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A Fluorogenic Histone Deacetylase Assay Well Suited for High-Throughput Activity Screening

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Summary

Histone deacetylases (HDACs) are important enzymes for the transcriptional regulation of gene expression in eukaryotic cells. Recent findings suggest that HDACs could be key targets for chemotherapeutic intervention in malignant diseases. A convenient and sensitive fluorogenic assay for HDAC activity would therefore expedite studies of HDAC in transcriptional regulation and in vitro screening for drug discovery. In this study, novel fluorogenic substrates of HDACs were synthesized with an ε-acetylated lysyl moiety and an adjacent MCA moiety at the C terminus of the peptide chain. Upon deacetylation of the acetylated lysyl moiety, molecules became substrates for trypsin, which released highly fluorescent AMC molecules in a subsequent step of the assay. The fluorescence increased in direct proportion to the amount of deacetylated substrate molecules, i.e., HDAC activity. The nonisotopic, homogeneous assay is well suited for high-throughput HDAC inhibitor screening.

Introduction

The reversible acetylation of histones is crucial for the transcriptional regulation of gene expression in eukaryotic cells. Discovered nearly 40 years ago [1], it has only recently become clear that acetylation of the ε amino group of specific lysine residues within the positively charged N-terminal tail of core histones H2A, H2B, H3, and H4 results in localized chromatin relaxation and a change in both histone-DNA and histone-nonhistone protein interaction. In general, acetylation activity is correlated with transcriptional activation, whereas deacetylation activity is accompanied by transcriptional repression [2–4]. Two classes of enzymes, histone acetyltransferases (HAT) and histone deacetylases (HDAC), have been shown to maintain the delicate dynamic equilibrium in the acetylation level of nucleosomal histones and may very well be active in the regulation of other cellular processes as well [5].

A growing body of experimental evidence indicates that the activity of HAT, and in particular, HDAC, affects cell cycle arrest, terminal differentiation of different cell types, and the pathogenesis of malignant disease [6]. The histone acetyltransferase BRCA2, for example, was shown to be a tumor suppressor gene whose mutation leads to a high risk of breast and ovarian cancer [7]. The role of HDAC was particularly highlighted by the recent finding that mutant retinoid receptors recruit HDAC in acute promyelocytic leukemia [8–10]. Interestingly, HDAC inhibitors such as trichostatin A (TSA) and trapoxin (TPX) were shown to induce cell differentiation, cell cycle arrest, and reversal of transformed cell morphology [11–13]. Not surprisingly, therefore, a number of HDAC inhibitors show a potency as promising antitumor agents [14–19]. The recent discovery of antimalarial effects of certain HDAC inhibitors [20] further supports the idea that HDACs could be key targets for chemotherapeutic intervention in a variety of human diseases.

The discovery of novel HDAC inhibitors as new drugs for transcription therapy and cancer chemoprevention, however, is currently obstructed by the lack of suitable assay systems. By far, the most widely distributed assay of HDAC activity depends on the incubation of the enzyme with acetate-radiolabeled histones [21] or peptide substrates [22–27] followed by extraction with organic solvents such as ethyl acetate and then the quantification of the released radiolabeled acetic acid by liquid scintillation count. ^{3}H-histones are obtained by a laborious procedure that relies on the sacrifice of animals. The degree of acetylation of prelabeled core histones changes within different preparations and it is therefore difficult to standardize the substrate properties. In constrast, labeled oligopeptides are synthesized by solid-phase technology, and postlabeling HPLC purification is required. Although the classical radioactive assay has been successfully used to measure HDAC activities from various sources, the need to separate product from substrate limits assay throughput. In addition, the use of scintillation cocktails makes these assays costly in terms of time, labor, and radioactive waste. Hence, assays of this type are not readily amenable to automation and high-throughput screening.

The scintillation proximity assay [28] in part solved the problem in that it does not require separation of product and substrate nor does it use scintillation cocktails. However, since radioactivity is still involved, problems with exposure of laboratory personnel, monitoring, and decontamination of screening equipment as well as disposal of radioactive waste remained unsolved.

