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Bioorganic & Medicinal Chemistry Letters 18 (2008) 1814–1819
Bioorganic & Medicinal Chemistry Letters

Probing the elusive catalytic activity of vertebrate class IIa histone deacetylases

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Received 18 November 2007; revised 7 February 2008; accepted 9 February 2008
Available online 14 February 2008

Abstract—It has been widely debated whether class IIa HDACs have catalytic deacetylase activity, and whether this plays any part in controlling gene expression. Herein, it has been demonstrated that class IIa HDACs isolated from mammalian cells are contaminated with other deacetylases, but can be prepared cleanly in *Escherichia coli*. These bacteria preparations have weak but measurable deacetylase activity. The low efficiency can be restored either by: mutation of an active site histidine to tyrosine, or by the use of a non-acetylated lysine substrate, allowing the development of assays to identify class IIa HDAC inhibitors.

The acetylation status of lysine residues on histone tails, and an increasing number of non-histone substrates (including transcription factors, heat shock and structural proteins), is tightly controlled by two counteracting enzyme families, histone acetyl transferases (HATs) and histone deacetylases (HDACs).[1,2] This dynamic process has a crucial role in chromatin structure and hence gene transcription, whereby the presence of acetyl groups (Ac-) on these lysine residues neutralizes the positive charges of the histone tails thereby decreasing their interaction with DNA, relaxing the chromatin, and allowing access to transcription factors. In contrast, removal of the Ac-groups condenses the chromatin, leading to transcriptional repression. Interest in the HDACs has been stimulated due to discovery that they control key processes such as skeletal and muscle formation, cardiac hypertrophy, T-cell differentiation and neuronal survival, and are deregulated in neoplasias.[3] Consequently, HDAC inhibitors (HDACi) are being investigated and vorinostat (Zolinza®, formerly known as SAHA) recently became the first clinically approved HDACi.[4]

The HDAC superfamily of enzymes can be divided into two large groups (classes I + II) based on characteristic conserved sequence motifs within a domain of about 350 amino acids harbouring the known or putative HDAC catalytic domain.[5,6] These two classes can be distinguished from a third, mechanistically distinct class, the sirtuin family.[7] Class II HDACs are further divided into sub-classes IIa and IIb, the former (HDACs 4, 5, 7 + 9) contain an N-terminal regulatory domain.[6] Although the role of class IIa HDACs in tissue-specific gene regulation is well documented,[8–11] the contribution of the catalytic domain to this activity is controversial. A substantial body of evidence illustrates that class IIa HDACs exert transcriptional repression through protein–protein interactions using the N-terminal domain, while the catalytic domain may not be required.[3,6,12] For instance, a natural HDAC9 splice variant lacking the catalytic domain retains full transcriptional repressive functions.[13] In parallel, several groups have questioned whether class IIa HDACs possess any intrinsic deacetylase activity, hypothesizing that any activity may be due to the presence of associated HDACs in multi-protein complexes.[6,12] Indeed, HDACs 4, 5 + 7 have been shown to associate with HDAC3.[14,15] Furthermore, attempts to impair the activity of HDAC4 by site-directed mutagenesis in the catalytic site were inconclusive as mutants showing impaired deacetylase activity lost the ability to interact with HDAC3.[15] Also, attempts to obtain active recombinant class IIa HDACs have been unsuccessful.[16]

Concurrently with work to develop subtype selective HDACi's (see following paper), we became interested in establishing whether class IIa HDACs demonstrate

Keywords: Histone deacetylase; HDAC.
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doi:10.1016/j.bmcl.2008.02.025

