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Active site tyrosine is essential for amidohydrolase but not for esterase activity of a class 2 histone deacetylase-like bacterial enzyme

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SHORT TITLE: *Histone-deacetylase-like enzyme with esterase activity*

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The abbreviations used:

AMC	7-amino-4-methylcoumarin
HAT	histone acetyltransferase
HDA1	histone deacetylase 1 (fission yeast)
HDAC	histone deacetylase
HDAH	histone deacetylase-like amidohydrolase (from strain FB188)
HDLP	histone deacetylase-like protein
HMC	7-hydroxy-4-methylcoumarin
IC50	Inhibitor concentration at 50 % inhibition
MCA	4-methylcoumarin-7-amide
NMM	4-methylmorpholine
RPD3	histone deacetylase from budding yeast (reduced potassium dependency)
RT	room temperature
SAHA	suberoylanilide hydroxamic acid

KEY WORDS: histone deacetylase, amidohydrolase, esterase, mechanism, FB188 HDAH, Bordetella/Alcaligenes, fluorogenic assay, chromogenic substrates, lipase

ABSTRACT

Histone deacetylases (HDACs) are considered to be among the most important enzymes that regulate gene expression in eukaryotic cells acting through deacetylation of ϵ -acetyl-lysine residues within the N-terminal tail of core histones. In addition, both eukaryotic HDACs as well as their bacterial counterparts were reported to also act on nonhistone targets. However, we are still far from a comprehensive understanding of the biological activities of this ancient class of enzymes. Here, we studied in more detail the esterase activity of HDACs focussing on the histone deacetylase-like amidohydrolase (HDAH) from *Bordetella/Alcaligenes* strain FB188. This enzyme was classified as a class 2 HDAC based on sequence comparison as well as functional data. Using chromogenic and fluorogenic ester substrates we show that histone deacetylases such as FB188 HDAH have indeed esterase activity that is comparable to those of known esterases. Similar results were obtained for human HDAC1, 3 and 8. Standard HDAC inhibitors were able to block both activities with similar IC₅₀ (inhibitor concentration at 50 % inhibition) at values. Interestingly, HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) also showed inhibitory activity against porcine liver esterase and *Pseudomonas fluorescens* lipase. The esterase- and the amidohydrolase activity of FB188 HDAH both appear to have the same substrate specificity concerning the acyl moiety. Interestingly, a Y312F mutation in the active site of HDAH obstructed amidohydrolase activity but significantly improved esterase activity, indicating subtle differences in the mechanism of both catalytic activities. Our results suggest that, in principle, HDACs may have other biological roles besides acting as protein deacetylases. Furthermore, data on HDAC inhibitors affecting known esterases indicate that these molecules, which are currently among the most promising drug candidates in cancer therapy, may have a broader target profile requiring further exploration.

INTRODUCTION

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are key enzymes in the regulation of gene expression in eukaryotic cells. HATs and HDACs catalyze the addition and the removal of acetyl moieties from the ϵ -amino groups of lysines near the amino termini of histones, respectively. In general, increased levels of histone acetylation are associated with increased transcriptional activity, whereas decreased levels of acetylation are associated with repression of gene expression [1-3]. The activity of HATs and, in particular, of HDACs, both affect angiogenesis, cell-cycle arrest, apoptosis, terminal differentiation of different cell types and the pathogenesis of malignant disease [4]. Not surprisingly, a number of HDAC inhibitors show a potency as promising antitumor agents. Several drug candidates are currently in phase I – III clinical trials [5-8].

Four classes of deacetylase enzymes have been categorized on the basis of sequence homology and functional properties [9]: RPD (reduced potassium dependency)-like class 1 HDACs, HDA histone deacetylase)-like class 2 HDACs and class 4 enzymes (HDAC11) are structurally

related zinc-dependent enzymes, whereas class 3 enzymes comprise the structurally unrelated NAD-dependent sirtuins [10].

For a long time research on HDACs had focussed mainly on their role in transcriptional regulation. More recently, however, data became available indicating that HDACs may also act on non-histone proteins and small molecules. Examples comprise HDAC 6, which has tubulin deacetylase activity [11-13], and bacterial histone deacetylase-like amidohydrolases (HDAHs) [14, 15].

Recently, the crystal structures of two class 1 enzymes, HDLP (histone deacetylase-like protein from *A. aeolicus*) [16] and HDAC8 [17, 18], as well as that of one class 2 enzyme, FB188 HDAH [19] have been solved. Based particularly on enzyme-inhibitor co-complex structures (see for example [Figure 3A and B](#)), a mechanism has been proposed which includes features of those from metallo- and serine proteases [16]. By this mechanism ([Figure 3C-E](#)), the active site zinc ion would bind to the carbonyl oxygen of the acetyl moiety polarizing the carbonyl group and thereby increase the electrophilicity of the carbon. The zinc ion also binds to the oxygen of a water molecule such that the nucleophilicity of the water oxygen is increased. In analogy to the mechanism of serine proteases, the nucleophilicity of the water molecule is further increased by the negative charge of a buried Asp-His charge-transfer relay system, to which the water molecule is hydrogen bonded. The nucleophilic attack of the water molecule on the carbonyl carbon would lead to a tetrahedral oxyanion transition state which would be stabilized by the aforementioned zinc-oxygen contacts and by a potential hydrogen bond to the hydroxyl group of a tyrosine residue (Y312 in HDAH). Finally, the acetate would be released and the ϵ -nitrogen of the lysine residue would accept a proton from a second Asp-His charge-transfer-relay system not present in *FB188* HDAH or any other class 2 enzymes [20]. Confirmation of the important role played by the aforementioned active site amino acid residues came from mutagenesis studies [16, 19, 21, 22]. The proposed mechanism has recently been challenged in part by: (i) *ab initio* calculation studies [23], (ii) experiments with transition state analogue inhibitors designed to mimic the proposed oxyanion intermediate (10) and (iii) experiments with substrates containing different acyl leaving groups [20]. Despite the huge body of data generated throughout many years of HDAC research, relatively little is known about natural substrates of different HDACs. Indeed, studies so far focussed exclusively on amides as possible substrates. In this study, we demonstrate that HDAC enzymes such as HDAH, HDAC1, HDAC3 and HDAC8 also have a very pronounced esterase activity that can be inhibited by known HDAC inhibitors. On the other hand, HDAC inhibitors are also active against known esterases. Specificity towards acyl moieties is similar for both, amidohydrolase and esterase activities. However, mutation of active site tyrosine (Y312 in HDAH) into phenylalanine impairs only amidohydrolase but actually improves esterase activity. Altogether, our experimental results improve our understanding of the catalytic mechanism of HDACs. Furthermore, we provide first evidence suggesting that at least certain members of the HDAC family may assume the biological role of an esterase.