The first principally nonisotopic methods for the determination of HDAC activity relied on immunoblotting of hyperacetylated histones [29]. However, this approach has the drawback that rather than measuring enzyme activity in the presence or absence of inhibitor, these immunoblotting procedures more resembled functional tools that are not suited for assay throughput. The first true nonisotopic assay for HDAC activity [30] used MAL (N-(4-methyl-7-coumarinyl)-N-α-(tert.-butyloxy-carbonyl)-N-Ω-acetyllysinamide) as a substrate. Unfortunately, formation of the deacetylated product is monitored by HPLC and fluorescence detection after extraction with ethyl acetate. Therefore, this assay is not exceptionally well suited for high-throughput screening. In addition,

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the substrate does not really well resemble acetylated lysine residues in the original context of histones. Surprisingly, however, the artificial substrate had an even lower K_M value (0.86 μM) as compared to the natural one (20 μM) [30, 31]. Recent improvements of the assay include: (1) fluorescence-labeled octapeptide substrates [32] that bear some closer resemblance to the native substrate; and (2) the introduction of an internal standard [33, 34] for the quantification of fluorescence substrate by HPLC. Although the assay was applied successfully on a number of occasions, including the study of time- and site-dependent deacetylation [33], it still remains not readily amenable to automation and high-throughput screening due to the necessary separation steps.

In principle, an assay could also be designed that quantifies acetate enzymatically released from substrates using an Acetic Acid Standard Test (No. 148261, Roche Diagnostics, Mannheim, Germany). Briefly, acetate is converted in the presence of acetyl-CoA synthetase with ATP and CoA to acetyl-CoA. The latter reacts with oxaloacetate to form citrate in the presence of citrate synthetase. The oxaloacetate required for this reaction is formed from L-malate and NAD in the presence of L-malate dehydrogenase upon reduction of NAD to $NADH^+$. As a signal, the absorbtion of $NADH^+$ is monitored at 340 nm. The test was recently adapted as an assay system for acetate releasing enzymes [35] and has already been applied to the characterization of a putative acetylpolyamin amidohydrolase with similarities to HDACs (C. Hildmann and A.S., unpublished data). A similar assay has been introduced for HATs [36]. However, as yet, no application to HDAC activity measurements has been reported.

The only method, so far, that has indeed been reported as an assay for high-throughput screening of a compound library is a cell-based system using a stably integrated reporter construct [37]. This system, however, does not discriminate between HDAC inhibitors and any other regulators of a tumor-suppressive pathway.

Here, we present the synthesis and the in vitro evaluation of a series of novel HDAC substrates that allow monitoring of HDAC activity via a novel type of sensitive fluorogenic assay. The peptidic substrates contain an ε-acetylated lysine residue followed by a 4-methylcoumarin-7-amide moiety at their carboxy terminus. The assay is a two-step enzymatic reaction (Figure 1). In the first reaction catalyzed by HDACs, acetate is released from ε-acetylated lysine moieties. In the second reaction, the deacetylated peptides are recognized as substrates by trypsin, which only cleaves after deacetylation of, and then after, lysine residues. The assay is intrinsically nonradioactive and highly sensitive and does not demand the consumption of expensive material such as histones. Proof-of-concept experiments with TSA, a well-known inhibitor of HDAC, indicate that the new assay is well suited for high-throughput screening efforts in the process of identifying novel HDAC inhibitors in large collections of candidate molecules.

Results

Substrates were derived from the sequence context around acetylated lysine residues in the N-terminal tails of histone H4. The synthesis of Ac-Arg-Gly-Lys(Ac)-MCA **(8)** was carried out as shown in Figure 2. First, the dipeptide Arg-Gly was synthesized by standard solid-phase chemistry using H-Gly-2-chlorotrityl resin **(1)** and Fmoc-Arg(Pbf)-OH **(2)** preactivated with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and N-methylmorpholine (NMM). The immobilized dipeptide **(3)** was Fmoc-deprotected with piperidine followed by acetylation of the N terminus with acetic anhydride. The product **(4)** was cleaved from the support using TFA and subsequently purified by reversed phase HPLC. The purified product was reacted with H-Lys(Ac)-MCA **(6)**, previously generated by Boc-deprotection of Boc-Lys(Ac)-MCA **(5)**. The final product **(8)** was obtained from **(7)** by arginine deprotection using TFA and subsequent semipreparative LC-MS. Ac-Gly-Gly-Lys(Ac)-MCA, Ac-Leu-Gly-Lys(Ac)-MCA, and Ac-Gly-Ala-Lys(Ac)-MCA were prepared in a similar way. For comparison, versions of all substrates with deacetylated lysine residues were also synthesized using the same procedure, except that instead of **(6)**, H-Lys(Z)-MCA was used, and final deprotection of Z was carried out in HBr/HOAc.