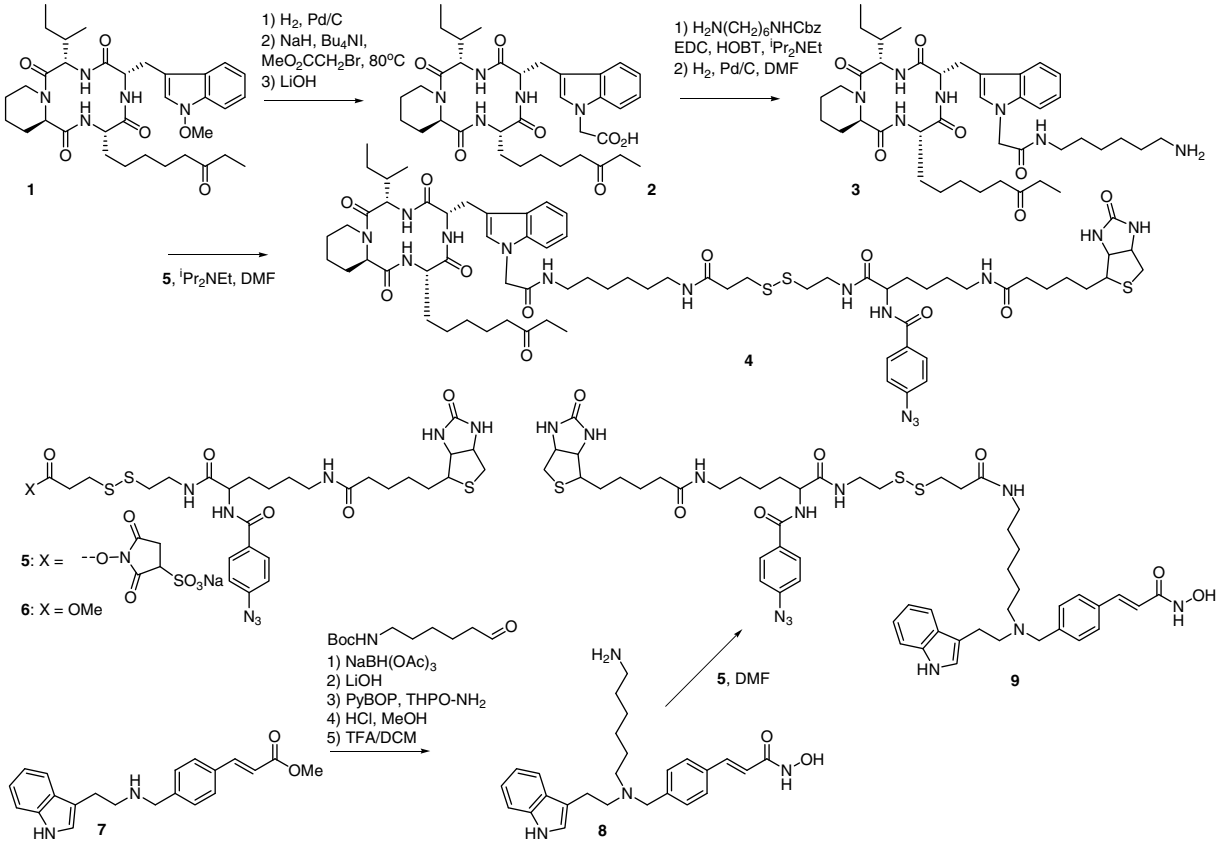
deacetylase activity, using HDAC4 as a representative example, with a view to establishing a screening platform if activity was detected.[17]

Initially, flag-tagged HDACs were prepared and purified from mammalian cells. C-terminally flag-tagged HDACs 1, 4 and 6 were expressed and purified from HEK293 cells, while in order to obtain functional HDAC3 it was necessary to co-express and co-immunopurify flag-tagged HDAC3 together with a GAL4 DBD-fusion of the N-CoR deacetylase activation domain from the same cell line.[17]

In order to demonstrate that indeed we were measuring and inhibiting purified flag-tagged HDAC isoforms two structurally distinct activity probes, **4** and **9**, were prepared, based on HDACi's Apicidin and NVP-LAQ824 (Scheme 1). Each probe carried a photo-activable cross-linking moiety and a biotin residue. The probes were prepared appending long side chains to portions of the molecule which were expected to be away from the active site and solvent exposed. The hydroxamic acid probe **9** was synthesized by replacing the hydroxylethyl side chain of NVP-LAQ824 by an aminohexyl chain and then coupling the hydroxy-succinimide ester **5** bearing both the phenyl azide cross-linking group and a biotin residue. In contrast, the Apicidin based probe **4** was prepared by firstly alkylating the indole moiety with methyl bromoacetate, followed by hydrolysis and addition of the aminohexyl chain. Coupling with the activated ester **5** completed the synthesis. The two compounds displayed nanomolar affinities against class I HDACs 1 and 3, whilst also inhibiting flag-tagged HDAC4 preparation with similar potency (Table 1). In contrast, both compounds lost some activity against HDAC6, with **4** displaying substantially reduced activity on this isoform having IC_{50} = 230 nM. An inactive control **6** was also prepared and revealed not to inhibit any isoforms.