EXPERIMENTAL

Synthesis of fluorogenic substrates

Boc-L-Lys-4-methylcoumarin-7-amide (MCA) and Boc-L-Lys(ϵ -acetyl)-MCA were purchased from Bachem (Weil am Rhein, Germany). 4-nitrophenyl acetate and all other reagents for organic synthesis were obtained from Sigma (Taufkirchen, Germany). The ϵ -Propionyl-derivative of Boc-L-Lys(ϵ -acyl)-MCA was synthesized as described [20]. The acetyl- and propionyl-esters of 7-hydroxy-4-methylcoumarin were synthesized using standard protocols. Briefly, 0.5 mmol of 7-hydroxy-4-methylcoumarin was suspended in 2 ml of dioxane. After addition of 90 μ l of N-methylmorpholine (NMM) and 180 μ l of acetyl chloride (or propionyl chloride) the suspension was incubated for 10 min at room temperature (RT). After addition of 2 ml of acetonitrile and 180 μ l NMM the solution was shaken overnight at RT. The solvent was removed under reduced pressure and the residue was redissolved in ethyl acetate. After extraction with 3 x 20 ml of water the solvent was removed under reduced pressure. The product was analyzed by reversed phase HPLC/Mass spectroscopy using a Waters Alliance/MicromassZQ system and a 250 x 10 mm-C18 column (Jupiter, Phenomenex, Aschaffenburg, Germany). Purities were > 90 % with no educt present. LC-MS (ESI): 4-methylcoumarin-7-acetate calculated for C₁₂H₁₀O₄ (M+H): 219.2. Found: 219.2. 4-methylcoumarin-7-propionate calculated for C₁₃H₁₂O₄ (M+H): 233.2. Found: 233.2.

Assays

HDAC1, HDAC3 and N-COR2 were obtained from BPS Bioscience Inc. (San Diego, USA). HDAC8 was expressed in *E.coli* and purified as described [20]. Porcine liver esterase (EC 3.1.1.1.) was from Sigma (Taufkirchen, Germany), *Pseudomonas fluorescens* lipase was purchased from Fluka (Taufkirchen, Germany). Mutant HDAH genes were generated by the Quik™-Change method using the protocols of the supplier (Stratagene, La Jolla, CA, USA). FB188 HDAH and its mutants were prepared as described [14]. For fluorometric assays, all pipetting and fluorescence detection steps were carried out with the help of a robotic workstation (CyBi™-Screen-Machine, CyBio AG, Germany) including a Polarstar fluorescence reader (BMG, Germany). Reactions were conducted in black 96-well microplates. For assays with chromogenic substrates, a UV-1601 spectrophotometer (Shimadzu, Duisburg, Germany) was used.

Fluorogenic assays. Amidohydrolase assays were performed similarly to the standard fluorogenic HDAC assay [24]. Briefly, 10 μ l of diluted enzyme solution (corresponding to 50 ng HDAH), 50 μ l substrate solution in HD buffer (15 mM Tris-HCl, 250 μ M EDTA, 250 mM NaCl, 0.1 % (w/v) PEG8000), pH 8.0, including 50 mM potassium phosphate and 80 μ l

trypsin solution (1 mg/ml in HD buffer) were mixed and incubated at 30 °C. The release of AMC (7-amino-4-methylcoumarin) was monitored by measuring the fluorescence at 460 nm ($\lambda_{\text{ex}} = 390$ nm). Fluorescence intensity was calibrated using free AMC. For HDAC1 and HDAC3, the two-step variant of the assay was used. Briefly, 10 μl of diluted enzyme solution and 50 μl substrate solution in HD buffer were mixed and incubated at 30 °C. After 45 min incubation 80 μl trypsin solution were added. After another incubation for 15 min at 30 °C the released AMC was monitored by measuring the fluorescence at 460 nm. K_M and V_{max} values as well as IC_{50} concentrations were determined as previously described [25]. Substrate concentrations between 0.5 and 50 μM were used for standard K_M and V_{max} determinations. For IC_{50} determinations a substrate concentration of 2 μM was used.

Fluorescence-based esterase assays were performed by mixing on ice 10 μl of enzyme solution, 180 μl HD buffer, pH8 and 10 μl substrate solution (from a 40 μM stock of either substrate C or D, Figure 1). After switching the reaction temperature to 20 °C the release of HMC (7-hydroxy-4-methylcoumarin) was monitored by measuring the fluorescence at 460 nm. Fluorescence intensity was calibrated using free HMC. Note that the esterase substrates (C and D, Figure 1) showed substantial hydrolysis at higher temperature.

Chromogenic esterase assay. In the standard chromogenic assay 10 μl of enzyme were mixed on ice with 980 μl HD buffer and 10 μl substrate solution. After switching the reaction temperature to 25 °C the release of 4-nitrophenol was monitored by measuring the absorbance at 405 nm. Absorbance was calibrated using free 4-nitrophenol ($\epsilon_{405} = 18500 \text{ M}^{-1} \text{ cm}^{-1}$).

RESULTS

The present study was initiated to examine a possible esterase activity of HDACs. Esters and amides are rather similar in structure and indeed a number of enzymes have been reported to exhibit dual functionalities accepting either substrate classes (e.g. [26-29]). The work presented herein concentrates particularly on the HDAC-like amidohydrolase (HDAH) from *Bordetella* (Alcaligenes strain FB188 [14] as an HDAC homologue which can be easily produced in large quantities. FB188 HDAH exhibits 30% identity over 291 residues with the first domain of human HDAC6 (HDAC6a), 35% identity with the second domain (HDAC6b), and both enzymes share a number of functional similarities. They are zinc-dependent amidohydrolases that are able to efficiently deacetylate chicken histones, and they show the same substrate specificity towards small peptidic substrates [20, 30]. In addition, FB188 HDAH is also inhibited by common HDAC inhibitors such as SAHA. Furthermore, the crystal structure of FB188 HDAH reveals the canonical fold of HDACs [19].