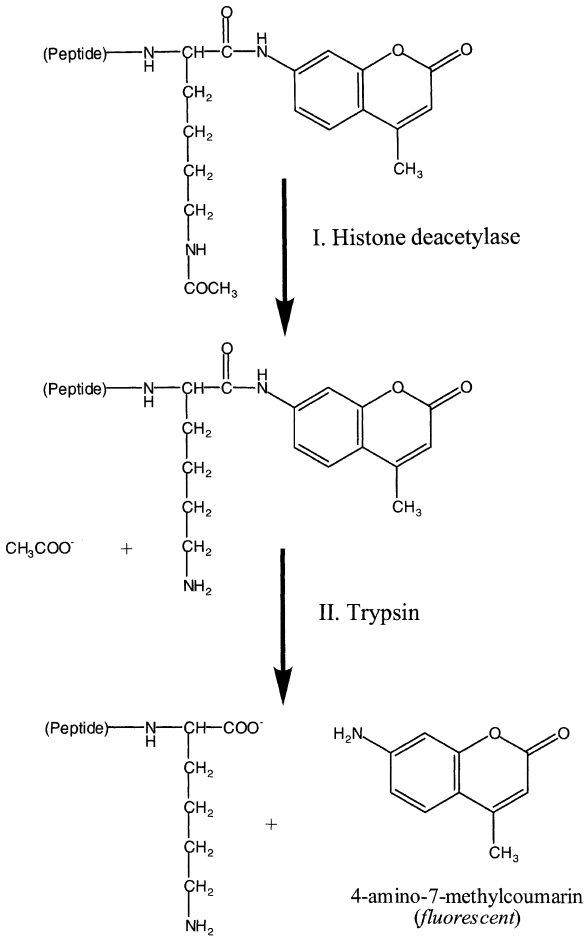

Figure 1. Principle of the Histone Deacetylase Assay

The assay comprises two steps. Step I: deacetylation of ε-acetylated lysyl moieties in peptidic substrates. Step II: cleavage of deacetylated substrates by trypsin and subsequent release of fluorescent 7-amino-4-methylcoumarin. Fluorescence measurement is done at λ_{ex} = 390 nm and λ_{em} = 460 nm.