With these reagents in hand, two parallel experiments were conducted: UV cross-linking to the HDAC isoforms and analysis of the covalently bound proteins, and pull-down experiments using streptavidin-coated beads pre-adsorbed with these biotinylated probes to capture the HDACs.[17,18]

Unsurprisingly, given their potency both probes cross-linked HDACs 1 and 3 (Table 1), whereas the negative control **6** lacking the HDACi failed to cross-link to either of these deacetylases. In contrast, only the hydroxamic acid probe **9** cross-linked significantly with HDAC6. The Apicidin based probe **4** only showed weak cross-linking, in line with its reduced potency on HDAC6, IC_{50} = 230 nM compared to **9**. Instead, the cross-linking and pull-down data obtained with flag-tagged HDAC4 expressed in HEK293 cells did not reflect the inhibition constants. While both probe compounds showed similar inhibition of this enzyme preparation, only the hydroxamic acid probe **9** effec-



Scheme 1. Activity probes **9** and **4**, based on NVP-LAQ824 (hydroxyethyl has been replaced by an aminohexyl chain) and Apicidin (**1**), respectively.

Table 1. IC_{50}'s of HDACi probes on HDAC isoforms from mammalian cells, and results of UV cross-linking and pull-down experiments[17]

	IC_{50} (nM)				UV cross-linking				Pull-down of HDAC4
	HDAC1[b]	HDAC3[b]	HDAC4[c]	HDAC6[b]	HDAC1	HDAC3	HDAC4	HDAC6	
LAQ Probe **9**	73	42	4	98	Yes	Yes	Yes	Yes	**Yes** Competed by NVP-LAQ824, but not Apicidin or MS-275
Apicidin Probe **4**	20	6	5	230	Yes	Yes	No	Weak	**No**
Negative Control **6**	NA at 1 μM	NA at 1 μM	NA at 1 μM	NA at 1 μM	No	No	No	No	**No**

[a]Values are means >2 experiments (std. dev. were within 30% of the IC_{50} values) measured with flag-tagged enzyme from HEK293 cells, using [b]'*Fluor de Lys*' substrate[21] or [c][^{3}H]-Ac histones.

tively cross-linked HDAC4.[17] No cross-linking was seen with the Apicidin probe **4**. Similarly, the hydroxamic acid probe **9**, but not **4**, was able to pull-down HDAC4. Pre-incubation of the HDAC4 preparation with a 10-fold excess of NVP-LAQ824 was able to prevent pull-down of HDAC4 by **9**, but this could not be competed with an excess of either Apicidin or MS-275. These data suggest that only the hydroxamic acid **9** binds to HDAC4. The lack of correlation between cross-linking, pull-down and inhibition experiments also points to the conclusion that HDAC4 does not, or does only to a marginal extent, contribute to the deacetylase activity of the HDAC4 complex from HEK293 cells.

Having demonstrated that purified HDAC class IIa isoforms could not be isolated from mammalian cells attention turned to *Escherichia coli*, that lack histones and endogenous HDACs. Accordingly the N-terminally truncated HDAC4 catalytic domain (CD) (starting at Thr653) was expressed, purified to homogenity and tested for activity on [^{3}H]acetyl histones cores.

Although deacetylase activity was seen with this enzyme, it was only modest, requiring 1 μM enzyme to see substantial conversion (Table 2). Intriguingly this activity could be inhibited with NVP-LAQ824 but not Apicidin, substantiating the previous findings in the cross-linking and pull-down experiments.