Histone deacetylase-like amidohydrolase is an efficient esterase. Previously, we have shown that FB188 HDAH is an amidohydrolase that catalyzes the release of acetate from ϵ -acetylated lysine residues in the context of various substrates including fluorogenic peptides [14, 20, 25, 31, 32] as well as eukaryotic histones [14]. In addition, we demonstrated that the enzyme also converted nonpeptidic amides such as *cis*-(+/-)-N-[4-(hydroxymethyl)cyclopent-2-enyl] acetamide [14]. For Boc-L-Lys(ϵ -acyl)-MCA substrates (Figure 1, A and B) the acetyl

derivative was the superior substrate with K_M and V_{max} values of $14.4 \pm 3.0 \mu\text{M}$ and $136 \pm 22 \text{ pmol sec}^{-1} \text{ mg}^{-1}$, respectively [20]. Boc-L-Lys(ϵ -propionyl)-MCA, however, did not show a significant conversion [20]. To test the possibility that FB188 HDAH also could act as an esterase we studied esterase activity in comparison to that of porcine liver esterase and *Pseudomonas fluorescens* lipase using 4-methylcoumarin-7-acetate as a substrate (Figure 1, C; Table 1). Cleavage of this substrate resulted in the release of fluorescent 7-hydroxy-4-methylcoumarin, which was monitored by measuring the fluorescence at $\lambda_{em} = 460 \text{ nm}$ and $\lambda_{ex} = 390 \text{ nm}$. HDAH indeed proved to exhibit specific esterase activity that is comparable or even superior to those of porcine liver esterase and *Pseudomonas fluorescens* lipase (Table 1), which were used as controls. For 4-methylcoumarin-7-acetate K_M and V_{max} values of $0.90 \pm 0.13 \mu\text{M}$ and $14100 \pm 2100 \text{ pmol sec}^{-1} \text{ mg}^{-1}$ were measured, respectively. For porcine liver esterase the corresponding values were $2.84 \pm 0.84 \mu\text{M}$ and $2150 \pm 630 \text{ pmol sec}^{-1} \text{ mg}^{-1}$. For *Pseudomonas fluorescens* lipase we obtained K_M and V_{max} values of $1.66 \pm 0.30 \mu\text{M}$ and $183 \pm 41 \text{ pmol sec}^{-1} \text{ mg}^{-1}$, respectively.

Esterase activity is dependent on the acyl moiety. In the aforementioned experiments with amide substrates FB188 HDAH exhibited a clear preference for acetamides over the corresponding propionyl derivatives. We therefore also tested 4-methylcoumarin-7-propionate as a substrate (Table 1). For the latter substrate K_M and V_{max} values of $31.2 \pm 3.7 \mu\text{M}$ and $2220 \pm 260 \text{ pmol sec}^{-1} \text{ mg}^{-1}$ were measured, respectively. Thus, the enzyme again showed a preference for the acetyl derivative, reflected by both a 34-fold increase in K_M and a 6-fold decrease in V_{max} for 4-methylcoumarin-7-propionate.

Esterase activity is modulated by the nature of the alcoholic moiety. To show if the alcohol moiety of the ester has a significant influence on the kinetic parameters of the ester substrates we compared the conversions of the aforementioned 4-methylcoumarin-7-acetate and 4-nitrophenyl acetate (Figure 1, E; Table 1). For the latter, we measured K_M and V_{max} values of $11.8 \pm 1.8 \mu\text{M}$ and $33000 \pm 4900 \text{ pmol sec}^{-1} \text{ mg}^{-1}$, respectively, indicating a 13-fold increase in K_M and a 2-fold increase in V_{max} .

Esterase activity can be blocked by HDAC inhibitors. To confirm that the esterase activity resides in the same active site of FB188 HDAH that is used to catalyze amidohydrolase reactions we tested esterase activity in the presence of known HDAC inhibitors (Table 2). For SAHA and CypX IC_{50} values of $0.43 \mu\text{M}$, $0.59 \mu\text{M}$ were obtained using 4-methylcoumarin-7-acetate as a substrate. Comparing these IC_{50} values to that obtained in the fluorogenic HDAC assay ([24]; Table 2) it appeared that the inhibition of amidohydrolase and esterase activities were comparable for both hydroxamate inhibitors.

Interestingly, HDAC inhibitors also showed a significant inhibition of control esterases. For *Pseudomonas fluorescens* lipase SAHA showed an IC_{50} values of 3.82. For porcine liver esterase no inhibitory activity was observed for the hydroxamic acids (SAHA, CypX). Here, however, MS275 proved to be a rather good inhibitor (data not shown). Furthermore, for a trifluoromethylketone derivative which we recently had identified in a screening program as a

submicromolar inhibitor of HDAH and eukaryotic HDACs, we also could detect submicromolar inhibition of porcine liver esterase (data not shown).

Y312F mutation impairs amidohydrolase but not esterase-activity. To further confirm that both amidohydrolase and esterase activities reside in the same active site and to study possible similarities in the catalytic mechanism between FB188 HDAH acting as amidohydrolase or esterase we analyzed the effect of mutations on enzyme activity (Table 1). Whereas mutant H142N (inner charge transfer relay system) proved to be inactive for both enzyme activities, Y312F only decreased amidohydrolase activity. Surprisingly, esterase activity was even improved in the mutant as compared to the wild-type enzyme. Using 4-methylcoumarin-7-acetate as a substrate K_M and V_{max} values of $1.68 \pm 0.32 \mu\text{M}$ and $513000 \pm 97700 \text{ pmol sec}^{-1} \text{ mg}^{-1}$, respectively, were measured. This is less than a 2-fold increase in K_M but a 36-fold increase in V_{max} compared to the wild-type enzyme. For 4-methylcoumarin-7-propionate as a substrate K_M and V_{max} values of $0.22 \pm 0.02 \mu\text{M}$ and $52400 \pm 4700 \text{ pmol sec}^{-1} \text{ mg}^{-1}$ were measured, respectively. This is a 4-fold decrease in K_M and a 23-fold increase in V_{max} as compared to the wild-type enzyme and the same substrate.

Human HDAC1, HDAC3 and HDAC8 also exhibit esterase-activity. Next, we were interested to know whether human HDACs are also capable, in principle, of executing esterase activity. For (recombinant) human HDAC1, 3 and 8 substrate conversions in pmol/min were compared using the same enzyme and substrate concentrations but different substrates. In Figure 2 the relative substrate conversions are depicted by arbitrarily setting the conversion of the HDAC substrate (Boc-L-Lys(ϵ -acetyl)-MCA) to 1. Using both 4-methylcoumarin-7-acetate and 4-methylcoumarin-7-propionate as substrates, we clearly demonstrated that HDAC1, HDAC3/N-COR2 and HDAC8 also have esterase activities. However, different specificities concerning the acyl moiety were observed. HDAC1 appeared to have a preference for the propionate derivative of the ester. As compared to Boc-L-Lys(ϵ -acetyl)-MCA the ester substrates were more efficiently converted by a factor of 3 and 43 for the acetate and the propionate derivative, respectively. For HDAC3/N-COR2 and HDAC8, similar to FB188 HDAH, a preference for the acetate derivative of the ester was observed. Here, as compared to the conversion of Boc-L-Lys(ϵ -acetyl)-MCA the ester substrates were more efficiently converted by a factor of 48 and 20 for the acetate and the propionate derivative, respectively.

