology

HDACs are a family of enzymes found in bacteria, fungi, plants, and animals that remove the acetyl group from the ϵ-amino groups of lysine residues present within the N-terminal extension of the core histones. This has the consequence that the positive charge density on the N-termini of the core histones increases, strengthens the interaction with the negatively charged DNA and blocks the access of the transcriptional machinery to the DNA template. Moreover, it has been hypothesized that histone modifications represent a code that can be recognized by non-histone proteins involved in the regulation of gene expression. The pattern of histone acetylation, regulated together with HDACs by histone acetyl trasferases (HAT), is the most extensively studied among these epigenetic mechanisms. Finally it has been found that many non-histone proteins that are part of transcription-factors complexes and regulate the cell-cycle and the cell death undergo lysine acetylation and deacetylation by HDACs and HATs. These modifications have profound effects on their biological function and/or their metabolic stability.

The HDACs belong to four structurally and functionally different phylogenetic classes: class I (HDAC-1, -2, -3, and -8) compounds are closely related to yeast RPD3; class IIa (HDAC-4, -5, -7, and -9) and class IIb (HDAC-6 and -10) share domains with yeast HDAC-1; class IV, recently described (comprising HDAC-11), exhibits properties of both class I and class II HDACs.[5] All the above HDACs are zinc dependent proteases.

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[a] Abbreviations: APL, acute promyelocytic leukemia; CAP, aromatic surface recognition motif; CTCL, cutaneous T-cell lymphoma; CU, connection unit; DMBA-TPA, 9,10-dimethylbenz[*a*]anthracene 12-*O*-tetradecanoylphorbol-13-acetate; DMSO, dimethyl sulfoxide; 5-FU, 5-fluorouracil; HAT, histone acetyl transferase; HDAC, histone deacetylase; HDLP, histone deacetylase-like protein; HIF-1α, hypoxia-inducible factor 1α subunit; PLZF, promyelocytic leukemia zinc finger; RA, retinoic acid; RAR, retinoic acid receptor; SAHA, suberoyl hydroxamic acid; TS, thymidylate synthase; TSA, trichostatin A; UBHA, uracil-based hydroxamates; ZBG, zinc-binding group.

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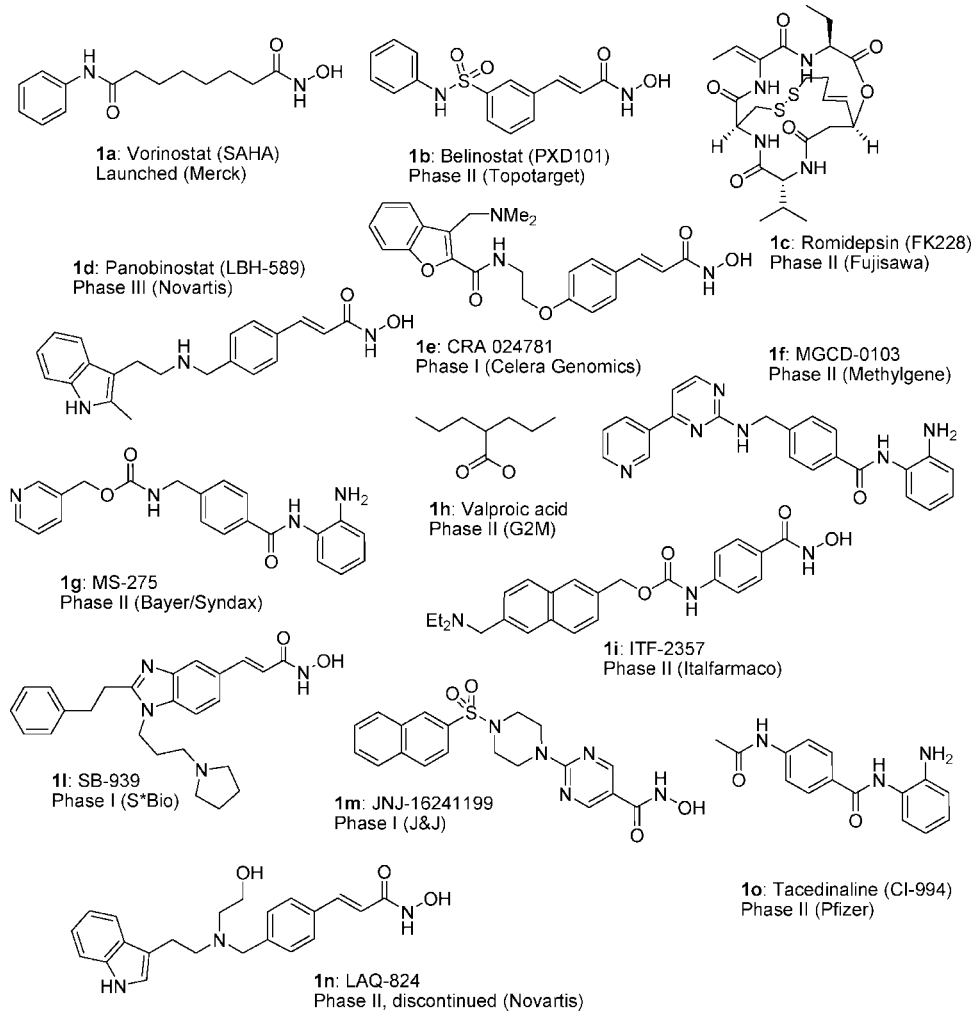


Figure 1. HDAC Inhibitors that have entered clinical trial.[4]

Class III HDACs have been identified on the basis of sequence similarity with Sir2, a yeast transcription repressor, and require the cofactor NAD^+ for their deacetylase function; up to now seven different sirtuins have been identified in mammals.[6]

Classes I and IV HDACs, mainly found in the nucleus, are expressed in many cell types, while the expression of class II HDACs, which are able to shuttle in and out of the nucleus, is restricted to some tissues. Sirtuins can have different cellular localization, depending on the isoform (cytoplasm, mitochondria and nucleus).

The exact physiological role of HDACs in cells is far from being completely elucidated, but a number of studies do exist that throw some light on the subject and many research groups are very active in the field. It was demonstrated first in yeast that the different HDACs have distinct biological functions and are recruited to specific regions of the genome.[7] Subsequently, by use of RNAi techniques, it has been proved that differentiation of function also occurs in higher eukaryotes such as *Drosophila*.[8] The division of labor among HDACs in mammals has been studied using knockout and transgenic mice and, again, RNAi techniques. The disruption of both HDAC-1 alleles in mice resulted in embryonic lethality due to proliferation defects and development retardation. Concomitantly, the expression of HDAC-2 and -3 was induced but could not compensate for HDAC-1 deficiency, thus suggesting a unique function for this enzyme.[9] In contrast, mice lacking HDAC-2 survive until the perinatal period when they succumb to a spectrum of cardiac defects.[10] Among other functions, HDAC-3 has been reported to be a transcriptionally independent critical regulator of mitosis.[11] In normal human tissues, HDAC-8 expression was found in smooth muscle cells, where it associates with the actin cytoskeleton and regulates the contractile capacity.[12] In contrast to class I HDACs, class IIa HDACs display tissue-specific expression and have a very long N-terminal extension that confers responsiveness to a variety of signal transduction pathways.[13] Knockout mice have been obtained for HDAC-4, -5 and -9. Mice lacking HDAC-4 display a remarkable phenotype characterized by chondrocyte hypertrophy leading to ectopic bone formation.[14] It has been demonstrated that in normal neurons HDAC-4 is a mediator of neuronal cell death.[15] HDAC-7 is a thymus-specific HDAC and inhibits the expression of Nur77 involved in apoptosis and negative selection during developing thymocytes.[16] HDAC-5 and -9 are instead involved in cardiac development because knockouts for these enzymes display cardiac hypertrophy.[17]

Class IIb subtypes 6 and 10 are characterized by a duplication of their catalytic domain; the better known isoform is HDAC-6, which deacetylates tubulin, microtubles,[18] and HSP90[19] (Table 1). Very little is known about HDAC-11.

Some of the biological functions of sirtuins have been elucidated and range from DNA repair to metabolism, but many questions remain to be answered.[4]

Table 1. Biological Functions of Class I and Class II HDACs

isoform	biological functions	model (ref)
	Class I	
HDAC 1	proliferation, regulation gene, expression apoptosis	knockout (mouse) (7), siRNA in tumor cells
HDAC 2	proliferation, cardiac morphogenesis	siRNA in tumor cells, knockout (mouse) (8)
HDAC 3	proliferation mitosis, regulation of INF expression	siRNA in tumor cells (9), siRNA in monocytic
HDAC 8	contractile capacity, proliferation regulation, telomerase activity	siRNA in human smooth muscle cells (10), siRNA in tumor cells
	Class IIa	
HDAC 4	skeletogenesis chondrocyte hypertrophy, mediator of neuronal death, repression of PLZF−RAR, HIF-1 stability	knockout (mouse) (12), siRNA in neuron, siRNA in promyelocytic leukemia, siRNA in renal carcinoma
HDAC 5	suppression of cardiac stress, signal, cardiac development	knockout (mouse) (15)
HDAC 7	regulation of apoptosis in developing thymocytes	siRNA in thymocytes (14)
HDAC 9	cardiac development	knockout (mouse) (15)
	Class IIb	
HDAC 6	status of tubulin acetylation, status of hsp90 acetylation	gene disruption in embryonic stem cells (16, 17), siRNA in telomerase immortalized normal human cells, siRNA in human embryonic kidney cells

3. HDAC Pharmacology

At present the main pharmacological application for HDAC inhibitors is the treatment of cancer.

The current research in cancer therapy focuses on the design of drugs specific against molecular alterations found only in the transformed cells. The aim is to associate a specific tumor type with a specific gene expression profile, thus defining the alteration responsible for each cancer. The molecular processes leading to the activation or repression of transcription are considered as possible targets for anticancer therapy.

HDACs have been implicated for the first time in cancer while studying acute promyelocytic leukemia.[20] Since then, HDAC silencing or inhibition has been shown to have an impact on cell cycle, cell growth, chromatin decondensation, cell differentiation, apoptosis, and angiogenesis in several cancer cell types. As examples, HDAC-dependent aberrant transcriptional repression as a pathogenic mechanism is implicated in non-Hodgkin's lymphomas[21] and in M2 subtype of myeloid leukemia.[22] Aberrant HDAC activity has also been identified in solid tumors.[23] The role of HDAC-1 was investigated in tumor cells using RNAi-mediated protein knockdown, and the involvement of this isoform in growth inhibition and in apoptosis was confirmed.[24] HDAC-2 has been associated with proliferation,[25] and increased expression of HDAC-2 has been found in human gastric cancer.[26] An antiproliferative effect was reported in cancer cells treated with siRNA for HDAC-3.[20] Inhibition of HDAC-4 by siRNAs in human renal cell carcinoma showed that the enzyme, together with HDAC-6, is a partner of hypoxia-inducible factor α-subunit (HIF-1α), probably regulating the acetylation level of the protein and consequently its stability.[27] In promyelocytic leukemia there is evidence that HDAC-4 represses the fusion protein PLZF-RARα.[28]

Multiple mechanisms have been described in tumor cells in an attempt to explain the anticancer activity of HDAC

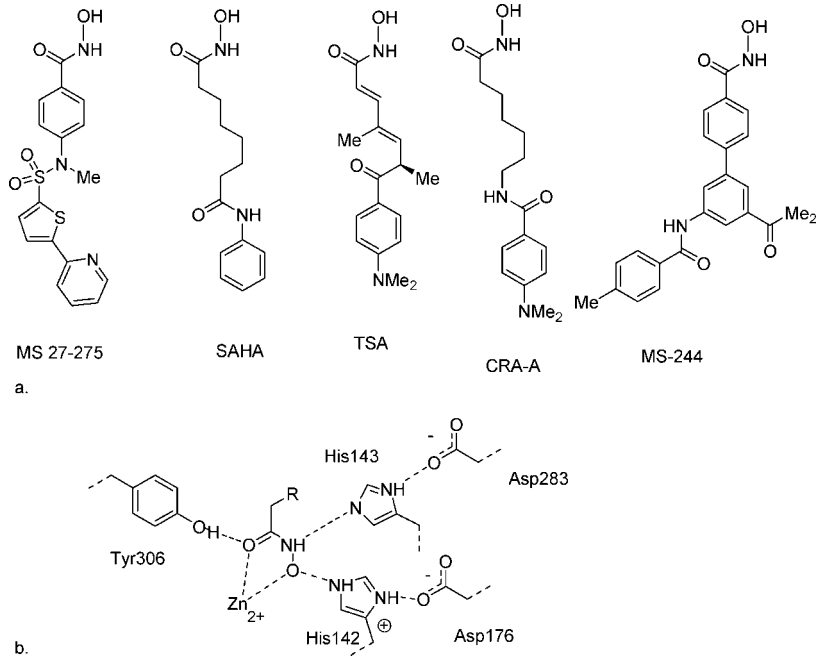


Figure 2. (a) The five inhibitors cocrystallized with HDAC-8. (b) Schematic diagram for the binding of the hydroxamate group into HDAC-8 active site.

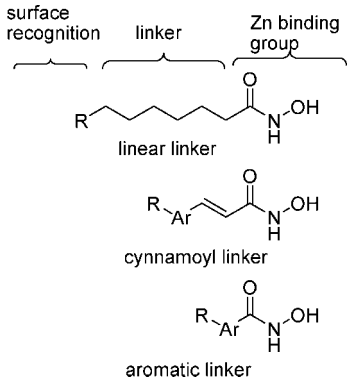


Figure 3. Linkers commonly found in the hydroxamate family of HDAC inhibitors.