Following this observation that HDAC4 does indeed have catalytic activity, albeit weak, interest arose in developing a screening platform to be able to identify isoform selective HDACi's. However, routine screening with high enzyme concentrations would not be practical and necessitated the evolution of more efficient screening processes. Potentially two scenarios could be envisaged to account for this inefficiency in deacetylase activity: lack of an optimal transition state in the active site of the enzyme, or the use of an inappropriate substrate.[17]

Table 2. Deacetylation of [^{3}H]acetyl histones cores by HDAC4WT or HDAC4(H976Y) 'Gain of function' catalytic domains from *E. coli*

Conditions	% Deacetylation in 4 h[a]
1 μM HDAC4	12
1 μM HDAC4WT + 10 μM NVP-LAQ824	3
1 μM HDAC4WT + 10 μM Apicidin	12
1 nM HDAC4(H976Y)	33
10 nM HDAC4(H976Y)	45
100 nM HDAC4(H976Y)	65

[a] Values are means >2 experiments.

Beside from the presence of N-terminal extension of class IIa HDACs, another significant difference is the substitution of a tyrosine residue present in the catalytic domain of class I HDACs with a histidine residue (Fig. 1a). This tyrosine residue is crucial to the proposed deacetylase mechanism and is believed to act as a transition-state stabilizer.[19] Indeed, in crystal structures with bound inhibitors this residue makes a H-bond with the carbonyl of the HDACi.[19,20] This Y–H substitution is surprising as molecular modeling suggests that class IIa enzymes would be unable to make a similar H-bond without substantial structural reorganization. Indeed, such an H-bond would be substantially longer, and therefore the class IIa HDACs should be expected to be less effective in processing Ac-lysines substrates (Fig. 1b).

In an effort to restore activity to HDAC4 it was decided to reintroduce the tyrosine residue in the hope of developing a 'gain of function' mutation. Accordingly, HDAC4 CD containing this H976Y mutation was generated in *E. coli* and its activity tested on a [^{3}H]acetyl histone substrate. A 1000-fold gain in activity was observed and activity of this mutant could be observed with nanomolar levels of enzyme (Table 2). Furthermore, optimization of the protocol allowed use of the commercial '*Fluor de Lys*' substrate, [21] and this HDAC4 'gain of function' (GOF) permitted IC_{50}'s to be determined, albeit using a mutated enzyme but a natural Ac-lysine substrate (Table 3).[22]

The second hypothesis to explain the weak of activity of HDAC4 is that Ac-lysine residues are not the natural substrates. Indeed HDACs have been proposed and/or reported to process a number of other substrates.[23,24] Consequently a small library of potential substrates was prepared from Boc-L-Lys-MCA by routine acylation reaction (Fig. 2), and the conversion of these by HDACs 1, 3, 4, 5, 6 and 7 was measured. The acyl groups selected were based on those known to be activated by coenzyme A (CoA), the rationale being that HATs use Ac-CoA to acetylate lysine residues. Incuba-

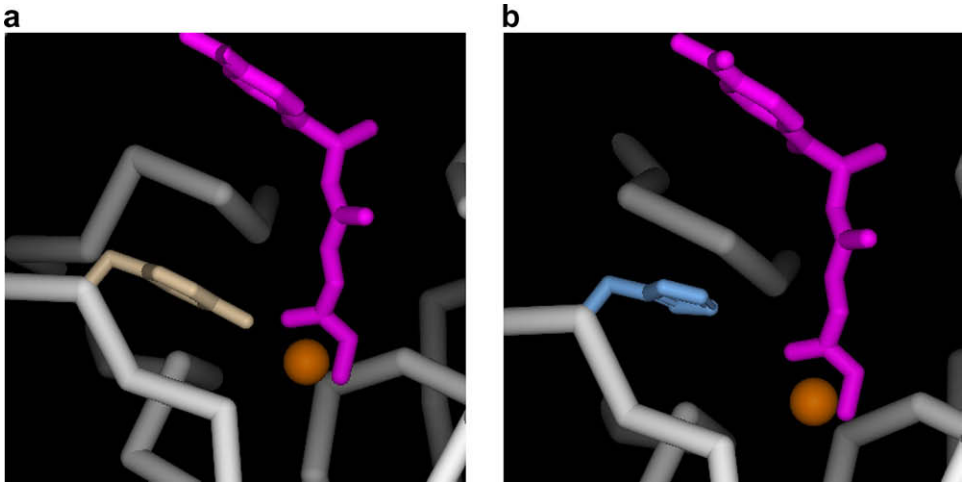
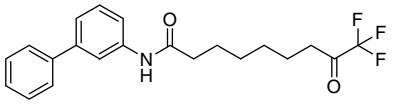
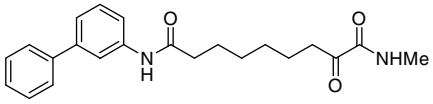