DISCUSSION

Esterase activity of FB188 HDAH. The major biological function of histone deacetylases is believed to be the deacetylation of ϵ -acetylated lysines in histones and other proteins [33]. For their bacterial counterparts the biological role is much less clear. Experimental data available so far supports the idea that these enzymes may also function as deacetylases of proteins with ϵ -acetylated lysine residues [14] as well as polyamines [15]. Here we present experimental evidences that histone deacetylase-like enzymes are also very catalytically efficient esterases. Using chromogenic and fluorogenic substrates we demonstrated that FB188 HDAH indeed has esterase activity comparable or superior to that of well-known lipases and esterases. In terms of

specific substrate conversion, the catalytic properties of HDAC-like enzymes conform to the general reactivity of amides and esters: in contrast to esters, amides are known to be resonance-stabilized as reflected by a more than 10³ times slower reaction in OH⁻-catalyzed reactions [34-36]. In our study we observed that the bacterial FB188 HDAH hydrolyzed specific ester substrates with deacylation rates that exceeded those of amides by up to 2 orders of magnitude. Other researchers have reported on similar results obtained with certain serine proteases which also show amidohydrolase and esterase activity [34-38].

In both ester and amide substrates, FB188 HDAH has as significant preference for acetyl over propionyl moieties presumably due to space limitations in the active site cleft [25]. In line with this assumption the larger 4-methylcoumarin-7-propionate is only bound well in the larger active site of the Y312F mutant, as reflected by a 142-fold decrease in the *K_M* value. On the other hand, comparing substrates based on 4-nitrophenol or 7-hydroxy-4-methyl coumarin it appears that enzyme activity is less dependent on the alcohol moiety. However, as shown for amide substrates [14, 16], it cannot be excluded that there may be more dramatic effects with other alcohol moieties.

Implications for the catalytic mechanism. Since standard HDAC/HDAH inhibitors such as SAHA and CypX inhibited not only amidohydrolase but also esterase activity, we tend to believe that both catalytic activities reside in the same active site cleft. Although differences between *IC*₅₀ values measured in different assays in general can be fairly large [39], for SAHA and CypX amidohydrolase and esterase assays the *IC*₅₀ data differed only by a factor of 2. In addition, both the amidohydrolase and the esterase activities show the same preference concerning acyl moieties, thus arguing against the possibility of two independent and rather different mechanisms. The view that both catalytic activities reside in the same active site gained further support from the fact that a H142N mutation in the “inner charge transfer relay system” was detrimental to both, amidohydrolase activity and esterase function.

In consistency with the aforementioned facts we like to propose a mechanism for ester cleavage (Figure 3C-E) that very much resembles the proposed mechanism for amide cleavage. In both cases a water molecule as the attacking nucleophile is activated by interaction with an Asp-His142 charge-transfer relay system and a zinc ion (Figure 3C). To increase the electrophilicity of the carbonyl carbon the carbonyl bond of the ester or amide is polarized by interactions of the carbonyl oxygen with the zinc ion and with the hydroxyl group of Tyr312 (Figure 3C). Given the higher reactivity of esters compared to that of amides it seems plausible to assume that part of this carbonyl polarization is dispensable in the case of ester cleavage. This would explain the fact that a Y312F mutation did impair the energetically more demanding amidohydrolase activity but still allowed the enzyme to work as an esterase. Furthermore, in the originally proposed mechanism it was stressed that tyrosine would stabilize the tetrahedral oxyanion transition-state by hydrogen bonding to the carbonyl oxygen (Figure 3D). At least in the case of ester cleavage this function of tyrosine is obviously also non-essential. Surprisingly, however, a Y312F mutation did even stimulate esterase activity, particularly *V*_{max}. As pointed out earlier, FB188 HDAH shows space limitations in the active site cleft. These structural constraints may force the

substrate and side chains of the enzyme into unfavorable conformations. In line with this assumption, the absence of a hydroxyl group in mutant Y312F would provide the active site with additional space, i.e. conformational freedom that could stimulate catalytic activity at least in the case of ester cleavage. Future crystallographic studies have to specify the structural differences between wild-type HDAH and the Y312F mutant that presumably causes the observed differences in catalytic activity.

The fact that amidohydrolase activity is more sensitive to small structural perturbations in the active site as compared to esterase activity has also been observed in other systems. It is for example a well-known fact that amidohydrolase activity of serine proteases can be easily damaged by site-directed mutagenesis [40] or chemical modifications [41-45]. In contrast, esterase activity was much less affected and even increased in some cases, although never to the extent reported herein for the Y312F mutant of FB188 HDAH. Thus, it appears that hydrolases, in general, require a more finely-tuned and stronger catalytic activity to hydrolyze energetically more demanding amides as compared to ester cleavage.

Implications for possible biological roles of histone deacetylase-like enzymes and HDACs. In line with previous findings indicating that FB188 HDAH structurally and functionally resembles eukaryotic HDACs we observed that not only FB188 HDAH but particularly also recombinant human HDAC1 and HDAC3 exhibited pronounced esterase activity that far exceeded their amidohydrolase activity in terms of molar substrate conversion. In regard of specificity, however, we noticed some differences. Whereas HDAC3/N-CoR2 and HDAC8 resembled FB188 HDAH in favoring the acetyl derivative of the ester substrate, HDAC1 showed a clear preference for the propionyl derivative. It can be speculated that esterase activity may have preceded amidohydrolase activity, which later in evolution may have been acquired through mutation of an active site residue (Tyr312 in HDAH). The mere existence of a pronounced esterase activity neither proves nor disproves that members of the histone deacetylase family at present have biological roles as esterases. The discovery of esterase activity, however, could be taken as an opportunity to reconsider our current view of histone deacetylases as specialized amidohydrolases and to motivate further research to identify biologically important esters as possible biological substrates of histone deacetylases.

Implications for HDAC inhibitor development. It is a well-known fact that inhibitors of HDACs affect angiogenesis, cell-cycle arrest, apoptosis, terminal differentiation of different cell types and the pathogenesis of malignant disease [4]. The development of HDAC inhibitors has become a very promising therapeutic approach in modern cancer research [8]. However, mass screening for HDAC inhibitors, particularly in miniaturized formats, is still impeded by the relatively modest activities of the recombinant enzymes available. Furthermore, the most common fluorogenic assay in primary screening [24] usually has to be performed as a two-step endpoint assay due to the susceptibility of most HDACs to trypsin [39]. In the light of our results it may be worthwhile to consider the esterase assay as a possible alternative [46]. In the esterase assay, substrate conversion rates are much more favorable for highly sensitive detection of small amounts of enzyme, particularly in miniaturized sample carriers.

Furthermore, the esterase-assay, e.g. with substrates C or D (Figure 1), permits continuous monitoring of enzyme activity. At least for all hydroxamate and trifluoromethylketone inhibitors tested so far the results of both assays, for example the IC₅₀ values, are comparable. However, compound classes may exist for which the transferability of results is not guaranteed. Whatever assays will be used for HDAC inhibitor screening though, it appears mandatory to include major esterases into the inhibitor profiling. Our results indicate that at least certain hydroxamates and trifluoromethylketone inhibitors of HDACs are also very efficient lipase or esterase inhibitors and therefore could cause unwanted side effects in the treatment of patients.