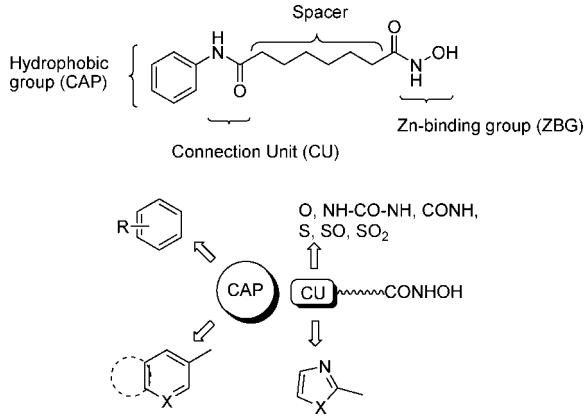


Figure 4. Structural components of SAHA and general structure of SAHA derived compounds.

inhibitors.[2c] Blocking HDAC activity should favor chromatin decondensation and a global increase in gene transcription; this could be a common mechanism for the induction of gene expression by HDAC inhibitors. In reality it appears that as many genes are transcriptionally up-regulated as are transcriptionally down-regulated. In this second case multiple pathways could be involved, employing many accessory proteins. Moreover, it has been proved that the antitumor activity of HDAC inhibitors is also related to the acetylation of non-histone proteins, in an often complex way. It also seems that the biological functions of HDACs are not always strictly dependent on their enzymatic activity.[2d]

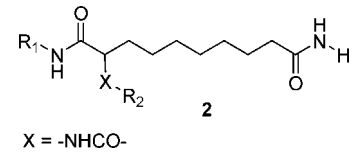
As will be shown later, the rationalization of these findings is extremely important because it may provide a foundation for the clinical trials and the development of combination therapies with classical cytotoxics as well as the new targeted agents.

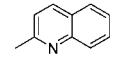
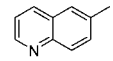
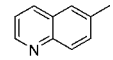
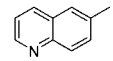
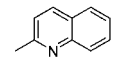
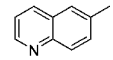
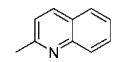
Beyond cancer, modulators of HDAC activity may be of interest in a number of novel therapeutic areas. One of them is immunomodulation. HDAC inhibitors have effects on the acetylation of key factors that regulate immune cell function. These acetylations are known to regulate the function of these inflammatory transcriptions. The net overall result is the suppression of proinflammatory cytokines such as TNFα and IL-1.[29] Another possible therapeutic effect is in stroke because of the fact that HDAC inhibitors increase the expression of neuroprotective proteins such as Hsp70 and Bcl-2 in the ischemic brain.[30]

With the use of partially purified enzymes and catalytically active recombinant HDACs, it has been shown that structurally diverse HDAC inhibitors can have a different activity on different HDAC classes or even specific isoforms. Considering inhibitors that have entered clinical trials (Figure 1), it has been reported that **1a**, **1n**, and **1b** are potent class I and class II HDAC inhibitors, while **1c**[31] and the 2-aminophenylamide derivatives (**1g**, **1o**, and **1f**) are somewhat selective versus class I HDACs.[32]

Concerning sirtuins, modulation of their activity could be perhaps useful to alleviate age-associated changes, since preliminary results indicate they have a role in human health and longevity. Nevertheless, the studies on their biological functions are still in a too early stage to provide clear therapeutic applications.[4]

Table 2. HDAC-1 and SC9 Cell Growth Inhibition of Selected SAHA Analogues from Ref 40



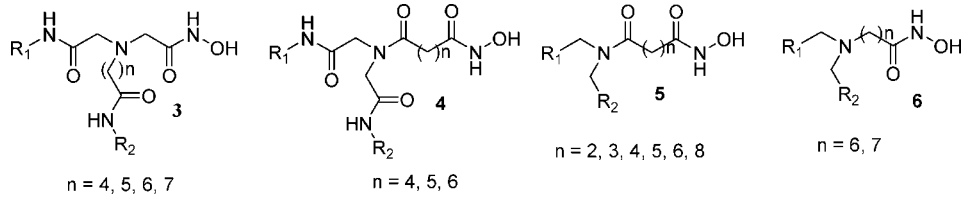
Cmpd	R_1	R_2	HDAC-1[a] IC_{50} (nM)	SC9 MTS[b] IC_{50}(nM)
SAHA	-	-	48	606
2a	C_6H_5	C_6H_5	12.1	73
2b	C_6H_5-CH_2	C_6H_5-CH_2O	48	412
2c	C_6H_5-CH_2		51.3	374
2d	C_6H_5-CH_2	C_6H_5	93	1039
2e	C_6H_5-CH_2		120	3423
2f		C_6H_5-CH_2O	7.7	110
2g		C_6H_5	3.5	90
2h			54	107
2i			1.3	756
2j			4	367
2k			42	165
2l		C_6H_5	9.4	43

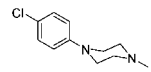
[a] Epitope-tagged human HDAC-1 complex immunopurified from stably expressing mammalian cells. [b] Murine erythroleukemia cells (SC-9).

4. Structural and Mechanistic Studies

The first structural information on Zn-dependent HDACs was derived from the crystal structure of histone deacetylase-like protein (HDLP), an HDAC-like protein from the bacterium *Aquifex aelicus*, complexed with SAHA and trichostatin A (TSA, Figure 2).[33] Only in 2004, 5 years later, did two independent groups publish the structure of human HDAC-8

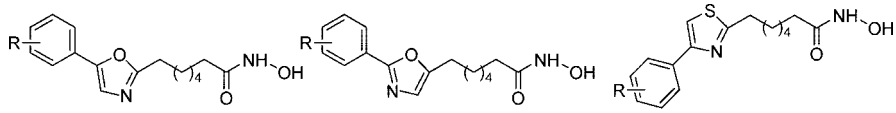
Table 3. HDAC and SC9 Cell Inhibition of Selected Diamine and Iminodiacetic Hydroxamic Acid Inhibitors



Cmpd	n	R_1	R_2	HDAC-1[a] IC_{50} (nM)	SC9 MTS[b] IC_{50} (μM)
SAHA	-	-	-	200	ND
3a	6	C_6H_5	C_6H_5	13±1.4	317
4a	5			1	ND
4b	5			9.5±4.9	50±27.7
4c	5	C_6H_5		25±21.2	46±10.9
4d	5			26.5±6.3	52±24.9
5a	6			36.5±4.9	555±164
6a	6	C_6H_5-CH_2	C_6H_5-$(CH_2)_2$	159±14.1	934

[a] Epitope-tagged human HDAC-1 complex immuno-purified from stably expressing mammalian cells. [b] Murine erythroleukemia cells (SC-9). ND = not determined.

Table 4. HDAC Inhibition of Selected Linear Chain Hydroxamic Acids Having Oxazole and Thiazole as CU



compd	R	IC_{50} (nM) HDAC[a]	IC_{50} (nM) HT1080[b]	compd	R	IC_{50} (nM) HDAC[a]	IC_{50} (nM) HT1080[b]
SAHA		140	2.4				
7a	H	9.8	8.41	**9a**	H	19	5.07
7b	*p*-CH_3O	2.1	0.31	**9b**	*p*-CH_3O	2.5	3.63
7c	*p*-Br	2.7	7.7	**9c**	*p*-Br	8.5	5.68
7d	*p*-ClC_6H_4	6.8	0.66	**9d**	*p*-Ph	11	1.32
8a	H	13	23% @ 50	**9e**	*p*-CF_3	42	2.39
8b	*p*-Br	4.4	27	**9f**	*p*-CF_3O	74	100% @ 50
8c	*p*-CF_3O	89	5.14	**9g**	*m*-Br	5.8	10.1
8d	*p*-Ph	12	15.3	**9h**	2,6-(CH_3O)	4.3	2.0
8e	*p*-F	23	76% @ 50	**9i**	β-naphthyl	4.3	2.39

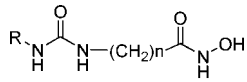
[a] Partially purified nuclear extracts from K562 erythroleukemia cells. [b] Human HT1080 fibrosarcoma cell line.

cocrystallized with five different inhibitors (Figure 2), finding similar results.[34] Very likely this delay was due to the fact that functional HDACs, except for HDAC-8, are never found as single monomeric polypeptides. They generally form high molecular weight multiprotein complexes where different subtypes are associated with specific coregulators as well as with other chromatin modifying enzymes.

The crystal structure shows a single compact α/β domain composed of a central eight-stranded parallel β-sheet and 11 or 14 α-helices. Approximately half of the amino acids are contained within the canonical secondary structure elements, while the other half forms loops that link the various elements of the regular secondary structure. A comparison of the overall structures of HDAC-8 and HDLP, the bacterial homologue of class I HDACs very often used as reference for homology models of human HDACs, reveals that major differences reside in the loop regions. It is very likely that structural variability in the loop regions emerging from the conserved core will be found across the entire family of HDACs, possibly reflecting the critical role of these regions in protein–protein interactions or in conferring substrate specificity to individual members of the family. Both research groups have found two monovalent cations in the HDAC-8 crystal structure, one near the active site and one at a distant site.

The HDAC-8 active site is a hydrophobic tunnel, about 12 Å long, that contains the catalytic site at the bottom. In every

Table 5. HDAC Inhibition for Selected SAHA Derivatives Having a Urea as the CU[45]



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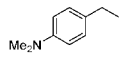
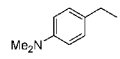
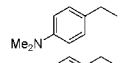
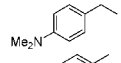
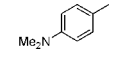
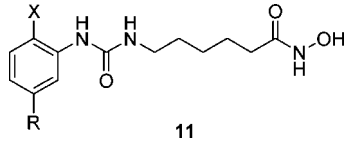
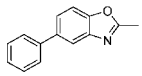
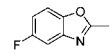
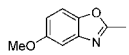
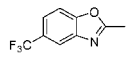
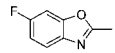
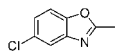
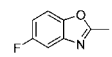
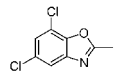
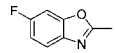
Cmpd	n	R	HDAC[a] IC_{50} (μM)	SQ-20B[b] IC_{50} (μM)
TSA	-	-	0.3	0.2
SAHA	-	-	0.7	3
10a	4		0.8	50
10b	5		0.8	25
10c	6		1	50
10d	7		0.7	50
10e	6		0.7	10

[a] Nuclear extracts from HeLa cells. [b] Human squamous carcinoma cells (SQ-20B).

Table 6. HDAC Inhibition for Selected SAHA Derivatives Having a Urea as the CU from Oxford Glycosciences

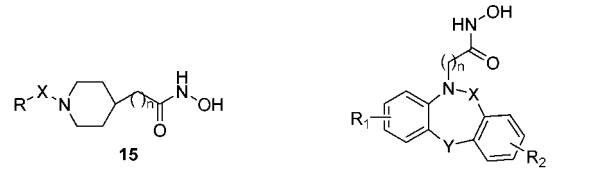


11

Cmpd	R	X	IC_{50} (nM)[a]
11a		H	8
11b		OMe	12
11c		OMe	400
11d		H	8
11e		H	12
11f		H	19
11g		H	9
11h		OMe	146
11i		OMe	15
11j		OMe	128
11k		OMe	18

[a] Nuclear extracts from HeLa cells.

crystal structure currently published this tunnel was filled with the aliphatic and lipophilic chains of the inhibitor. The amino acids forming the tunnel's wall (Phe152, Phe208, His180, Gly151, Met274, and Tyr306) are conserved across the class I HDACs except for Met274, which is a leucine in the other family members. Particularly interesting are the two side chains



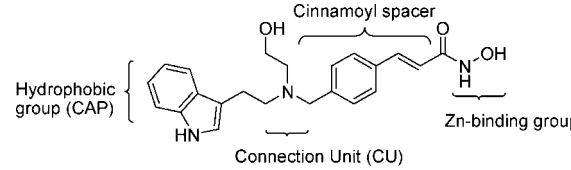
15

X = CO, NH-CO, SO_2, NH-SO_2
R = aromatic, heteroaromatic ring, alkylaryl
n = 1-3

16

X = CO, CS, SO_2, CH_2
Y = CH_2, $CH{=}CH_2$, $CHOR_3$, $CH{-}NR_4$, CO, O, S, SO, SO_2, NR_5
n = 2-9

Figure 5. Alkylpiperidino- and alkyltricyclohydroxamic acids.



1n

Figure 6. Structural components of **1n** and general structure of cinnamoyl derived compounds.

of the two phenylalanines, which lay almost parallel to one another. This has been exploited in inhibitor design, as shown below.

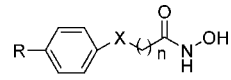
At the bottom of the tunnel lies a Zn^{2+} ion. The architecture of the active site is conserved between HDAC-8 and HDLP, and on the basis of the homology sequences, it must also be very similar to the other Zn-dependent HDACs. The Zn^{2+} is pentacoordinated with two sites occupied by the hydroxamic acid moiety of the inhibitors and the others by Asp178, His180, and Asp267. Immediately below the active site there is a cavity filled by several water molecules. Very likely it is used to eliminate the acetic acid molecule produced via amide hydrolysis.[35] In HDLP this pocket has a different shape and is 14 Å long. It has been proposed that this cavity may be used both to improve binding potency of the known inhibitors and to gain isoform selectivity.

The modes of binding of the inhibitors are quite similar for all five molecules. The hydroxamate is complexed by the Zn^{2+} ion, and the central hydrophobic portion (the so-called linker) fits into the hydrophobic tunnel. The terminal aromatic portion (the capping group) can either interact with the external portion of the enzyme or be completely solvent exposed. In effect, there are structural differences in the protein surface in the different complexes; they are essentially mediated by the loop 30–36 and suggest a certain flexibility for this region.

More recently the structure of a bacterial homologue of class IIb HDAC-6 (PDB code FB188) has been solved.[36] It exhibits the canonical fold of class I HDACs and contains a Zn^{2+} ion in the catalytic site. The highest structural diversity compared to class I enzymes is found in the loop regions, especially in the area around the entrance to the active site, indicating significant differences among the acetylated proteins binding to classes I and II HDACs.