Figure 1. Active site geometries of (a) class I and (b) class IIa HDACs showing impact of the Y–H substitution and lengthening of H-bond to bound inhibitor.

Table 3. IC_{50}'s of HDACi probes on HDAC isoforms

	IC_{50} (nM)				
	HDAC1[b,d]	HDAC3[b,d]	HDAC4GOF[b,e]	HDAC4WT[c,e]	HDAC6[b,d]
Vorinostat	30	57	540	13% inhibition at 10 μM	43
NVP-LAQ824	2.6	3.6	4.4	420	8.3
PXD101	18	46	24	20% inhibition at 2 μM	15
MS-275	120	400	NA at 10 μM	NA at 10 μM	NA at 10 μM
MGCD-0103	130	610	45% inhibition at 5 μM	NA at 10 μM	32% inhibition at 2 μM
Apicidin	23	9.7	3600	8700	4300
 **11**	1400	120	200	33	1700
 **12**	12	10	NA at 10 μM	NA at 10 μM	ND

[a]Values are means >2 experiments, standard deviations were within 30% of the IC_{50} values. Measured using the [b]*Fluor de Lys* or [c]**10a** substrates. HDACs expressed in [d]HEK293 cells or [e]*E. coli*.

tions of these substrates revealed that only **10b**, **10c**, **10g**, **10n**, **10q** and **10r** where deacylated to a minor extent by HDACs 1, 3 and 6. However, trifluoroacetamide **10a** was efficiently processed not just by sub-nanomolar levels of HDAC4, but also by the other two class IIa HDACs 5 and 7 (Fig. 2). This substrate proved to be poorly processed by class I HDACs demonstrating the subtle differences between the classes. This finding permitted development of a second HDAC4 screening assay: HDAC4WT, albeit using the wild-type enzyme but an 'unnatural' substrate.[25]

The development of these two assays allowed the benchmark compounds to be profiled (Table 3). Unsurprisingly based on the previous data NVP-LAQ824 is able to inhibit both HDAC4 WT and 'gain of function', along with the other HDACs. Similarly the other hydroxamic acid HDACi's (vorinostat and PXD101) show lack of selectivity but interestingly appear to be more potent inhibitors of the H976Y mutant than the wild-type enzyme. The basis for this selectivity between HDAC4GOF and WT is not understood, but the rank-order of the inhibitors is maintained. Both Apicidin and aminobenzamides, like MS-275 and MGCD-0103, failed to inhibit either HDAC4 preparation in line with the cross-linking and pull-down experiments. However, an interesting observation is that the trifluoromethyl ketone class of HDACi, exemplified by **11**,[26] appears to inhibit both wild-type and 'gain of function' HDAC4. Interestingly, this appears to be a specific feature of the trifluoromethyl ketones, as a related class of electron deficient ketones, the ketoamides exemplified by **12**,[27] failed to inhibit either form of HDAC4.