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REFERENCES

- 1 Grunstein, M. (1997) Histone acetylation in chromatin structure and transcription. *Nature* **389**, 349-352
- 2 Peterson, C. L. (2002) HDAC's at work: everyone doing their part. *Mol. Cell* **9**, 921-929
- 3 Wade, P. A., Pruss, D. and Wolffe, A. P. (1997) Histone acetylation: chromatin in action. *Trends Biochem. Sci.* **22**, 128-132
- 4 Chung, D. (2002) Histone modification: the "next wave" in cancer therapeutics. *Trends in Molecular Medicine* **8**, S10-S11
- 5 Johnstone, R. W. (2002) Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nature reviews* **1**, 287-299
- 6 Rosato, R. R. and Grant, S. (2003) Histone deacetylase inhibitors in cancer therapy. *Cancer Biol. Ther.* **2**, 30-37
- 7 Villar-Garea, A. and Esteller, M. (2004) Histone deacetylase inhibitors: understanding a new wave of anticancer agents. *Int. J. Cancer* **112**, 171-178
- 8 Minucci, S. and Pelicci, P. G. (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nature reviews* **6**, 38-51
- 9 Gregoret, I. V., Lee, Y.-M. and Goodson, H. V. (2004) Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *JMB* **338**, 17-31

- 10 Grozinger, C. M. and Schreiber, S. L. (2002) Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. *Chem. Biol.* **9**, 3-16
- 11 Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X.-F. and Yao, T.-P. (2002) HDAC6 is a microtubule-associated deacetylase. *Nature* **417**, 455-458
- 12 Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y. and Seigneurin-Berry, D. (2002) In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBO J.* **21**, 6820-6831
- 13 Zhang, Y., Li, N., Caron, C., Matthias, G., Hess, D., Khochbin, S. and Matthias, P. (2003) HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. *EMBO J.* **22**, 1168-1179
- 14 Hildmann, C., Ninkovic, M., Dietrich, R., Wegener, D., Riester, D., Zimmermann, T., Birch, O. M., Bernegger, C., Loidl, P. and Schwienhorst, A. (2004) A new amidohydrolase from *Bordetella/Alcaligenes* strain FB188 with similarities to histone deacetylases. *J. Bacteriol.* **8**, 2328-2339
- 15 Sakurada, K., Ohta, T., Fujishiro, K., Hasegawa, M. and Aisaka, K. (1996) Acetylpolyamine amidohydrolase from *Mycoplana ramosa*: gene cloning and characterization of the metal-substituted enzyme. *J. Bacteriol.* **178**, 5781-5786
- 16 Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R. and Pavletich, N. P. (1999) Structures of a histone deacetylase homologue bound to TSA and SAHA inhibitors. *Nature* **401**, 188-193
- 17 Vannini, A., Volpari, C., Filocamo, G., Casavola, E. C., Brunetti, M., Renzoni, D., Chakravarty, P., Paolini, C., De Francesco, R., Gallinari, P. and Steinkühler, C. (2004) Crystal structure of a eukaryotic zinc-dependent histone deacetylase, human HDAC8, complexed with a hydroxamic acid inhibitor. *PNAS* **101**, 15064-15069
- 18 Somoza, J. R., Skene, R. J., Katz, B. A., Mol, C., Ho, J. D., Jennings, A. J., Luong, C., Arvai, A., Buggy, J. J., Chi, E., Tang, J., Sang, B.-C., Verner, E., Wynands, R., Leahy, E. M., Dougou, D. R., Snell, G., Navre, M., Knuth, M. W., Swanson, R. V., McRee, D. E. and Tari, L. W. (2004) Structural snapshots of human HDAC8 provide insights into the class I histone deacetylases. *Structure* **12**, 1325-1334
- 19 Nielsen, T. K., Hildmann, C., Dickmanns, A., Schwienhorst, A. and Ficner, R. (2005) Crystal structure of a bacterial class 2 histone deacetylase homologue. *J. Mol. Biol.* **354**, 107-120
- 20 Riester, D., Wegener, D., Hildmann, C. and Schwienhorst, A. (2004) Members of the histone deacetylase superfamily differ in substrate specificity towards small synthetic substrates. *Biochem. Biophys. Res. Com.* **324**, 1116-1123
- 21 Hassig, C. A., Tong, J. K., Fleischer, T. C., Owa, T., Grable, P. G., Ayer, D. E. and Schreiber, S. L. (1998) A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. *Proc. Natl. Acad. Sci. USA* **95**, 3519-3524

- 22 Kadosh, D. and Struhl, K. (1998) Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo. *Genes & Dev.* **12**, 797-805
- 23 Vanommeslaeghe, K., VanAlsenoy, C., DeProft, F., Martins, J. C., Tourwé, D. and Geerlings, P. (2003) Ab initio study of the binding of trichostatin A (TSA) in the active site of histone deacetylase like protein (HDLP). *Org. Biomol. Chem.* **1**, 2951-2957
- 24 Wegener, D., Wirsching, F., Riester, D. and Schwienhorst, A. (2003) A fluorogenic histone deacetylase assay well suited for high-throughput activity screening. *Chem. Biol.* **10**, 61-68
- 25 Hildmann, C., Wegener, D., Riester, D., Hempel, R., Schober, A., Merana, J., Giurato, L., Guccione, S., Nielsen, T. K., Ficner, R. and Schwienhorst, A. (2006) Substrate and inhibitor specificity of class 1 and class 2 histone deacetylases. *J. Biotechnol.* **124**, 258-270
- 26 Thompson, R. C. and Blout, E. R. (1970) Evidence for an extended active center in elastase. *Proc Natl Acad Sci U S A* **67**, 1734-1740
- 27 Hui, D. Y., Hayakawa, K. and Oizumi, J. (1993) Lipoamidase activity in normal and mutagenized pancreatic cholesterol esterase (bile salt-stimulated lipase). *Biochem. J.* **291**, 65-69
- 28 Alkema, W. B. L., Dijkhuis, A.-J., de Vries, E. and Janssen, D. B. (2002) The role of hydrophobic activ-site residues in substrate specificity and acyl transfer activity of penicillin acylase. *Eur. J. Biochem.* **269**, 2093-2100
- 29 Komeda, H. and Asano, Y. (2005) A DmpA-homologous protein from *Pseudomonas* sp. is a dipeptidase specific for -alanyl dipeptides. *FEBS J.* **272**, 3075-3084
- 30 Heltweg, B., Dequiedt, F., Marshall, B. L., Brauch, C., Yoshida, M., Nishino, N., Verdin, E. and Jung, M. (2004) Subtype selective substrates for histone deacetylases. *J. Med. Chem.* **47**, 5235-5243
- 31 Wegener, D., Hildmann, C., Riester, D. and Schwienhorst, A. (2003) Improved fluorogenic histone deacetylase assay for high-throughput-screening applications. *Anal. Biochem.* **321**, 202-208
- 32 Riester, D., Hildmann, C., Grünwald, S., Beckers, T. and Schwienhorst, A. (2006) Profiling of histone deacetylase specificity based on combinatorial fluorogenic substrate libraries. *subm.*
- 33 Kouzarides, T. (2000) Acetylation: a regulatory modification to rival phosphorylation? *EMBO J.* **19**, 1176-1179
- 34 Bender, M. L., Schonbaum, G. R. and Zerner, B. (1962) Spectrophotometric investigation of the mechanism of α -chymotrypsin catalysed hydrolases. *J. Am. Chem. Soc.* **84**, 2540-2550
- 35 Zerner, B., Bond, R. P. M. and Bender, M. L. (1964) Kinetic evidence for the formation of acyl-enzyme intermediates in the α -chymotrypsin-catalyzed hydrolyses of specific substrates. *J. Am. Chem. Soc.* **113**, 2259-2263