A certain amount of structural work has also been devoted to gaining an insight into the isoforms' differences. In particular the Wiest group has built three-dimensional models of the four class I HDACs using the three-dimensional structure of HDLP (PDB code 13CR) as template.[37] The work has been validated by comparing it with the structure of HDAC-8, which became available during their studies. The four HDACs share the same active site and zinc binding

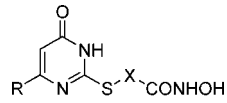
Table 7. Selected Arylsulfinyl- and Arylsulfanylhexanoic Hydroxamic Acids Hydroxamides



12

compd	X	*n*	R	HDAC IC_{50} (μM) purified liver	HDAC IC_{50} (μM) CEM cells	IC_{50} (μM) 50% viability in CEM cells
SAHA				0.44 ± 0.03	0.33 ± 0.05	1.9 ± 0.1
12a	S	5	H	0.12 ± 0.11	9.4 ± 1.9	>25
12b	SO	5	H	0.06 ± 0.02	7.4 ± 1.4	>25
12c	SO_2	5	H	0.04 ± 0.01	15.5 ± 7.4	>25
12d	S	5	Cl	0.26 ± 0.07	>25	>25
12e	SO	5	Cl	0.15 ± 0.01	1.3 ± 0.4	2.6 ± 0.2
12f	SO	5	Me_2N	0.18 ± 0.01	2.24 ± 0.1	3.4 ± 1.0
12g	SO	6	Cl	0.38 ± 0.01	1.6 ± 0.2	12.3 ± 3.0
12h	S	5	4Cl-$C_6H_4SO_2NH$	0.11 ± 0.01	9.4 ± 4	>25

Table 8. Mouse HDAC-1 Inhibitory Activity of Selected UBHAs

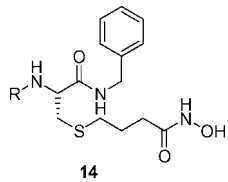


13

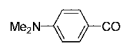
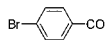
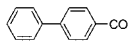
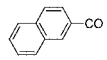
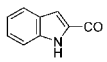
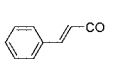
compd	R	X	mHDAC-1[a] IC_{50} ± SD (nM)
TSA			1.8 ± 0.07
SAHA			112 ± 4.0
13a	Ph	$(CH_2)_4$	40 ± 2.4
13b	Ph	$(CH_2)_5$	39 ± 1.6
13c	$PhCH_2$	$(CH_2)_5$	103 ± 4.1
13d	$PhCH(CH_3)_2$	$(CH_2)_5$	121 ± 4.8

[a] Partially purified HDAC-1 from mouse liver.

Table 9. Activity of L-Cysteine Hydroxamic Acid Analogues



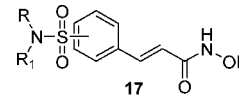
14

Cmpd	R	IC_{50} (μM) MM96L[a]	IC_{50} (μM) NFF[b]	Selectivity[c]
TSA	-	0.03	0.20	6.7
14a		0.14	0.35	2.5
14b		0.2	0.83	4.2
14c		0.9	2.8	3.1
14e		0.6	1.14	1.9
14f		0.13	0.8	6.2
14g		0.2	0.8	4

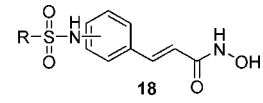
[a] MM96L, melanoma cells. [b] NFF, neonatal foreskin fibroblasts. [c] Ratio of IC_{50} values.

residues with HDLP. The difference between HDAC-1 and -2 is very small (93% sequence similarity), whereas the similarity between HDAC-1 and -3 is 73% and that between

Table 10. Cinnamoyl Derivatives Having a Sulfonamide as the CU



17 "reverse"



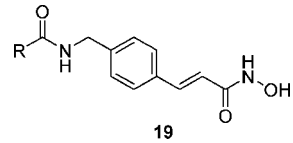
18 "forward"

compd		R	R_1	IC_{50} (nM)[a]
17a	meta	Ph	H	28
18a	meta	Ph		188
17b	meta	4-Me-C_6H_4	H	18
18b	meta	4-Me-C_6H_4		123
17c	meta	3,4-dimethoxyphenyl	H	23
18c	meta	3,4-dimethoxyphenyl		137
17d	meta	[1,1′-biphenyl]-4-yl	H	54
18d	meta	[1,1′-biphenyl]-4-yl		106
17e	meta	$PhCH_2$	H	34
18e	meta	$PhCH_2$		23%@1000
17f	para	Ph	H	29
18f	para	Ph		59
17g	para	[1,1′-biphenyl]-4-yl	H	66
18g	para	[1,1′-biphenyl]-4-yl		30
17h	ortho	Ph	H	na[b]
17i	ortho	naphthalen-2-yl	H	n.a.
17k	meta	Ph	Me	476

[a] Nuclear extracts from HeLa cells. [b] na: nonactive.

HDAC-1 and -8 is 31%. In all four models studied there is a single α/β domain that contains an eight-stranded parallel β sheet sandwiched between 13 and 17 α-helices. As already observed for the X-ray structure of HDAC-8, half of the residues are contained in this secondary structure and the other half resides in loops and turns. The residues critical for catalysis are highly conserved, leading to practically identical active sites. This suggests that in order to find selective inhibitors it would be better to concentrate on the external part of the binding site, which shows some small differences. An interesting overall difference is recognizable in the surface electrostatic potential. HDAC-1 and -2 display considerably more positive charge on the face containing the active site opening than HDAC-3 and -8, with these differences probably relating to target selectivity. The results from these efforts seem to point toward quite similar active sites for all the isoforms, apart from the aforementioned cavity behind the active site, and different flanking loop regions. Very likely, these regions, together with substrate discrimination, have the function to mediate the interaction with the proteins in the multiprotein complexes formed by HDACs (except isoform 8). Since we do not have structural information about these complexes, it can be expected that the

Table 11. Cinnamoyl Derivatives Having a Benzylamide as the CU

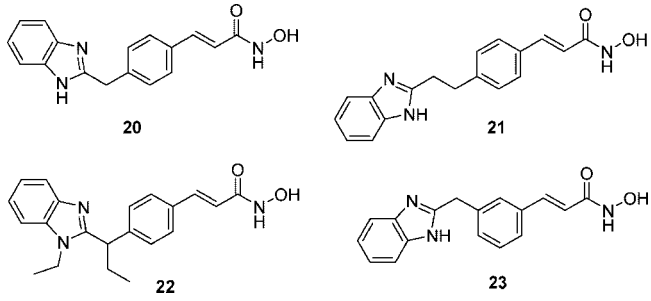


19

compd	R	IC$_{50}$ (μM) A549[a]	SK-BR-3[b]	MKN45[c]	HDAC[d] IC$_{50}$ (nM)
19a (SK-7041)	4-(dimethylamino)phenyl	0.48	0.16	0.83	172
19b	4-(pyrrolidin-1-yl)phenyl	0.35	0.11	0.80	205
19c	3-pyridyl	2.89	1.28	4.98	370
19d	2-pyridyl	2.23	0.87	3.64	941
TSA		0.08	0.02	0.10	53
1g		3.58	0.87	4.16	233

[a] A549, human lung cancer. [b] SK-BR-3, breast cancer. [c] MKN45, stomach cancer. [d] Nuclear extract from SNU-16 (human gastric adenocarcinoma).

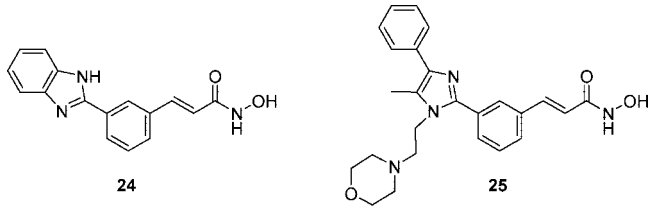
Table 12. Cinnamoyl Derivatives Having a Methylene as the CU and a Benzimidazole as the CAP



20 **21** **22** **23**

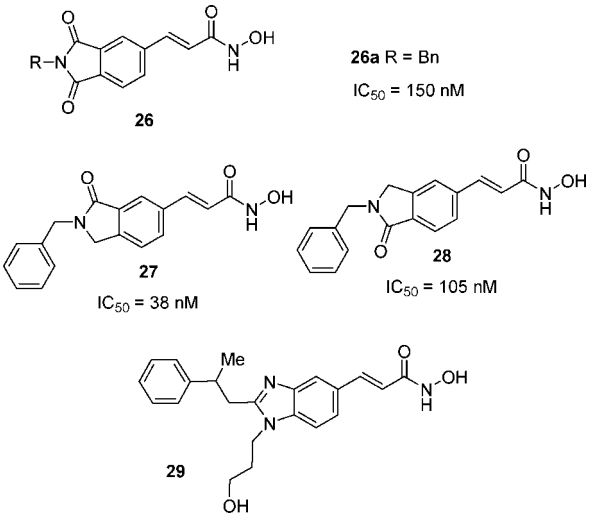
compd	IC$_{50}$ (nM) test 1[a]	test 2[b]
20	28	69
21	140	160
22	96	310
23	150	150

[a] Purified extract from human T-cell leukemia Jurkat cells. [b] T-cell growth inhibition.



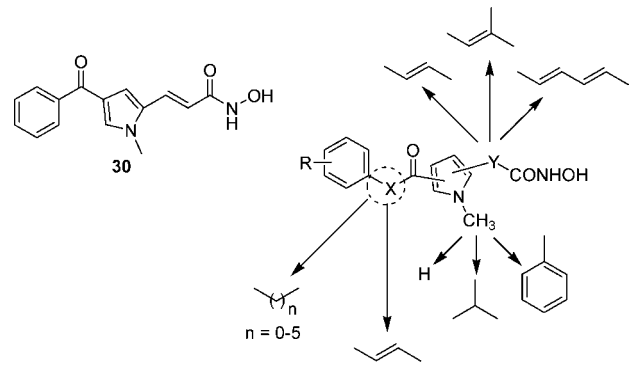
24 **25**

Figure 7. Benzimidazole (**24**) and imidazole (**25**) derivatives from Syrrx.



26 **27** **28** **29**

Figure 8. Compounds containing cyclic moieties as the CU.



30

Figure 9. Cinnamoylpyrrole derivatives.

planning of isoform selectivity from simple modeling of the external part would be quite a difficult task.

Some mechanistic hypotheses have also been put forward. Comparing the catalytic profile of some HDAC-8 mutants, Fierke and co-workers[38] concluded that His143 is used both as the general base and as the general acid necessary for amide cleavage. His142 appears to function as an electrostatic catalyst to stabilize the formation of the oxyanion intermediate.

The same research group has studied the catalytic activity of HDAC-8 with cations other than Zn^{2+}, putting forward the hypothesis that the in vivo counterion could be Fe^{2+} instead of Zn^{2+}.[39]

5. Small-Molecule Class I and Class II HDAC Inhibitors

Inhibitors of classes I and II HDAC enzymes have a general structure consisting of a zinc-chelating moiety, a linker, and an external motif, the so-called "surface recognition motif". They can be roughly divided into two main groups depending on the zinc-chelating moiety: the hydroxamic acid derivatives and the non-hydroxamic acid derivatives.

The first group is by far the richest. From recent literature (both papers and patents) it is clear that a further subdivision can be done depending on the nature of the linker: linear chain, cinnamoyl and aromatic, or heteroaromatic (Figure 3).

Considerable efforts have been done in the search for hydroxamic acid replacements, although the amount of published material is far less abundant. Three classes of putative zinc binding moieties can be distinguished: thiols and thiol derivatives, benzamides, and ketones. In addition, few examples of

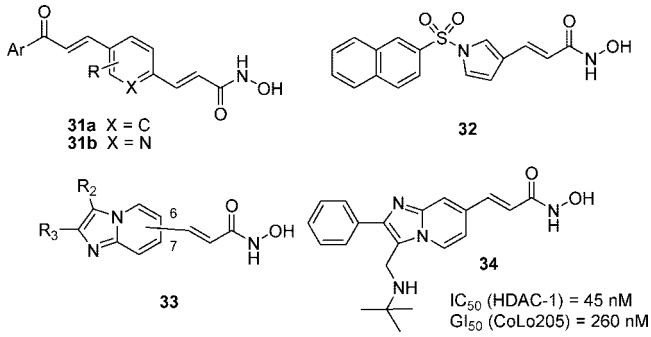


Figure 10. Miscellaneous aromatic cinnamoyl linkers.

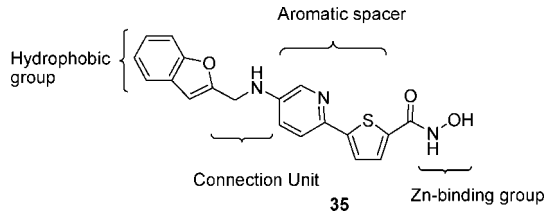


Figure 11. Structural components for inhibitors having a heteroaromatic linker.

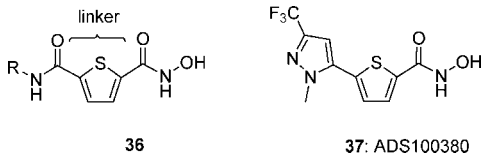


Figure 12. Early compounds containing a thiophene as linker.

compounds not possessing a canonical zinc binding group, but able to inhibit at least one HDAC isoform, have appeared in literature.

5.1. Hydroxamic Acid Inhibitors with a Linear Chain as Linker. A large part of the HDAC inhibitors reported to date consist of a hydroxamic acid and a five- or six-carbon hydrophobic linear spacer attached to an aromatic surface recognition motif (CAP) via a connection unit (CU) (Figure 4). SAHA is the earliest well-known example. Most of its binding energy comes from the polar interactions of the hydroxamic acid with the Zn^{2+} ion and from the hydrophobic interactions of the linear carbon chain with the wall of the binding cavity.

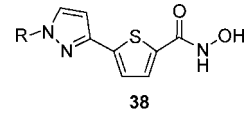
Many teams have worked on the hypothesis that modifications to the hydrophobic group and the connection unit, which are assumed to interact with the entrance area of the catalytic pocket, will provide opportunities for discovering more potent and possibly isoform-selective HDAC inhibitors.

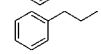
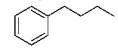
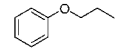
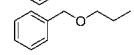
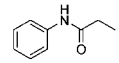
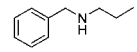
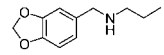
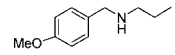
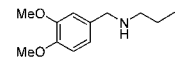
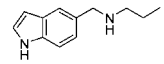
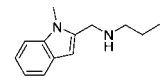
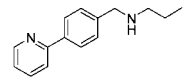
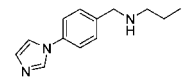
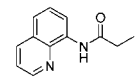
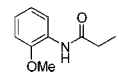
The same research group that discovered SAHA claimed SAHA-like compounds with α substitution on the terminal amide (**2**, Table 2).[40] Anilide and aminoquinolide containing compounds generally showed improved enzymatic and proliferation inhibitory activity compared to the corresponding benzylamine derivatives, and the 8-aminoquinoline component appeared to be optimal in the cellular assay.