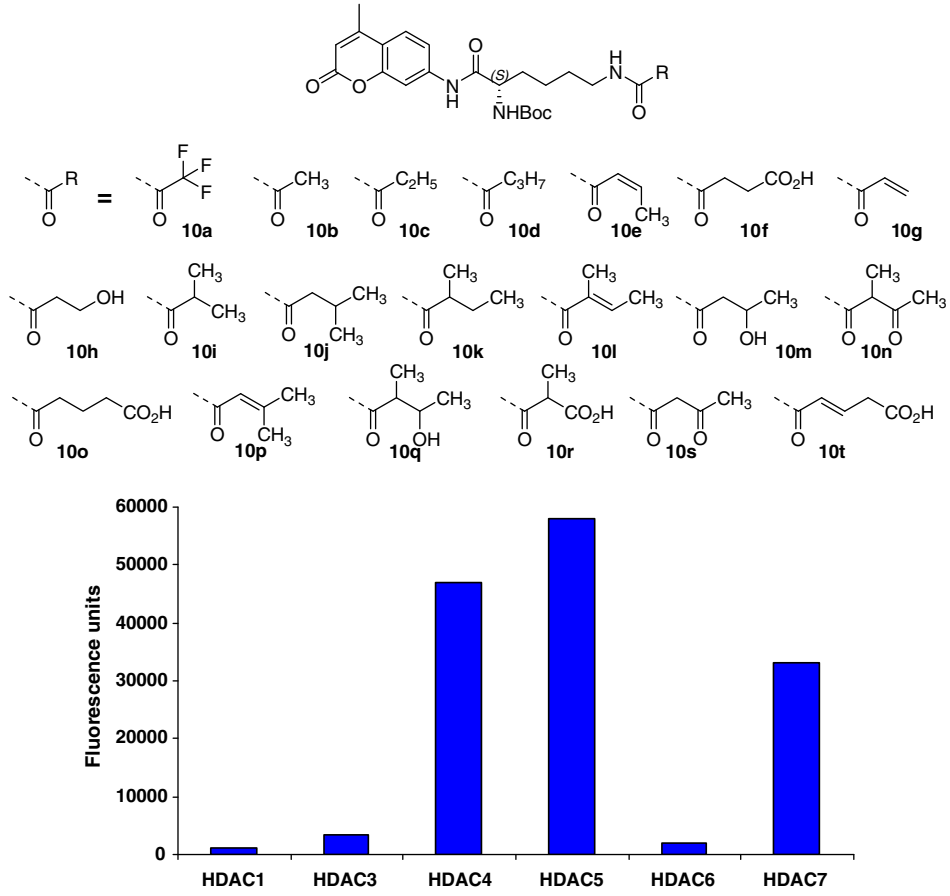


Figure 2. (a) Synthetic substrates from Boc-L-Lys-MCA; (b) conversion of **10a** by HDAC isoforms showing class IIa selectivity.

In conclusion, it has been demonstrated that the enzymatic activity of class IIa HDACs expressed in mammalian cells is due to the presence of contaminating deacetylases, likely to be endogenous class I HDACs present in the class IIa complex. When pure class IIa HDACs can be isolated from bacteria they possess a weak but measurable intrinsic deacetylase activity, the low catalytic efficiency of which is due to the presence of a unique H residue. Finally the low efficiency can be restored and measured either by an H–Y mutation in the active site to give a 'gain of function', or by the use of an non-Ac-lysine 'unnatural' substrate and the wild-type enzyme.

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PAGE and analyzed by Western blot using alkaline phosphatase conjugated ExtrAvidin. While in the latter, streptavidin-coated magnetic beads pre-treated with the probes were incubated for 3 h at RT with flag-tagged HDAC4. The bound proteins were detached from the bead, separated and visualized by Coomassie-Blue staining.

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22. DMSO/compound solution were incubated for 10 min with His-tagged HDAC4GOF (653-1084, H976Y) from *E. coli* in assay buffer (20 mM Hepes, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.1 mg/ml BSA), *Fluor-de-Lys* substrate[21] solution was added and left for 1 h at 37 °C and the reaction stopped by adding developer[21]/TSA solution. Measure the fluorescence at ex.360 nM/em.460 nM.
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25. DMSO/compound solution was incubated for 10 min with His-tagged HDAC4 CD(653–1084) from *E. coli* in assay buffer (25 mM Tris/HCl, pH 8, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.1 mg/ml BSA), **10a** substrate solution was added and left for 1 h at 37 °C and the reaction stopped by adding developer[21]/TSA solution. Measure the fluorescence at ex.360 nM/em.460 nM.
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