- 36 Bender, M. L. and Kezdy, J. (1965) Mechanism of action of proteolytic enzymes. *Ann. Rev. Biochem.* **34**, 49-76
- 37 Walsh, C. (1979) *Enzymatic reaction mechanisms*, Freeman, San Francisco
- 38 Bennet, A. J. and Brown, R. S. (1998) in *Comprehensive Biological Catalysis: A Mechanistic Reference*, vol. 1 (Sinnott, M., ed.), pp. 293-326, Academic Press, San Diego
- 39 Wegener, D., Hildmann, C. and Schwienhorst, A. (2003) Recent progress in the development of assays suited for histone deacetylase inhibitor screening. *Mol. Gen. Metab.* **80**, 138-147
- 40 Abrahamsén, L., Tom, J., Burnier, J., Butcher, K. A., Kossiakoff, A. and Wells, J. A. (1991) Engineering subtilisin and its substrates for efficient ligation of peptide bonds in aqueous solution. *Biochemistry* **30**, 4151-4159
- 41 Nakatsuka, T., Sasaki, T. and Kaiser, E. T. (1987) Peptide segment coupling catalyzed by the semisynthetic enzyme thiolsubtilisin. *J. Am. Chem. Soc.* **109**, 3808-3810
- 42 West, J. B., Scholten, J. D., Stolowich, N. J., Hogg, J. L., Scott, A. I. and Wong, C.-H. (1988) Modification of protease to esterase for peptide synthesis: methyl chymotrypsin. *J. Am. Chem. Soc.* **110**, 3709-3710
- 43 Zhong, Z., Bibbs, J. A., Yuan, W. and Wong, C. H. (1991) Active-site-directed modification of subtilisin. *J. Am. Chem. Soc.* **113**, 2259-2263
- 44 Plettner, E., DeSantis, G., Stabile, M. R. and Jones, J. B. (1999) Modulation of esterase and amidase activity of subtilisin *Bacillus lentus* by chemical modification of cysteine mutants. *J. Am. Chem. Soc.* **121**, 4977-4981
- 45 Lloyd, A. C., Davis, B. G. and Jones, J. B. (2000) Site-selective glycosylation of subtilisin *Bacillus lentus* causes dramatic increases in esterase activity. *Bioorg. & Medicinal Chemistry* **8**, 1537-1544
- 46 Gronlund, J. L., Yu, M. K., Concepcion, A. M., He, S., Venkataramani, R., Marmorstein, R. and McCafferty, D. G. (2002) in *Peptides: The Wave of the Future* (Lebl, M. and Houghten, R. A., eds.), pp. 541-542, Kluwer Academic Publishers, Norwell

FIGURE CAPTIONS

Fig. 1. Small molecule substrates of FB188 HDAH. (A) Boc-L-Lys(ϵ -acetyl)-MCA, (B) Boc-L-Lys(ϵ -propionyl)-MCA, (C) 4-methylcoumarin-7-acetate, (D) 4-methylcoumarin-7-propionate, (E) 4-nitrophenyl-acetate.

Fig. 2. Bar diagram of amidohydrolase and esterase activities of different HDACs. Fluorogenic amide Boc-L-Lys(ϵ -acetyl)-MCA (black bars), and esters 4-methylcoumarin-7-acetate (gray bars) and 4-methylcoumarin-7-propionate (white bars) were used as substrates, respectively. For (recombinant) human HDAC1, 3 and 8 substrate conversions in pmol/min were compared using the same enzyme and substrate concentrations but different substrates. Relative substrate conversions are depicted by arbitrarily setting the conversion of the HDAC substrate Boc-L-Lys(ϵ -acetyl)-MCA to 1 for each enzyme.

Fig. 3. Structure of the active site of FB188 HDAH and proposed catalytic mechanism. (A) Crystallographic structure of inhibitor SAHA bound to the active site. (B) Schematic representation of interactions between SAHA and active site residues of FB188 HDAH. (C-E) Proposed mechanism for the deacetylation of amides ($X = \text{NH}$) and esters ($X = \text{O}$). HDAH residues are labelled.