Compounds **2a** and **2l**, when tested in an HCT-116 xenograft model in nude mice, showed tumor growth inhibition (TGI) greater than 50% at doses of 12.5 and 50 mpk, respectively, with less than 20% of body weight loss.

In subsequent patents, modified compounds of general formula **2**, with X = sulfonamide, urea, thiourea, and carbamate groups, have been claimed and reported to have generally

Table 13. HDAC Inhibition and Antiproliferative Assay Results for Pyrazolylthiophene Hydroxamates **37** and **38a**−**p**



Cmpd	R	HDAC (IC_{50}, μM)[a]	MCF-7 (IC_{50}, μM)	MKN45 (IC_{50}, μM)
37		0.750	11.4	31.5
38a		0.153	2.06	4.54
38b		0.100	1.24	2.91
38c		0.034	0.52	2.19
38d		0.022	0.50	1.50
38e		0.020	0.35	0.75
38f		0.021	0.12	0.84
38g		0.033	0.46	1.80
38h		0.029	0.30	0.63
38i		0.013	0.20	0.55
38l		0.107	1.58	4.50
38k		0.005	0.08	0.28
38j		0.006	0.17	0.37
38m		0.005	0.06	0.12
38n		0.009	0.19	0.56
38o		0.007	0.06	0.20
38p		0.008	0.12	0.23

[a] Nuclear extracts from HeLa cells.

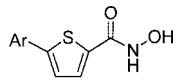
antiproliferative activity on the SC9 cell line in the concentration ranges of 1–10 and 40–100 nM.[41]

An additional patent from Aton Pharma (Merck & Co.) reported a novel class of hydroxamic acids still structurally related to SAHA (Table 3).[42]

Compounds of general structure **3** and **4** afforded the best results on the isolated enzyme with **3a** and **4a** being, respectively, 20- and 200-fold more potent than SAHA. The presence of two aromatic moieties seems to increase binding probably through additional hydrophobic interactions with the external part of the active site cavity.

Abbott (**7**−**9**, Table 4)[43] and SK Corporation[44] have both published compounds with a linear linker where the connection unit is a five-membered heteroaromatic ring. These compounds are generally very potent on the isolated enzyme with many examples in the low nanomolar range. Unfortunately, they show a large drop in potency on passing to cell systems. Reported IC_{50} values are, in the best cases, comparable to those of SAHA.

Table 14. HDAC and Antiproliferative Assay Results for Compounds **39a−c**



39

Cmpd	Ar	HDAC (IC_{50}, μM)[a]	MCF-7 (IC_{50}, μM)
37		0.750	11.4
39a		0.243	2.25
39b		1.13	-
39c		1.26	-

[a] Nuclear extracts from HeLa cells.

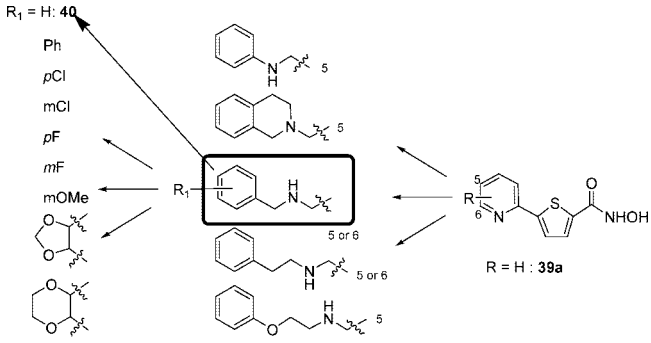


Figure 13. Optimization of lead compound **39a**.

SAHA derivatives where the CU is a urea have been published by two different research teams. The compounds from the Kozikowski group[45] (**10**, Table 5) have submicromolar potency on the isolated enzyme, while in human squamous carcinoma cells (SQ-20B) they are from 3 to more then 10 times less efficient than SAHA (10−50 μM). Phenylureas from Oxford GlycoSciences in many cases had low nanomolar potency on the isolated enzyme (**11**, Table 6) probably because of the large size of the hydrophobic CAP.[46]

Sulfur has also been used as the CU in the form of sulfoxide/sulfone. Marson and co-workers described the synthesis and the efficacy of arylsulfinyl and arylsulfanyl hexanoic acid hydroxamides.[47] Table 7 shows that most of the novel sulfur containing compounds exhibited IC_{50} values less than 400 nM in the purified enzyme assay. The optimal linker length consisted of five methylenes, and sulfoxides were generally found to be more potent than the corresponding sulfides in the enzymatic assay. The cell activity was somewhat disappointing.

Another class of compounds with sulfur in the CU are the uracil-based hydroxamates (UBHAs, **13**).[48] These derivatives were first tested on three maize deacetylases HD2, HD1-B (class I), and HD1-A (class II). In this case the optimal length for the linear chain was found to be four or five methylene units and the introduction of phenyl, benzyl, and 2-phenylethyl moieties at the C6 position of the uracil was highly beneficial. Selected UBHAs were tested on mouse HDAC-1 (Table 8). Most of the compounds, after incubation for 48 h at 1 μM with human myeloid leukemia U937 cells, caused the cell cycle to arrest in G1 phase.

Glenn and co-workers described a series of compounds based on a cysteine scaffold (**14**, Table 9).[49] A total of 6 of the 37 compounds tested had high nanomolar activity on a particularly aggressive human melanoma cell line (MM96L) and 4 had IC_{50} values of ≤200 nM, comparable to TSA on this cell line.

On normal human cells (NFF, neonatal foreskin fibroblasts) they showed a certain degree of selectivity. No data are reported on isolated enzyme inhibition, and on the basis of only on cell activity, it is not easy to establish a useful SAR.

The Menarini group concentrated part of its effort on a previously unreported linker, the 4-alkylpiperidine (**15**, Figure 5).[50] The hydroxamic acid was preserved at one end of the molecule, and a simple aromatic moiety was linked to the other end. The 3D geometry of the aromatic ring was varied through the use of different kinds of CUs such as amide, sulfonamide, carbamate, and urea. The compounds were assessed for enzymatic inhibition at 0.1 μM on nuclear extracts from HeLa cells (values from 42% to 82%) and then on human colon carcinoma cells (HCT-116) where the IC_{50} values ranged from 0.03 to 0.95 μM.

The same company worked on a new structural class of SAHA derivatives: *n*-alkylhydroxamic acids ω-substituted with tricyclic systems characterized by a central seven-membered ring (**16**, Figure 5).[51] The compounds were also tested on nuclear HeLa cells extracts at 0.1 μM for enzyme inhibition (reported values from 40% to 83%) and on HCT-116 cell line showing IC_{50} values that ranged from 0.03 to 2 μM.

5.2. Hydroxamic Acid Inhibitors with a Cinnamoyl Linker. The cinnamoyl spacer was revealed to be highly beneficial for inhibitory activity very early on in the history of HDAC inhibitors.

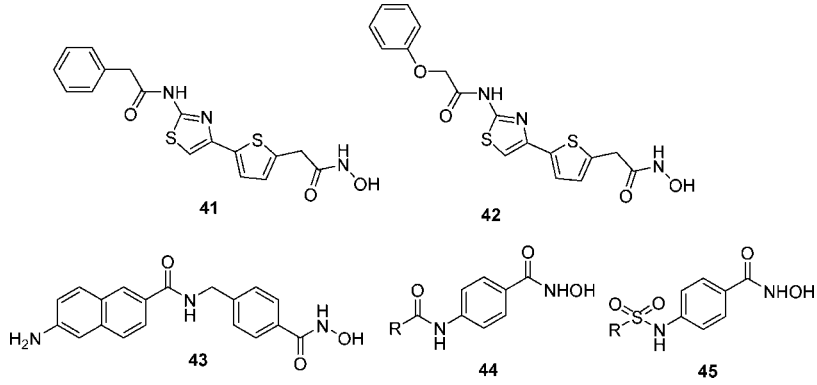


Figure 14. Thiazolyl−thiophene and phenyl hydroxamates.

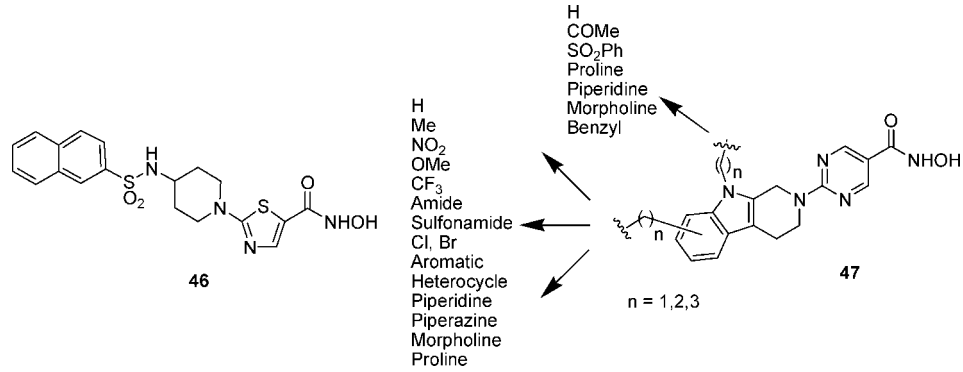


Figure 15. HDAC inhibitors from Mikana Therapeutics.

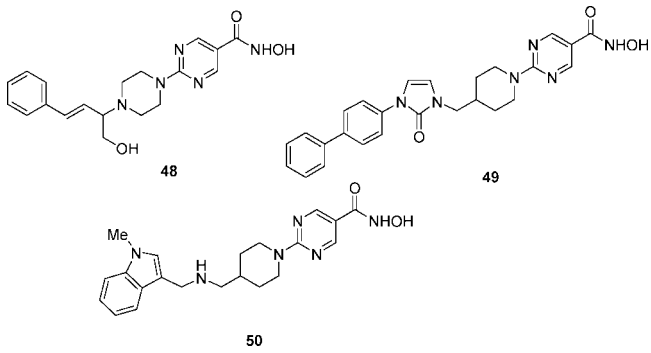


Figure 16. Selected examples of Janssen inhibitors.

In 2002 Novartis claimed the invention of a library of aminomethyl substituted cinnamoyl hydroxamates including **1n** (Figure 6) and **1b**, both of which have entered clinical trials. **1b** is at present in phase III, while **1n** was discontinued after phase II.

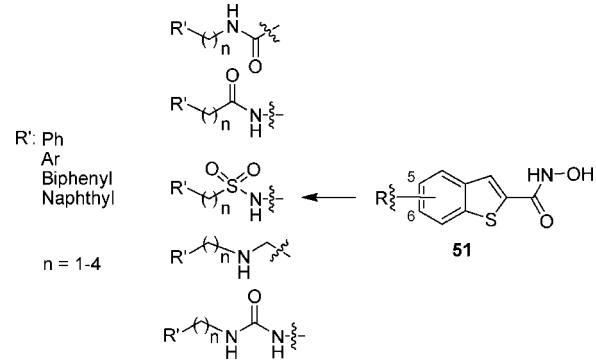
Later, Topo Target (Prolifix) reported the design and synthesis of a new class of HDAC inhibitors containing the cinnamoyl linker and a sulfonamide as the CU.[52]

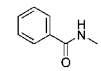
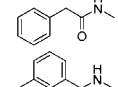
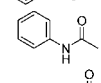
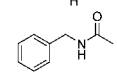
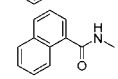
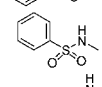
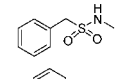
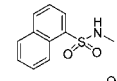
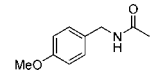
Forward- and reverse-sulfonamide refer to the structures with the N-atom of the sulfonamide respectively nearer (**18**) or further (**17**) from the hydroxamic acid bearing moiety (Table 10). As shown in Table 10, compounds having "reverse" sulfonamide functionality in the meta position (**17a**, **17b**, **17c**, and **17d**) showed a small but consistently better HDAC-inhibitory activity than their analogues with a "forward" sulfonamide functionality (**18a**, **18b**, **18c**, and **18d**). The same trend was shown when a methylene was introduced between the aryl group and the sulfonamide moiety (**17e** vs **18e**). In para-substituted compounds the difference between reverse and forward sulfonamides was very small (**17f** vs **18f**; **17g** vs **18g**). Ortho substitution led to inactive compounds (**17h** and **17i**).

Saturation of the C═C bond of the cinnamic moiety significantly reduced enzyme inhibition. From the extensive SARs, only partially reported in this context, compound **17a**, otherwise known as PXD101, was selected for clinical investigation and is currently in phase II.

SK Chemicals and In2Gen Co reported a series of cinnamoyl derivatives where the CU is a benzylamide (**19**, Table 11).[53] Antiproliferative activities of compounds **19a**−**d** against human lung cancer (A549), breast cancer (SK-BR-3), and stomach cancer (MKN45) cell lines were evaluated and compared to those of TSA and **1g**. An HDAC inhibition assay was also performed using a nuclear extract from SNU-16 (human gastric adenocarcinoma). On the basis of the encouraging results, **19a** and **19b** were selected as preclinical candidates.

Table 15. Selected Examples of Benzothiophene-2-hydroxamic Acids from Aton Pharma (Merck)



Cmpd	Position	R	HDAC-1 IC_{50} nM[a]
51a	-	H	672±98.9
51b	5		106.5±13.4
51c	6		203.5±48.7
51d	5		31±7
51e	6		11.5±2.1
51f	5		140±14.1
51g	6		79±11.3
51h	5		582±9.8
51i	5		76.0±21.2
51j	6		53.0±7.0
51k	5		1069.5±21.9
51l	6		46.5±23.3
51m	6		41±1.3
51n	6		26±1.4
51o	5		36±1.4

[a] Epitope-tagged hHDAC-1 complex immunopurified from stably expressing mammalian cells.

Fujisawa claimed the IP on a large set of cinnamic-type hydroxamates bearing benzimidazole residues as the hydrophobic terminal and methylene groups as connection units.[54] Four compounds were tested for human HDAC inhibitory activity

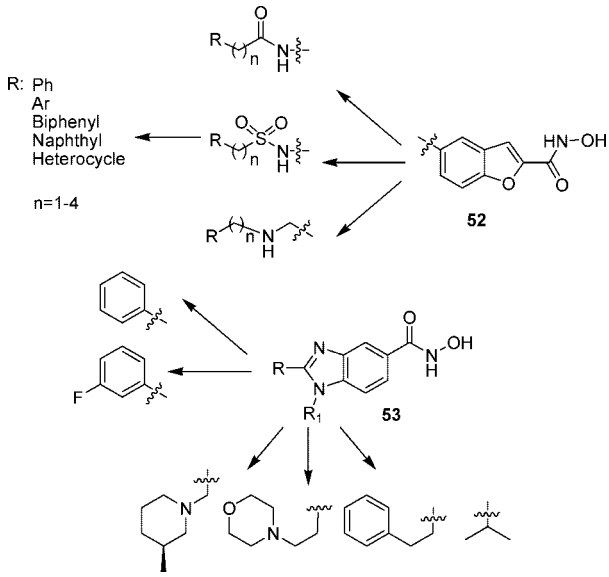


Figure 17. General structure of benzofuran-2-hydroxamic acids (**52**) and benzimidazole-5-hydroxamic acids (**53**) from Syrrx.

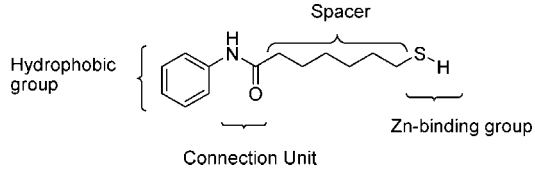
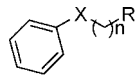


Figure 18. Structural components of thiol derivatives.

Table 16. HDAC Inhibition Data for Thiol-Based SAHA Analogues



54

compd	X	*n*	R	IC_{50} (μM)[a]
SAHA				0.28
54a	−NHCO−	6	−SH	0.21
54b	−NHCO−	6	−SAc	7.1
54c	−NHCO−	6	−SMe	>100
54d	−NHCO−	4	−SH	6.2
54e	−NHCO−	5	−SH	0.37
54f	−NHCO−	7	−SH	1.5
54g	−NHCO−	5	$-NHCOCH_2SH$	0.39

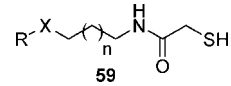
[a] Nuclear extracts from HeLa cells.

using HDAC purified from human T-cell leukemia Jurkat cells (test 1) and for T-cell growth inhibition in a blastogenesis test

Table 17. Growth Inhibition of Various Cancer Cells Using SAHA and **58**

	EC_{50} (μM)	
cancer cell line	SAHA	**58**
MDA-MB-231 (breast)	1.5	2.3
SNB-78 (CNS)	16	9.1
HCT116 (colon)	0.58	3.0
NCI-H226 (lung)	2.6	2.6
LOX-IMVI (melanoma)	1.3	1.1
SK-OV-3 (ovarian)	2.5	4.5
RXF-631L (renal)	2.0	2.4
St-4 (stomach)	5.2	5.0
DU-145 (prostate)	1.6	4.5

Table 18. HDAC Inhibitory Activity of Selected Mercaptoacetamides



59

compd	R	X	*n*	IC_{50} (μM)
TSA				0.0035
SAHA				0.080
59a	*p*-(Me_2N)Ph	−CONH−	3	0.20
59b	*p*-(Me_2N)Ph	−CONH−	4	0.45
59c	*p*-(Me_2N)Ph	−CONH−	$(CH_2)_n$═*p*-Ph	0.20
59d	8-quinolinyl	−CONH−	3	0.25
59e	C_6H_5	−NHCONH−	3	0.75
59f	C_6H_5	−NHCONH−	4	0.63
59g	8-quinolinyl	−NHCO−	3	0.044
59h	3-quinolinyl	−NHCO−	3	0.048

in vitro (test 2) (Table 12). No further biological data or special advantages have been presented regarding this class of compounds.

A class of inhibitors represented by benzimidazole derivative **24** (Figure 7) has been claimed by Syrrx (now part of Takeda).[55] A year later a second patent was published with a new family of derivatives reported to be more effective than prior inhibitors and represented by substituted imidazole **25**.[56] In both patents no biological data are presented.

In 2005 Miyachi and co-workers published the design and synthesis of a novel class of phthalimide-derived HDAC inhibitors, represented by general formula **26** (Figure 8); the potency on the isolated enzyme ranged from 7.8 to 177 nM.[57]

More recently novel cyclic amide/imide-bearing hydroxamic acids, coming from the selective reduction of one of the carbonyl groups of **26**, were designed.[58] Interestingly, the analysis of the two isolated regioisomers (**27** and **28**) indicated that the derivative bearing the carbonyl group in the meta position was 3 times more potent than the para regioisomer.

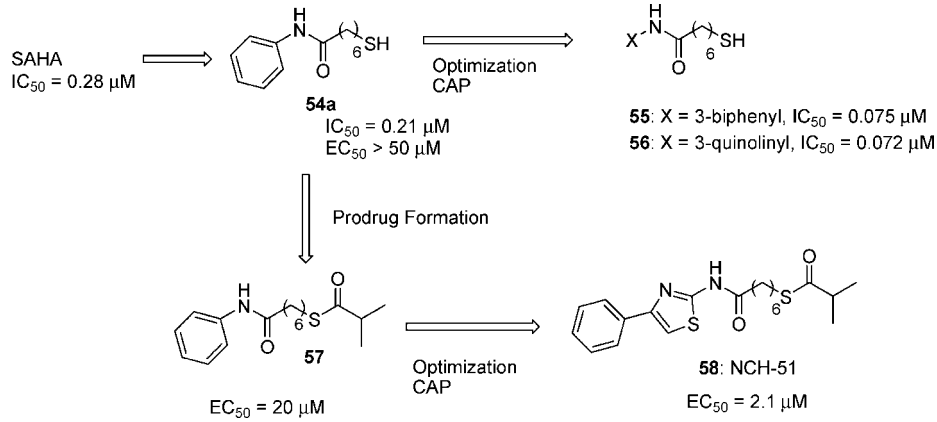
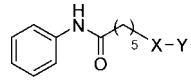


Figure 19. Thiol inhibitors and prodrug derivatives.

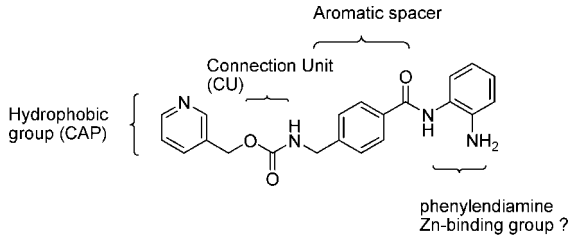
Table 19. IC_{50} Values for Mercaptoacetamide Analogues of SAHA on HDACs and HDAC-8



60

compd	X	Y	IC_{50} (μM) HDACs[a]	IC_{50} (μM) HDAC-8[b]
SAHA	$-CH_2CONH-$	$-OH$	0.37	0.82
60a	$-CH_2CSNH-$	$-OH$	>10	>10
60b	$-NHCOCH_2-$	$-OH$	>40	>40
60c	$-NHCOCH_2-$	$-SH$	2.44	3.89
60d	$-NHCSCH_2-$	$-SH$	13.3	>40
60e	$-CH_2COCH_2-$	$-SH$	0.15	0.69
60f	$-CH_2COCH_2-$	$-SAc$	0.081	0.19
60g	$-NHCOCH_2-$	$-SAc$	20.1	>40
60h	$-CH_2COCH_2-$	$-SCH_3$	>40	>40

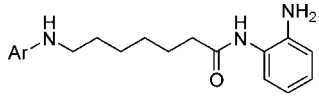
[a] Nuclear extracts from HeLa cells. [b] Purified human recombinant.



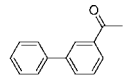
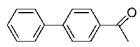
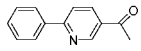
1g

Figure 20. Structural components of **1g** and general structure of compounds with a 2-aminophenylamide as the ZBG.

Table 20. Biological Activity of Selected 2-Aminophenylamides of ω-Substituted Alkanoic Acids



61

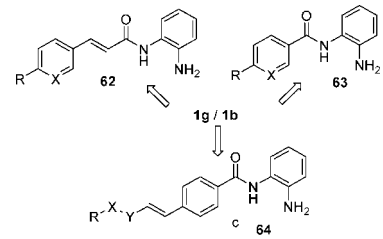
Cmpd	Ar	HDAC-1[a] IC_{50} (μM)	HCT-116 IC_{50} (μM)	H4-Ac EC_{50} (μM)
1g	-	1	0.5	1
61a		1	12	25
61b		2	7	10
61c		3	5	5

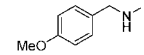
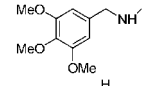
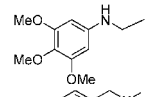
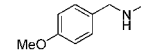
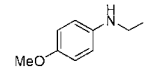
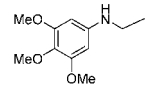
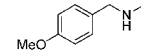
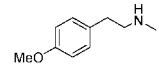
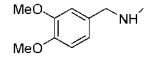
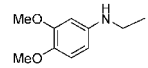
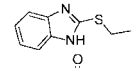
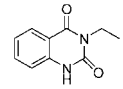
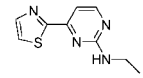
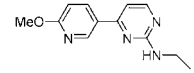
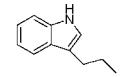
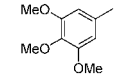
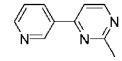
[a] Recombinant HDAC-1.

Cinnamoylhydroxamic acids containing a heteroaryl moiety have been proposed by various research groups. Benzimidazole derivatives, showing excellent anticancer activity and an ability to promote differentiation and apoptosis, have been disclosed by S*BIO.[59] Among more than 100 described examples, compound **29** showed IC_{50} = 51 nM on HDAC-1 and IC_{50} = 119 nM on HDAC-8. It is claimed that, after in vivo experiments, the compounds were generally well tolerated with no obvious sign of toxicity and showed a reduction in tumor volume relative to the control.

Massa and Mai reported the design and synthesis of a series of HDAC inhibitors where the aromatic portion of the cinnamoyl moiety is a pyrrole. Starting from compound **30** (Figure 9), a

Table 21. SAR of Selected Pyridin-3-ylacrylamides (X = N) and 2-Aminophenylcinnamides (X = CH) (**62**), 2-Aminophenylbenzamides (X = CH) and 2-Aminophenylnicotinamides (X = N) (**63**), and 4-Vinylbenzamides (**64**)



Cmpd	X	Y	R	IC_{50} (μM) HDAC-1	IC_{50} (μM) HCT-116
62a	N		$C_6H_5CH_2NH-$	3	1
62b	N			2	3
62c	N			4	2
62d	N			3	3
62e	CH			4	37
62f	CH			4	12
62g	CH			2	2
63a	N			4	0.5
63b	N			3	0.8
63c	CH			4	0.6
63d	CH			2	0.4
63e	CH			0.3	0.32
63f	CH			2	0.6
63g	CH				
63h	CH			0.6	0.1
64a	NH	CO		3	0.07
64b	NH	CO		1	0.3
64c	NH	CH_2		4	0.1
64d	NH	CH_2		2	0.2

very large amount of work has been published on the chemical manipulations.[60] It is worth noting that the optimization work has been done on the maize HD-2. This enzyme, albeit structurally quite different from the mammalian HDACs, has

Table 22. In Vivo Antitumor Activity of **62g** and **63d** in Nude Mice Xenograft Models

	% tumour growth inhibition relative to vehicle control					
	po route			ip route		
	A549[a]	PANC1[a]	SW48[a]	A549[a]	HCT116[a]	SW48[a]
1g				46 (20 mg/kg)	56 (20 mg/kg)	68 (20 mg/kg)
62g	40 (70 mg/kg)		16 (60 mg/kg)		80 (20 mg/kg)	73 (25 mg/kg)
63d	67 (50 mg/kg)	84 (50 mg/kg)	78 (30 mg/kg)	73 (40 mg/kg)	86 (75 mg/kg)	100 (75 mg/kg)

[a] A549 (non-small cell lung), PANC-1 (pancreatic), SW48 and HCT-116 (colon).

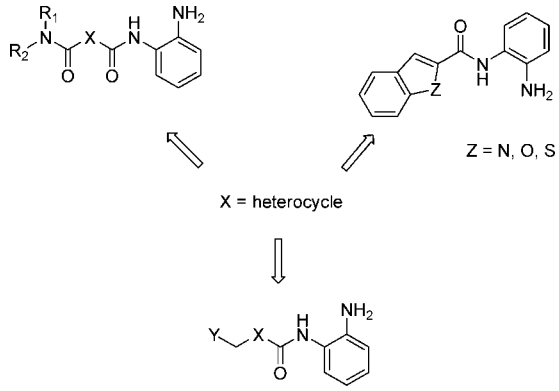


Figure 21. 2-Aminophenylamides from Hoffmann-La Roche.

been shown to be a good predictive model for the behavior of class I mammalian HDACs.

The same authors are the inventors of cinnamoyl HDAC inhibitors of a novel series recently claimed by DAC.[61] These compounds are analogues of **31**, where the aromatic portion is a pyridine or again a phenyl (Figure 10). Six compounds of this new class were shown to block in vivo papillomas formation induced by a mixture of 9,10-dimethylbenz[*a*]anthracene 12-*O*-tetradecanoylphorbol-13-acetate (DMPA-TPA).

Altana has released three patents relative to the invention of a novel class of sulfonylpyrrole derivatives represented by compound **32**. No specific results are provided.[62]

In summary, a series of structurally analogous molecules have been proposed by S*Bio in 2006.[63] In this case, the α,β-unsaturated hydroxamic acid functionality was linked to the pyridine ring of a imidazo[1,2-*a*]pyridine (**33**) system as shown in Figure 10. Many compounds were reported having an IC_{50} of <0.2 μM on the isolated enzyme, with the specifically claimed derivative **34** exhibiting a GI_{50} of 0.26 μM on the CoLo205 cell line.