Tab. 1. Kinetic parameter for chromogenic and fluorogenic substrates

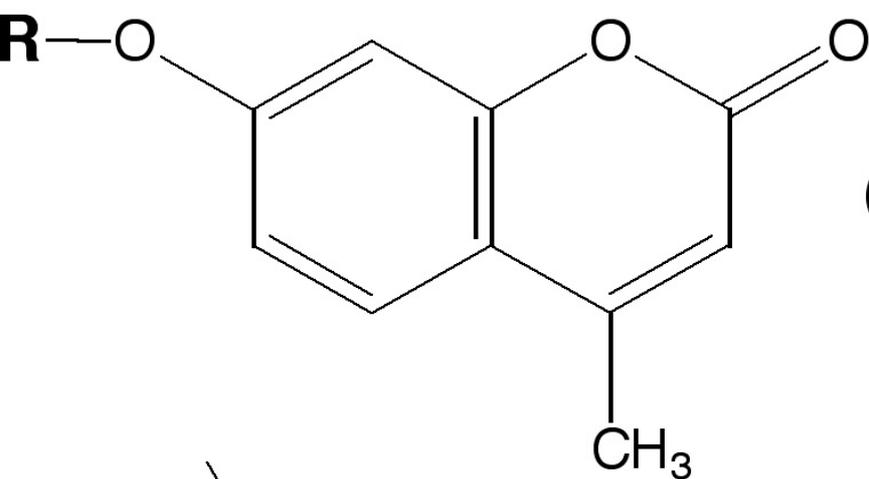
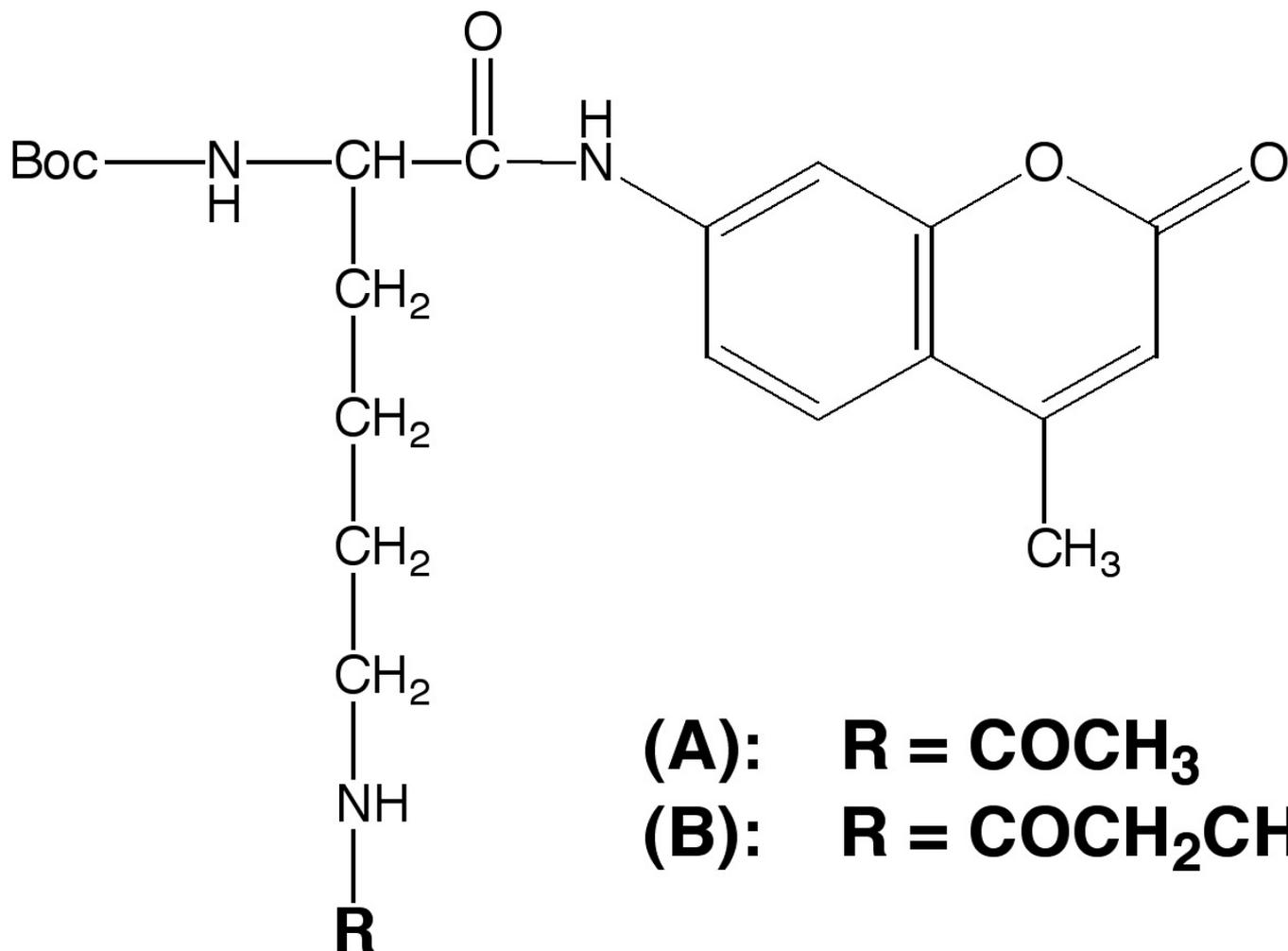
Substrate	HDAH		HDAH Y312F		P. fluorescens lipase		Porcine liver esterase		
	K_M [μM]	V_{\max} [pmol sec^{-1} mg^{-1}]	K_M [μM]	V_{\max} [pmol sec^{-1} mg^{-1}]	K_M [μM]	V_{\max} [$\text{pmol s}^{-1} \text{mg}^{-1}$]	K_M [μM]	V_{\max} [pmol sec^{-1} mg^{-1}]	
A*	Boc-L-Lys(ϵ -acetyl)-MCA	14.4 \pm 3.0	136 \pm 22	NA	NA	NA	NA	NA	NA
B	Boc-L-Lys(ϵ -propionyl)-MCA	NA	NA	nd	nd	nd	nd	nd	nd
C	4-methyl-coumarin-7-acetate	0.90 \pm 0.13	14100 \pm 2100	1.68 \pm 0.32	513000 \pm 97700	1.66 \pm 0.30	183 \pm 41	2.84 \pm 0.84	2150 \pm 630
D	4-methyl-coumarin-7-propionate	31.2 \pm 3.7	2220 \pm 260	0.22 \pm 0.02	52400 \pm 4700	2.54 \pm 0.23	313 \pm 28	nd	nd
E	4-nitrophenyl-acetate	11.8 \pm 1.8	33000 \pm 4900	nd	nd	47.0 \pm 0.8	4850 \pm 1220	nd	nd

* named like in Figure 1; P. fluorescens, Pseudomonas fluorescens; NA, no activity (background); nd, not determined

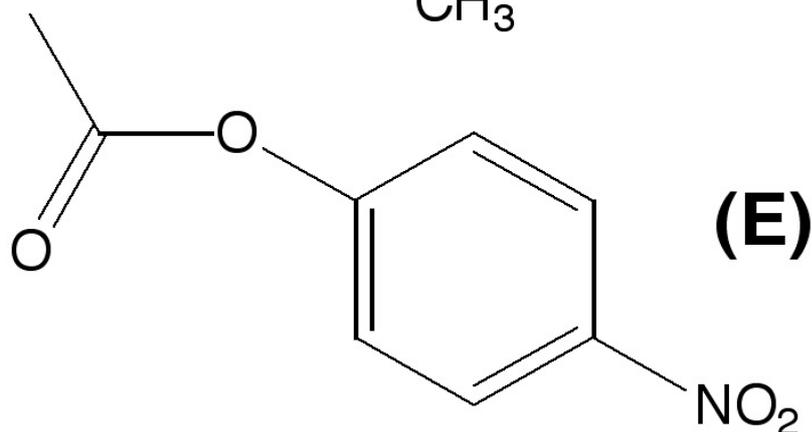
Tab. 2. IC₅₀ values of HDAC inhibitors monitored by effects on amidohydrolase and esterase activity