5.3. Hydroxamic Acids Inhibitors with an Aromatic or Heteroaromatic Linker. A number of HDAC inhibitors containing an aromatic or heteroaromatic moiety as linker unit (**35**, Figure 11) have appeared in the literature and patents.

One of the earlier examples of HDAC inhibitors belonging to this class was compound **36**, reported by Roche in 2004. The thiophene core scaffold used as linker (Figure 12) represented the main novelty. The potency on the isolate enzyme of the selected examples was in the low nanomolar range, with a couple of subnanomolar compounds, while the cytotoxicity on HT29 384 human carcinoma cells ranged from 920 to 10 nM. The same company released three additional patents in 2005, extending the previous studies.[64]

Still in 2004 Argenta Discovery published a patent describing examples of molecules with a thiophene as linker, which was then followed by an additional two.[65] In this case the thiophene was linked to a second heterocycle like pyridine, pyrimidine, imidazole, or pyrazole. The details of the identification and optimization of these compounds have recently been published.[66] On the basis of virtual screening studies, the first set of 75 compounds was prepared and tested in a HDAC enzyme assay, from which **37** was identified as a submicromolar HDAC inhibitor.

For the subsequent optimization, different lipophilic groups were introduced onto one of the pyrazole nitrogens (Table 13). By pursuit of this strategy, notable results have been obtained in the in vitro assays with low nanomolar compounds like **38k**−**p**. Experiments on compound **38h** revealed efficacy in mouse HCT116 xenograft studies. However, in vitro experiments showed that it had significant cytochrome P450 3A4 inhibition (IC_{50} = 0.51 μM) and lower than ideal Caco-2 permeability, the latter being consistent with the poor oral bioavailability observed. Replacement of the pyrazole ring with another aromatic or heteroaromatic ring was explored. Of the three possible isomers of pyridine (**39a**−**c**, Table 14), the ortho derivative (**39a**) gave better results than **37** on the isolated enzyme and in the MCF-7 cell line.

Optimization of the new lead **39a** was performed through the incorporation of additional substituents onto the pyridine ring (Figure 13). On the basis of the pharmacological results and structural simplicity, the first exploration led to the selection of compound **40** as prototype for further profiling. **40** was more active than both SAHA and ADS102550 in the enzyme inhibition test and in the MCF-7, MDA-MB231, and HCT-116 cell proliferation tests.

Novel biaryl linked hydroxamates as HDAC inhibitors have also been proposed by S*Bio in 2005.[67] Many of the claimed examples are heterobiaryl compounds bearing a thiazolyl−thiophene as the core element. Several compounds were selective for HDAC-8. For example, compound **41**(Figure 14) is claimed to have an IC_{50} value greater than 100 μM on HDAC-1 and an IC_{50} of 41 nM on HDAC-8 whereas compound **42** has an IC_{50} of 700 nM on HDAC-1 and 38 nM on HDAC-8.

A number of research groups have claimed the simple phenyl ring as linker.

Osaka Industrial Promotion proposed in 2003 a class of hydroxamate derivatives represented by compound **43**. IC_{50} values ranging from 30 to 240 nM are reported for the series, while **43** showed marked in vivo antitumor effects against mouse HT-29 tumor cells at a dose of 100 mg/kg.[68]

Merck in 2006 published derivatives **44** and **45**, where the CU is an amide and a sulfonamide, respectively, in the para position of the aromatic ring.[69] The capping hydrophobic group was mainly chosen from a large variety of aromatic, monocyclic and bicyclic heteroaromatic, biaryl, and naphthyl systems. The compounds were tested for HDAC inhibitory activity and exhibited an IC_{50} of ≤30 μM.

Others have introduced alternative connection units like benzylcarbamates, phenolethers, and alkynes[70] or alkenes.[71]

Mikana Therapeutics in 2004 claimed the 2-aminothiazole as a linker. Compound **46** had GI_{50} = 0.7 μM in MCF-7 cells and IC_{50} = 0.05 μM in a HDAC inhibition assay. Successively, the same group claimed a series of compounds containing a 2-aminopyrimidine as linker[72] (Figure 15). These compounds

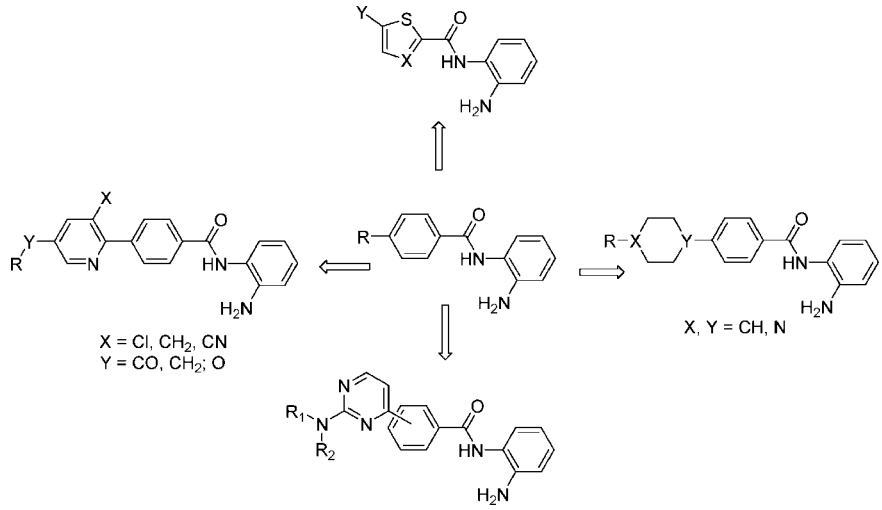


Figure 22. AstraZeneca SAR study.

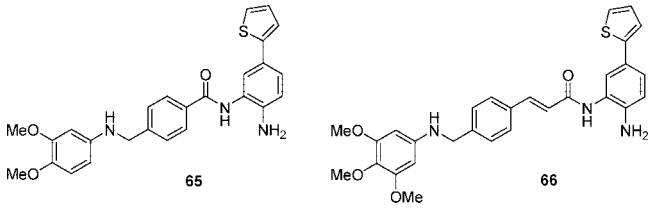


Figure 23. Selected examples of *p*-heteroaryl 2-aminophenylamides.

were assayed for antiproliferative activity using the human cell line MCF-7 and exhibited good results.

In the field of pyrimidines, from 2003 onward Janssen Pharmaceutica has released many patents claiming the use of this linker and the pyridine.[73] Janssen is actually developing compound **1m** (Figure 1). Other representative structures proposed are summarized in Figure 16.

HDAC inhibitors containing a benzothiophene as linker have been claimed by, among others, Aton Pharma (Merck & Co) in 2005 (Table 15).[74]

Simple benzothiophene 2-hydroxamic acid **51a** had an IC_{50} of 672 nM. Substitution at either position 5 or 6 with phenyl (**51b**,**c**), benzyl (**51d**,**e**), or naphthyl (**51f**,**g**) amide caused a moderate to strong increase of potency. Substitution with a reverse phenylamide (**51h**) resulted only in a poor improvement of potency versus **51a**, whereas a reverse benzylamide substitution was very well tolerated at both the 5 (**51i**) and 6 (**51j**) positions, inducing again a strong increase in potency. Substitution with a reverse naphthylamide was very deleterious to activity (**51k**). The sulfonamide connecting unit (**51l**, **51m**, and **51n**) at position 6 was beneficial.

Benzofuran (**52**) and benzimidazole linkers (**53**) have been reported by Syrrx (Figure 17).[75] It is claimed that the described compounds have been tested in HDAC-1, -2, -6, and -8 assays and that they showed <1000 nM activity against these enzymes. No detailed biological results are disclosed for the individual molecules.

5.4. Thiols and Thiol Derivatives. Thiols are well-known inhibitors of zinc-dependent enzymes such as ACE and matrix metalloproteinases,[76] and HDACs are no exceptions (Figure 18). The dithiol reduced form has been shown to be the active component of **1c** (Figure 1), currently in phase II.

The first substitution of the SAHA hydroxamate group with a thiol was reported by Suzuki and co-workers and yielded fruitful results.[77] In an enzymatic assay, thiol **54a** was as potent as SAHA. With ad hoc modifications they analyzed the importance of the free thiol group and the effect of the linker (Table 16).

Thiol conversion into thioacetate **54b** and methyl sulfide **54c** led to a 30-fold drop in potency in the first case and to an inactive compound in the second. These results suggest that the free thiol, generated under physiological conditions from **54b** but not from **54c**, is crucial for the interaction with the zinc ion in the active site. HDAC inhibition is dependent on chain length, with $n = 5$ or 6 being the optimal values. The similarity of the SAR between thiols and hydroxamates indicated that thiols have a binding mode analogous to that of hydroxamates.

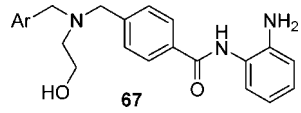
Substitutions on the aromatic group to increase potency were also investigated. When a second phenyl group was introduced at the meta position of the first one (compound **55**), the IC_{50} improved (0.075 μM), as it did with a quinoline (**56**, $IC_{50} =$ 0.072 μM) (Figure 19).

Compound **54a** was tested on human lung cancer NCI-H460 and found to be very weak ($EC_{50} > 50$ μM). The authors investigated then the possibility of improving cell potency by using disulfides and *S*-acyl derivatives as prodrugs. While the results with disulfides were quite disappointing (no effect was observed on NCI-H460 cells), the thioesters seemed more promising.[78] The *S*-isobutyryl derivative **57**showed $EC_{50} = 20$ μM in the cell test, which became 2 μM when a phenylthiazole was used in place of the simple phenyl group (**58**). **58** was evaluated in various human cancer cell lines and gave EC_{50} values ranging from 1 to 10 μM, therefore comparable to those of SAHA (Table 17).

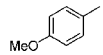
The same research group considered that, according to the proposed deacetylation mechanism, a zinc-chelating activated water molecule makes a nucleophilic attack on the carbonyl carbon of an acetylated lysine substrate. If the water molecule is removed from the active site, this would result in an increase in binding energy. Analogues with α-mercaptoacetamide at the linker terminus very likely would be taken into the active site, and once there, they would force the water molecule off the zinc ion. This was indeed the case with compound **54g** having an IC_{50} of 0.39 μM (Table 16). α-Hydroxy- or α-aminoacetamides analogous were inactive.

With this new zinc binding domain (ZBG), in an analogous way to work done on SAHA, various aromatic cap groups, spacers, and connection units have been prepared and tested (Table 18).[79]

Table 23. Hydrophilic Analogues of **1g**



67

		IC_{50} (nM)	IC_{50} (μM)[a]						
Cmpd	Ar	HDAC	HepG2	HCT116	SW620	SKBR3	MCF-7	A549	CCD-1059SK
SAHA	-	263	0.6	0.9	0.6	0.8	>100	12.6	43.5
1g		2700	0.9	0.7	3.2	3.7	4.3	23.3	65.0
67a		n.a.[b]	-	-	-	-	-	-	
67b		3700	1.2	0.9	3.3	3.9	10.0	20.5	19.4
67c		2400	3.5	5.7	4.5	6.9	8.4	20.8	28.3
67d		1800	0.9	0.8	2.2	3.5	3.8	20.0	41.7
67e		3132	1.0	0.8	3.6	3.6	10.4	24.2	94.8
67f		4987	0.9	0.8	2.1	3.4	4.9	17.3	18.4

[a] HepG2 (hepatoma), HCT1-116 and SW620 (colon), SKBR3, MCF-7 (breast), A549 (non-small-cell lung), CDD-1059SK (normal fibroblast cells). [b] n.a.: nonactive.

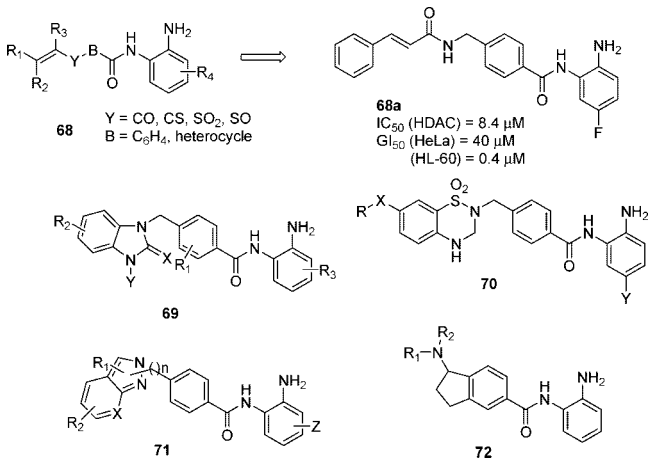


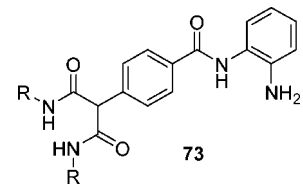
Figure 24. Shenzhen Chipscreen Biosciences, Takeda, and Bayer 2-aminophenylamides.

Concerning the chain length, $n = 3$ or 4 was the best while the amide CU was better than the urea. The reverse amide analogues **59g** and **59h** were particularly potent. Quite disappointing was the large drop in potency observed when testing the best inhibitors of this series for cytotoxicity on human cancer cell lines such as HeLa (cervix), SQ-20B (squamous carcinoma), MCF-7 (breast), and PC-3 (prostate), and the EC_{50} values were in the midmicromolar range.

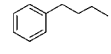
Gu and co-workers designed a series of SAHA analogues where the hydroxamic acid was replaced by sulfur-containing moieties such as thioamides, mercaptothioacetamides, etc. (Table 19).[80]

Unexpectedly, the α-acetomercapto ketone **60f** was more potent than the corresponding free thiol **60e** (almost 2-fold) and SAHA. Since the same relation did not occur with mercaptoacetamides (**60c** vs **60g**), it appeared that the ketone carbonyl is synergistic in binding with the α-thioacetoxyl group. S-

Table 24. SAR of *N*,*N*′-Diarylmalonamides

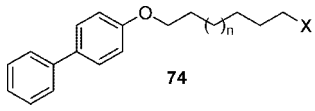


73

Cmpd	R	IC_{50} (nM) HDAC-1[a]	IC_{50} (nM) HCT-116
73a		41	171
73b		34	500
73c		63	222
73d		56	607
73e		35	383
73f		47	394
73g		199	596
73h		229	987

[a] Recombinant enzymes expressed in mammalian cells and affinity purified.

Table 25. Selected Examples of Electrophilic Ketones Having a Linear Linker and an Ether as the CU[95]

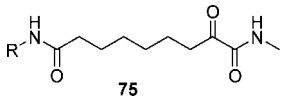


74

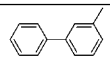
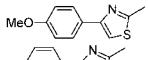
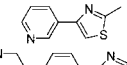
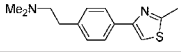
compd	*n*	X	IC_{50} (μM)[a]
74a	1	−CONHOH	0.02
74b	1	$-COCF_3$	2.9
74c	1	$-COCO_2H$	3.7
74d	1	$-COCO_2Me$	0.062
74e	0	$-COCO_2Me$	0.082
74f	2	$-COCO_2Me$	0.109
74g	1	$-COCONH_2$	>50
74h	1	−COCONHMe	0.11
74i	1	−COCONHEt	4.9
74j	1	$-COCONMe_2$	>5
74k	1	$-COCONH(CH_2)_2N(Me)_2$	>50
74l	1	$-COCONHCH_2CO_2H$	>50

[a] Mixture of HDAC-1 and HDAC-2 from nuclear extraction K562 erythroleukemia cells.

Table 26. Activity of Selected α-Ketoamides[93b]



75

Cmpd	R	IC_{50} (nM)[a]	Cell Proliferation IC_{50}(μM) HT1080	MDA435
75a		3.7	6.9	14
75b		9.1	2.7	5.8
75c		4.3	0.67	2.0
75d		3.1	0.12	0.16

[a] Mixture of HDAC-1 and HDAC-2 from nuclear extraction K562 erythroleukemia cells.

Methylation of **60c** to give α-methylthioketone **60h** resulted in a loss of activity.

Argenta Discovery has introduced the mercapto ketone moiety on a compound having an heteroaromatic linker, but no pharmacological data on this new series have been disclosed up to now.[81]

5.5. 2-Aminophenylamides. Compounds belonging to the 2-aminophenylamide series have the general formula shown in Figure 20, exemplified by **1g**. The 2-aminophenylamide moiety is formally considered as the zinc chelating group, although no X-ray structure has ever been reported. Two docking studies have been published up to now on the subject. One claims that the best docked **1g** structure on the HDLP protein has the compound binding at the entrance to the active pocket.[82] The other, based on the homology model of HDAC-1, places the aromatic free amino group near the zinc atom.[83]

Suzuki and co-workers, from Mitsui Pharmaceuticals, identified **1g** from a set of synthetic benzamide derivatives.[84] **1g** inhibited HDAC with an IC_{50} of 4.8 μM and displayed antiproliferative activity in several cancer cell lines (breast, colorectum, leukemia, lung, ovary, and pancreas) and had a significant oral anticancer activity against solid tumors and lymphomas. It is currently undergoing phase II clinical evaluation.

Following the hypothesis that the diaminobenzene binds in the active site, Vaisburg and co-workers designed 2-aminophenylamides of ω-substituted alkanoic acids, a hybrid between **1g** and SAHA (Table 20).[85] The compounds showed no significant improvement over **1g**.

Compound **61b** was evaluated in several different human tumor xenograft models, and in the best case it gave a TGI of 53% when dosed as intraperitoneal injection (ip) at 40 mg/kg per day for 3 weeks without any associated body weight loss. The same authors carried out further work using **1g** and **1b** (Figure 1) as the structural starting points for the design of novel 2-aminophenylamides (Table 21).[86]

In spite of their modest in vitro potency, **62g** and **63d** had significant in vivo antitumor activity in several different tumor xenograft models when administrated daily by either intraperitoneal injection (ip) or orally (po). Moreover, they showed acceptable pharmacokinetic profiles in the rat (intravenous (iv) half-life of 1.8 and 0.7 h and bioavailability of 65% and 20% respectively, Table 22).

Compounds belonging to the 2-aminophenylamide series having a heterocycle as linker have been described by Hoffmann-La Roche (Figure 21). Pharmacological data are not reported in the patents.[87,88]

AstraZeneca published an extensive SAR study on 2-aminophenylamides with an aromatic linker summarized in Figure 22.[89] The released patents include more than 200 compounds, but also in this case HDAC inhibition data are not reported.

Researchers from MethylGene, in an effort to explore the 14 Å internal cavity adjacent to the enzyme catalytic site, found that *o*-aminobenzamide inhibitors with para aromatic or para heteroaromatic substituents on the aniline ring, such as phenyl, furanyl, or thienyl, can have significant HDAC inhibitory potency. More than 300 examples are described in detail in two patents.[90] Compounds **65** and **66** (Figure 23) had a TVI (tumor volume inhibition) of 72% and 78%, respectively, when administered ip to nude mice bearing xenografts of the HCT-116 tumor cell line.

Since the low water solubility of these compounds prevented iv delivery and might also be the reason for their reduced cellular activity, Maeda and co-workers prepared more hydrophilic analogues of **1g** (Table 23).[91]

In this set of compounds, with the notable exception of **67a**, all the molecules have more or less the same HCT-116 cell growth inhibition as **1g**. **67b−f** were also tested on a panel of human cancer cells and for the toxicity against normal fibroblast cells. The activities were comparable to those of **1g**.

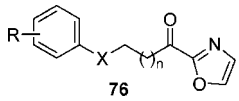
Additional 2-aminophenylamides have been claimed by Shenzhen Chipscreen Biosciences (**68**),[92] Takeda (**69−71**)[93] and Bayer Pharmaceuticals Corp (**72**) (Figure 24),[94] but with the exception of **68a** no specific biological data are presented in the corresponding patents.

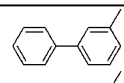
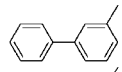
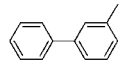
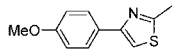
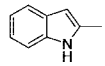
A systematic study on SARs, isoform selectivity, and PK data has recently been published by the Merck group for derivatives of the general structure **73** (Table 24).[95]

When tested against a panel of HDAC isoforms, compound **73a** was found to be inactive on HDAC-6 and -8 (IC_{50} > 10 000 nM), almost 10-fold selective for HDAC-1 (IC_{50} = 36 nM) over HDAC-2 (IC_{50} = 313 nM) and 20-fold selective over HDAC-3 (IC_{50} = 697 nM). Its bioavailability in the rat and dog was excellent (88%) but was a little bit low in the Rhesus monkey (27%). Plasma clearance was found to be low in all three species.

5.6. Ketones. Electrophilic or simple methyl ketones have been proposed more than once as protease inhibitors, and HDAC enzymes are no exception. Trifluomethyl ketones, α-ketoamides, α-keto esters, and α-keto heterocycles, opportunely functionalized, have been prepared and tested as HDAC inhibitors.[96]

Table 27. HDAC Inhibitory Activity of Selected α-Keto Oxazoles



Cmpd	n	X	R	IC_{50} (μM)[a]
76a	5	-O-		0.62
76b	5	-CONH-		0.06
76c	4	-CONH-		0.31
76d	5	-NHCO-		0.03
76e	5	-NHCO-		0.09

[a] Mixture of HDAC-1 and HDAC-2 from nuclear extraction K562 erythroleukemia cells.

In a systematic study with a linear linker, an ether as CU and a *p*-biphenyl group as the CAP, the α-keto esters (**74d**−**f**) and the α-ketoamide **74h** were found to be potent HDAC inhibitors (Table 25). The SARs correlated with those of the corresponding hydroxamates, indicating a similar binding mode. As happened with trifluoromethyl ketones, the α-keto esters and amides are also readily metabolized in vivo to the corresponding inactive alcohol. Modifications of the geometry and linkage of the aromatic moiety for potency increase resulted in **75a**, which was very good on the isolated enzyme (IC_{50} = 3.7 nM) but poorly active on the two selected cell lines. The increase in polarity and aqueous solubility obtained with the introduction of polar heteroatoms (**75b**−**d**) improved the cellular activity.

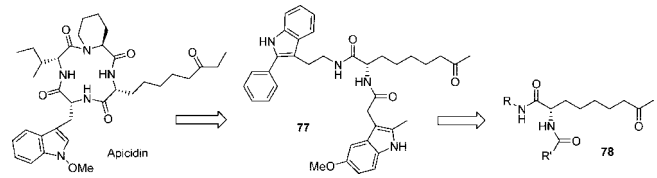
Compounds **75b**−**d**, containing a 4-phenyl or 4-pyridylthiazole moiety, showed an IC_{50} < 10 nM on the isolated enzyme and IC_{50} < 10 μM on human HT1080 fibrosarcoma cells and human MDA435 breast carcinoma cells (Table 26). Compound **75b** had a significant antitumor effect in a HT1080 flank xenograft mouse tumor model (100 mg/kg) in spite of having a short half-life in vivo in cell culture and in whole blood. This was due to its rapid reduction to the inactive α-hydroxyamide. However, the 100 mg/kg dose group showed some evidence of toxicity (rough coats, weight loss, lethality). Some α-heterocyclic ketones have also been reported to show SARs consistent with those observed for trifluoromethyl ketones, α-keto esters, and α-ketoamides.

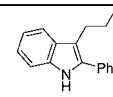
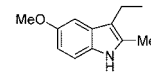
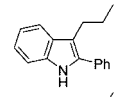
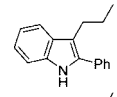
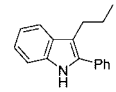
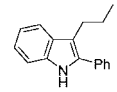
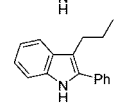
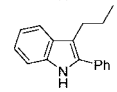
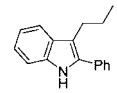
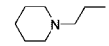
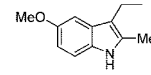
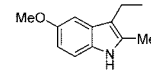
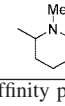
Heterocyclic ketones containing an oxazole (**76**) were particularly potent (Table 27).

Some of the more potent compounds were found to have antiproliferative activity in an MDA435 cell line in the micromolar range. Again, it is likely that their potency in cells was compromised by their rapid reduction to the inactive alcohol. It was demonstrated that in whole blood **76a** had a half-life of 0.21 h.[95]

Very recently, simple methyl ketone derivatives have been reported as HDAC inhibitors (Table 28). Jones and co-workers selected the natural product apicidin, a cyclic peptide containing an ethyl ketone, as a starting point for the design of new non-hydroxamate HDAC inhibitors.[97] After screening corporate sample collection looking for compounds that contained the L-AODA (2-amino-8-oxodecanoic acid) amino acid, they identified **77** as a promising starting point. Extensive SAR work was done around the two amide groups separately, and the most interesting substituents were combined and incorporated for a more extensive SAR around this series in a 2-D array. From this work small and potent HDAC inhibitors were identified. Interestingly, the results revealed that the SAR of the two separated amide groups were not additive: very likely binding to one portion of the surface influenced the binding of the second amide group.

Table 28. Selected Examples Methylketone HDAC Inhibitors



Cmpd	R	R'	HDAC-1 IC_{50}(nM)[a]	PRO (HeLa) IC_{50}(nM)[a]
78a			590	730
78b		-Me	930	7600
78c			590	3000
78d			480	3400
78e			540	2500
78f			200	2000
78g			220	2000
78h			190	<390
78i			18	230
78j			25	<390
78k			79	500
78l			55	720
78m			55	430

[a] Recombinant HDAC-1, affinity purified.

78m displayed an improved microsomal stability (human Cl_{int} = 54 μL min^{-1} mg^{-1} compared to >300 μL min^{-1} mg^{-1} for **77**) and was profiled on HDAC isoforms and tested for antiproliferation activity on a panel of cell lines (Table 29).

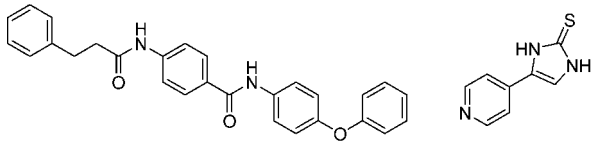
5.7. Miscellaneous. Some molecules that have escaped the chemical classification described in the previous paragraphs have been discovered through screening of compound collections and natural product mixtures or have been obtained from rational design.

In 2003, Hu et al., following an HTS campaign on a chemical compound collection, identified **79a** and **79b** (Figure 25) as

Table 29. Activity of **78m** on the HDAC Isoforms 1–8 and Various Cell Lines

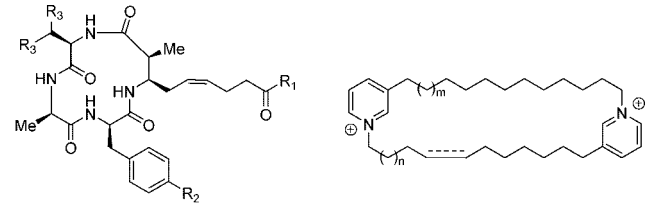
HDAC isoforms[a]	IC_{50} (nM)	cell line	IC_{50} (nM)
1	55	cervical-HeLa	430
2	170	colon HCT-116	670
3	14	lung A 549	2000
4	NA	myeloid U937	450
5	1900	ovarian A2780	1700
6	13	kidney G401	250
7	na[b]	breast MCF7	230
8	3000	human renal epithelial	>20000

[a] Recombinant affinity purified enzymes; [b] na: not active.



79a: SB-429201 **79b**: SB-379278A

Figure 25. Atypical HDAC inhibitors obtained from compound collection screening by Hu et al.



Azumamide	R_1	R_2	R_3
A	NH_2	H	Me
B	NH_2	OH	Me
C	OH	OH	Me
D	NH_2	H	H
E	OH	H	Me

Cyclostellettamines

80a m = 1, n = 1
80b m = 1, n = 4, Δ^{13}
80c m = 2, n = 4, Δ^{13}
80d m = 1, n = 2

Figure 26. HDAC inhibitors derived from Japanese marine invertebrates.