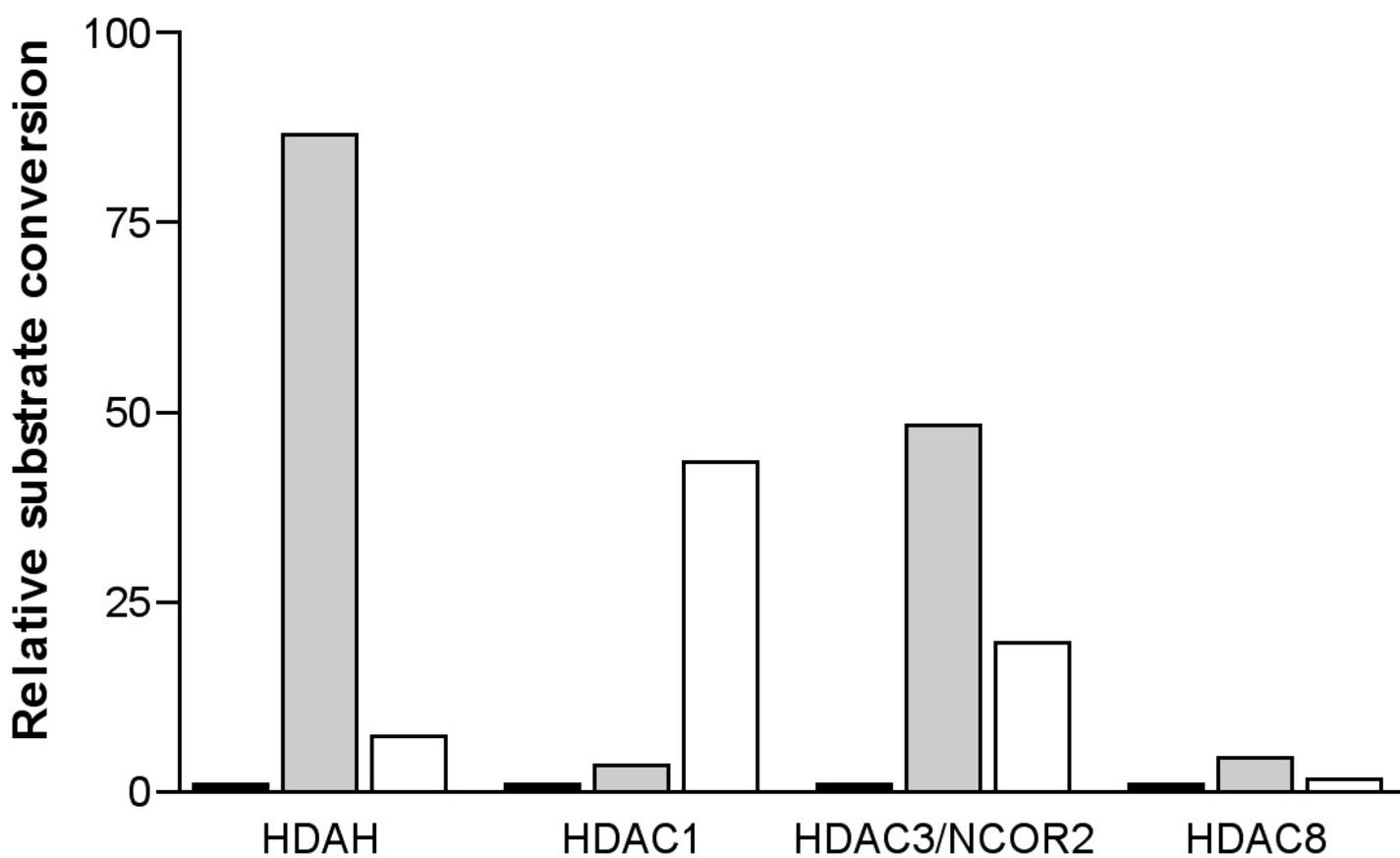
Inhibitor	IC₅₀ [μM]			
	<u>HDAC assay</u>*	<u>Fluorogenic esterase assay</u>[#]		
	HDAH	HDAH	Pseudomonas fluorescens lipase	Porcine liver esterase
SAHA	0.95	0.43	3.82	NA
CypX	0.29	0.59	–	NA

* [24] ; [#] based on 4-methylcoumarin-7-acetate as substrate; NA, no activity (background)

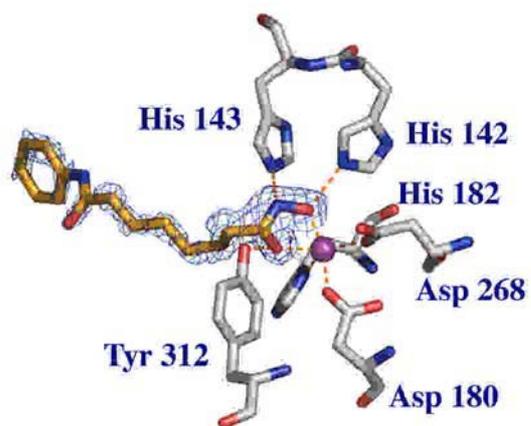


(C): R = COCH₃
(D): R = COCH₂CH₃

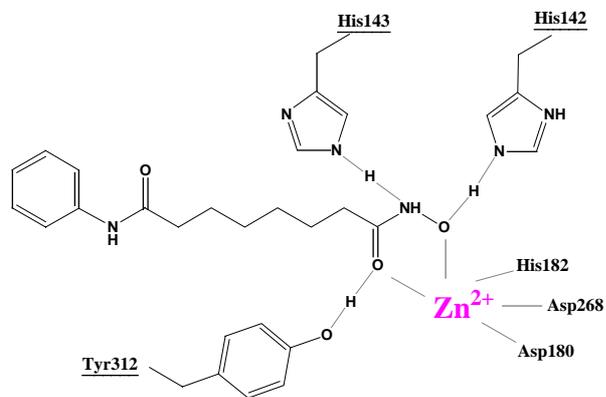




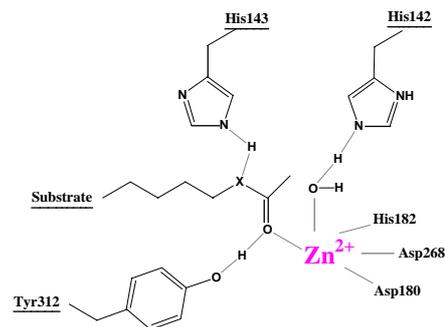
A



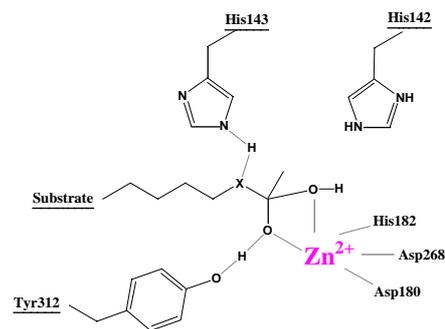
B



C



D



E