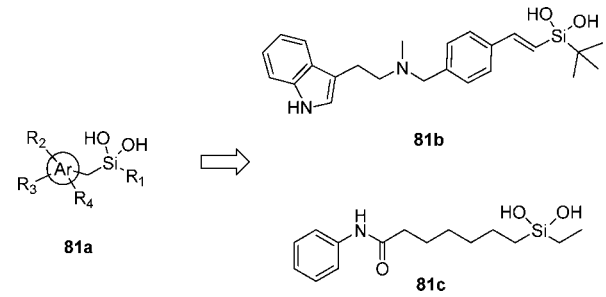
atypical HDAC inhibitors.[98] The first one was somewhat selective on HDAC-1, while the second was active on HDAC-8. No further development of these leads has been reported.

The group of Fusetani, involved in the search of antitumor leads from Japanese marine invertebrates, identified two classes of inhibitors from these sources: the azumamides A−E[99] and the cyclostellettamines (Figure 26).[100] The first ones showed IC_{50} values ranging from 1.3 μM (azumamide D) to 45 nM (azumamide A) when tested for enzyme-inhibitory potency on crude enzymes extracted from K562 human leukemia cells. Cyclostellettamines, when tested on the same cell extracts, were all in the micromolar range of activity, with the most potent being **80b** (IC_{50} = 27 μM).

Finally from a rational design by Axys Pharmaceutical (now Celera) the silanediols (Figure 27), which are supposed to be transition state mimics, emerged as interesting substituents for the hydroxamic acids.[101] In this case too, no biological activity data are available.

5.8. Isoform Selectivity. As observed at the beginning of this paper, molecules currently in clinical trial are generally pan-inhibitors or, in the best of cases, they show some selectivity between class I and class II isoforms (aminophenylamides).[102] With the new generation of compounds and the availability of recombinant HDAC isoforms, a large effort has been devoted to the search for selective inhibitors with some success (Table 30).

Compounds **19a** and **19b**, belonging to the class of hydroxamic acids with a cynnamoyl linker, have been found to be quite



81a **81b** **81c**

Figure 27. Silanediols from Axys Pharmaceutical: general formula and selected examples.

selective for HDAC-1 and -2.[104] The same selectivity profile was found by MethylGene researchers in compounds belonging to the class of aminophenylamides when substituted with an heteroaromatic ring in a position para to the aminogroup (Figure 28, **82a**). Because of the interaction with the 14 Å cavity near the zinc binding site, these derivatives showed nanomolar potency on HDAC-1 and -2 while being completely inactive on HDAC-3 to -8 (**82b**, Figure 28, Table 30). Selective compounds reported in Table 30 are generally preferentially active toward class I HDACs, but there are also few molecules selective toward class II enzymes. **83a** and **83b** (Figure 28), belonging to the class of cinnamoylpyrrole hydroxamic acids and initially found to be selective toward maize class II HDACs,[59] also showed the same selectivity on human HDACs.[105]

6. In Vivo Activity of Class I and Class II HDACs Inhibitors

HDAC inhibitors have shown antitumor activity as single agents, and as such, SAHA reached the market, while many others are undergoing clinical trials for a panel of tumor types. Nevertheless, the greatest promise probably lies in mechanism-based combinations with the classical cytotoxic agents or the more recent targeted agents.[106]

The ability of HDAC inhibitors to open the chromatin structure, thus facilitating access to the DNA, can be exploited to increase the antitumor effects of DNA-targeting agents. An increase in cytotoxicity was observed in combination with docetaxel, adriamycin, VP-16, and cisplatin in different tumor models.[107] Interestingly, the synergy with topoisomerase II inhibitors was found to be schedule-dependent in vitro,[108] and these findings were further confirmed by in vivo preclinical models.[109] In other cases the role of HDACs may be much more complex.[110]

Sometimes there are oncogenic proteins that incorporate HDACs. The most studied case is the acute promyelocytic leukemia (APL). Normally this disease responds to retinoic acid (RA) that induces cell differentiation and growth arrest.[111] When the receptor for the retinoic acid (RAR) is expressed as a fusion protein with the promyelocytic leukemia zinc finger (PLZF), the cells become resistant to RA. This effect is the result of the association of RAR−PLZF with HDACs and the consequent repression of genes normally induced by RA that trigger differentiation. When patients with APL not responding to RA are treated with RA and an HDAC inhibitor, the cellular responses to RA are restored. In general, when a tumor type is characterized by these kinds of fusion proteins, a combination of the original drug with an HDAC inhibitor may be highly beneficial.

A number of combination strategies with targeted agents have been proposed in different leukemic cells: treatment with SAHA

Table 30. Activity of Selected HDAC Inhibitors on HDAC Isoforms 1–9

	IC$_{50}$ (μM) of HDAC isoforms[a]								
	1	2	3	4	5	6	7	8	9
SAHA[101]	0.021	NT[b]	0.037	NT	NT	0.025	NT	1.2	NT
1g[101]	0.18	NT	0.74	NT	NT	>100	NT	44.9	NT
1f[101]	0.82	NT	0.62	NT	NT	>30	NT	>25	NT
1n[101]	0.0018	NT	0.0037	NT	NT	0.015	NT	0.14	NT
azumamide E[103]	0.050	0.100	0.080	30%, 50 μM	10.0	12.0	9.7	3.7	28.0
19a (SK-7041)[103]	***	***	*	*	*	*	NT	NT	NT
19b (SK-7068)[103]	***	***	*	*	*	*	NT	NT	NT
79b[97]	na[c]	NT	na	NT	NT	NT	NT	0.5	NT
79a[97]	1.5	NT	na	NT	NT	NT	NT	na	NT
82b[82]	0.04	0.1	>20	>20	>20	>20	>20	>20	NT

[a] Results in the original paper are reported as the enzyme inhibition at 1 μM concentration: *** indicates more than 90% inhibition, and * indicates less than 20%. [b] NT: not tested. [c] na: not active.

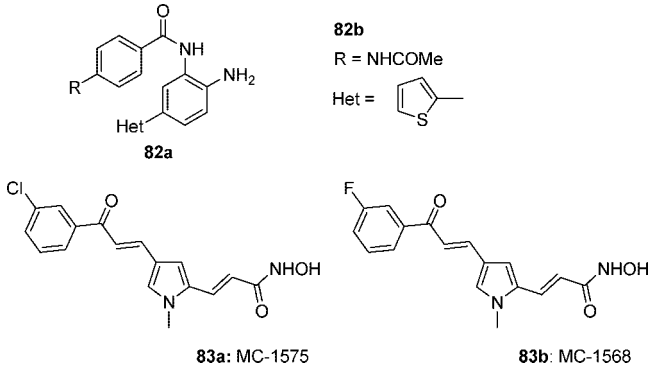


Figure 28. Aminophenylamides heterosubstituted selective for HDAC-1 and -2, with **82b** as selected example, and (aryloxopropenyl)pyrrolyl hydroxamates selective toward class II HDACs.

and imatinib (4-(4-methyl-piperazin-1-ylmethyl)-*N*-[4-methyl-3-(4-pyridin-3-ylpyrimidin-2-ylamino)phenyl]benzamide mesilate) during the advanced phases of chronic myeloid leukemia;[112] in vitro treatment with SAHA and dasatinib (2-{6-[4-(2-hydroxyethyl)piperazin-1-yl]-2-methylpyrimidin-4-ylamino}thiazole-5-carboxylic acid (2-chloro-6-methylphenyl)-amide) in primary imatinib sensitive or imatinib-resistant chronic myeloid leukemia;[113] in vitro treatment with the nucleoside analogue fludarabine;[114] in vitro treatment with the proteasome inhibitor bortezomib combined with SAHA in human leukemia cells;[115] and in vitro treatment with SAHA and an HSP 90 antagonist.[116] Concerning solid tumors, a synergistic effect has been demonstrated with tamoxifen in MDA-231 cells α-estrogen receptor (α-ER) negative[117] or with 5-fluorouracil (5-FU) in resistant cells.[118]

Finally, it has been demonstrated that all HDAC inhibitors to date synergize with ionizing radiation (γ-irradiation) to kill tumor cells in vitro and several have shown this synergy in vivo.[119] This effect may be due to multiple reasons, among which lie the down-regulation of genes and proteins involved in DNA damage response induced by HDAC inhibitors. Reduction of repair and survival proteins is also partially responsible for the synergistic effect with classical chemotherapeutic compounds.

7. Clinical Trials of HDAC Inhibitors

On the basis of these translational studies, many clinical trials are open: 41 trials for SAHA, 6 for **1d**, 15 for **1c**, 15 for **1b**, 9 for **1f**, and 3 for **1g**. Results from phase I and phase II clinical trials have already been published for SAHA[120] and **1c**.[121] Generally SAHA has been well tolerated and had antitumor activity. Interestingly, the major adverse events observed differed according to the route of administration, iv or po, probably because of differences in pharmacokinetics. **1c** too has been generally well tolerated with a toxicity profile similar to that of SAHA but in addition has an effect on QTc prolongation. However, this agent has shown significant clinical benefit in patients with cutaneous and peripheral T cell lymphoma.

The data from phase I clinical trials for **1g** in advanced solid tumors and lymphoma patients demonstrated that its half-life was much longer than predicted (39–80 h in humans), and so a daily dose was not tolerated.[122] Concerning **1b**, data from phase I demonstrated that it was well tolerated with transient antileukemic effects.[123] Phase I clinical trials results are also available for combination therapy between the HDAC inhibitor valproic acid and retinoic acid or decitabine that demonstrated that these combinations with HDAC inhibitors are safe and active.[124] Recently the first clinical study in which valproic acid has been combined with the cytotoxic agent epirubicin has been reported for patients with solid tumors. Antitumor activity was observed in heavily pretreated patients and also in patients with tumors resistant to anthracyclines such as melanoma.[125]

8. Conclusions

A large amount of work has been carried out in the past 5 years in the field of HDAC inhibitors, and more than 100 patents claiming new chemical series have been published.

The great potential of these epigenetic modulators was clear from the beginning, and this has favored an empirical approach to testing before a deeper knowledge of HDAC biology and the role of individual isoforms could be acquired. It has not yet been unambiguously established if, from a pharmacological point of view, it would be better to hit many HDAC isoforms (pan-inhibitors) at the same time or to have subtype-selective inhibitors. Supporters of both opinions exist. Isotype specific HDAC inhibitors (with few exceptions) are not yet available because the high homology among catalytic sites and the tendency of HDAC to complex with other proteins to become functionally active have made this a very hard task. Up to now the insights into the biological roles of isoforms have been obtained mainly with RNAi techniques, and highly selective compounds would be very useful for gaining a deeper insight into the biology and pharmacology of HDACs.

Cardiac toxicity has been observed sporadically. According to some researchers, the induced cardiac abnormalities is a class effect that shows up with the more potent HDACs, while others think that they are related to the chemical structure of the particular molecule under study. Nevertheless, almost all agree that this should not impede the development of these drugs.

The translation of preclinical results to clinical trials is another key point. Many trials involving a number of HDAC inhibitors, used either as a single agent or in combination, are currently

ongoing. However, the exact mechanisms by which these inhibitors lead to the observed biological effect is not known. The mode of action may differ from one inhibitor to another because of the chemical structure (leading to a particular modulation of the various HDAC isoforms) or because of the pharmacokinetic profile. Tracking histone deacetylation in the blood of patients has been a widespread method to measure drug activity but is clearly not sufficient because of the wide range of non-histone target proteins, and there is a strong need for opportune and more specific biomarkers.

The same inhibitor may be effective on different tumor types because of different mechanisms of action. A representative example may be the sensitization of resistant cells to 5-fluorouracyl (5-FU) action. In fact, in this particular case resistance is due to an up-regulation of thymidylate synthase (TS), and the 5-FU target and HDAC inhibitors are particularly effective because they down-regulate TS directly and indirectly through inhibition of HSP90, a chaperone for TS.

The overall direct consequence of these early observations is that an opportune selection of patients for clinical trials is required for epigenetic therapy in combination with other chemotherapeutic agents.

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Biographies

Marielle Paris received her Master's Degree in Chemistry from the Université Paul Sabatier (Toulouse III) in 1994 and a Ph.D. in Organic Chemistry in 1998 under the guidance of Prof. Jean Martinez at the Université des Sciences et Techniques du Languedoc in Montpellier (France). In 1999, she joined Menarini Ricerche in Florence as a postdoctoral fellow. In 2000, she began work at Bachem AG in Bubendorf (Switzerland), developing new methodologies in peptide synthesis. In 2001, she moved to Biopep S.A., at Montpellier, where she was appointed laboratory head and project leader. Since 2003, she has joint the medicinal chemistry team at Menarini Ricerche, Pomezia, Rome, Italy.

Marina Porcelloni received her undergraduate education in Chemistry at the University of Florence under the supervision of Prof. Giovanni Poli in 1998 and a Ph.D. in Organic Chemistry under the guidance of Prof. Varinder K. Aggarwal at the University of Bristol in 2001. In the same year she moved back to Italy where she joined Menarini Ricerche in Pomezia, Rome, Italy, as medicinal chemist working, among other projects, on the design and synthesis of NKA antagonists and HDAC inhibitors and coauthoring several publications and patents.

Monica Binaschi received her degree in Biology from University of Milan in 1988 with her thesis on the mechanisms of drug resistance to antitumor drugs and anthracyclines. She continued her studies at Istituto Nazionale dei Tumori in Milan in the laboratory of Professor Giovanni Capranico, after receiving a fellowship. In 1995 she was a Visiting Fellow in the laboratory of Caroline Austin in the Department of Biochemistry and Genetic at University of Newcastle-upon-Tyne, under a Concerted Action programme on "Anticancer Drug Action on Topoisomerase II" supported by European Commission. She moved to Rome in 1999 and joined the Menarini Research group where she studies therapeutics in discovery and development programs in the oncology area.

Daniela Fattori is the head of the chemistry department at Menarini Ricerche, Pomezia, Rome, Italy. After receiving her undergraduate degree in Chemistry at the University of Rome "La Sapienza", she moved to Lausanne University, Switzerland, where she obtained a Ph.D. in Organic Chemistry under the direction of Professor Pierre Vogel. Following an experience as "Maitre Assistante" at the same university, she joined the group of Prof. Maurizio Botta at Siena University, Italy, for a postdoctoral experience. In 1993 she joined the Merck Research Labs (IRBM) in Rome, Italy, where she worked in the field of classical medicinal chemistry and biorganic chemistry. In 2001 she joined Menarini as combinatorial chemistry laboratory head and after 1 year took the position of department head.

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