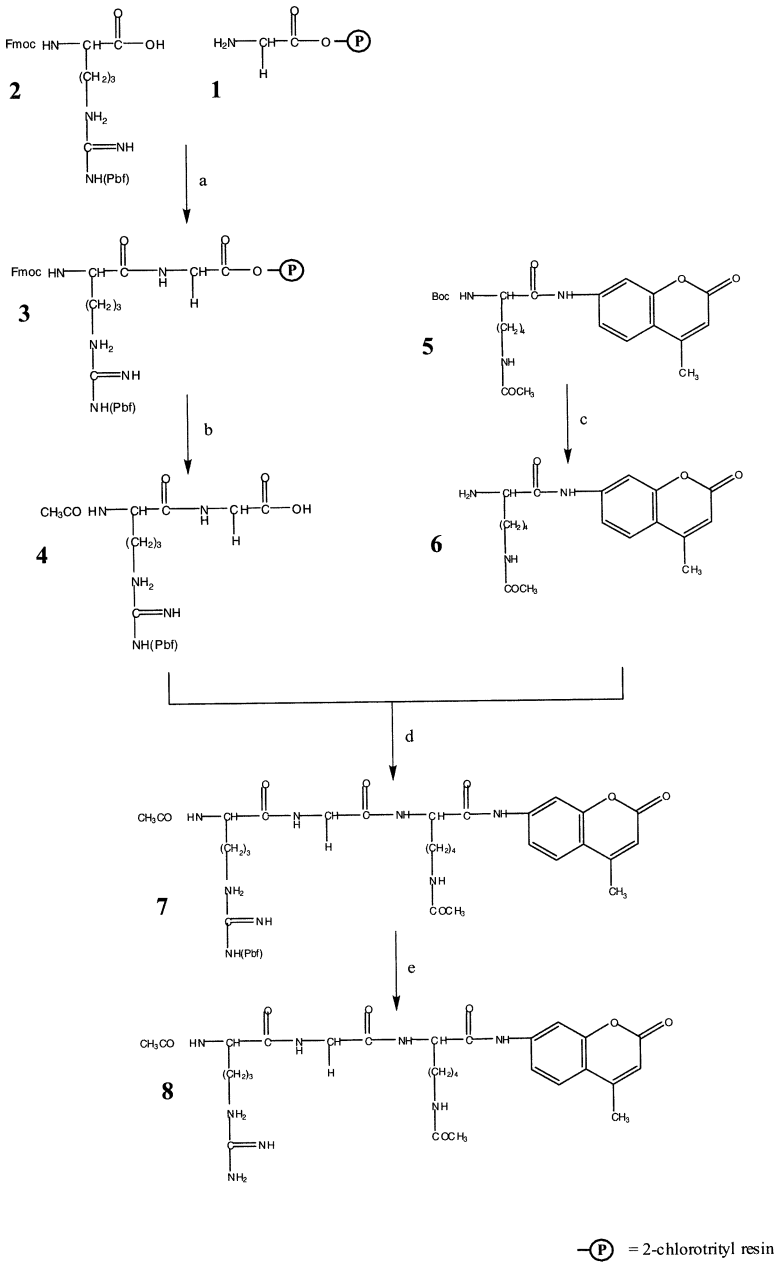

Figure 2. Synthesis of Fluorogenic Substrate Ac-Arg-Gly-Lys(Ac)-MCA

Reaction conditions: (a) Fmoc-Arg(Pbf)-OH preactivated with TBTU, NMM and DMF at RT for 1 hr; (b) piperidine, DMF, acetic anhydride, and DIPEA at RT for 30 min and, subsequently, DMF, DCM, ether, TFA, and DCM at RT for 1 hr; (c) TFA and DCM at RT for 1 hr; (d) DMF, TBU, and NMM at RT for 2 hr; (e) TFA at RT for 2 hr.

The HDAC assay was designed as a two-step enzymatic reaction. In the first step, HDAC releases the acetate moiety from ϵ-acetylated lysine residues of the substrate peptides. Reaction conditions optimal for HDAC activity are used. In the second step, the deacetylated peptides containing now unprotected lysine residues are recognized by trypsin and subsequently cleaved to release 7-amino-4-methylcoumarin (AMC). The fluorescence enhancement approached 30-fold relative to both the concomitant unhydrolyzed substrate and the substrate with an acetylated lysine residue.

First, the trypsin reaction was analyzed in greater detail (Figure 3). Digestion of peptides with ϵ-acetylated lysine residues did not result in any measurable cleavage within 24 hr. In contrast, the nonacetylated forms of substrate peptides were cleaved by trypsin, although with K_M values of ≥594 μM as compared to 7 μM for the known trypsin substrate Tos-Gly-Pro-Arg-MCA. Among the deacetylated versions of the substrates, Boc-Lys-MCA was digested the least efficiently and only at very high trypsin concentrations (≥0.5 μg/ml). At this concentration, all other substrates were quantitatively digested within less than 1 min. At lower trypsin concentrations (0.05 μg/ml), only Ac-Leu-Gly-Lys-MCA achieved a digestion to completion within 12 min. In the following and throughout this study, we used a final trypsin concentration of 5 mg/ml and an incubation time of 20 min to ensure the tryptic digest of even the least suitable substrate (i.e., Boc-Lys-MCA) going to completion. In the standard HDAC assay, TSA (final concentration of 1 μM) was added together with trypsin to completely prevent HDAC action within the period of tryptic digest.

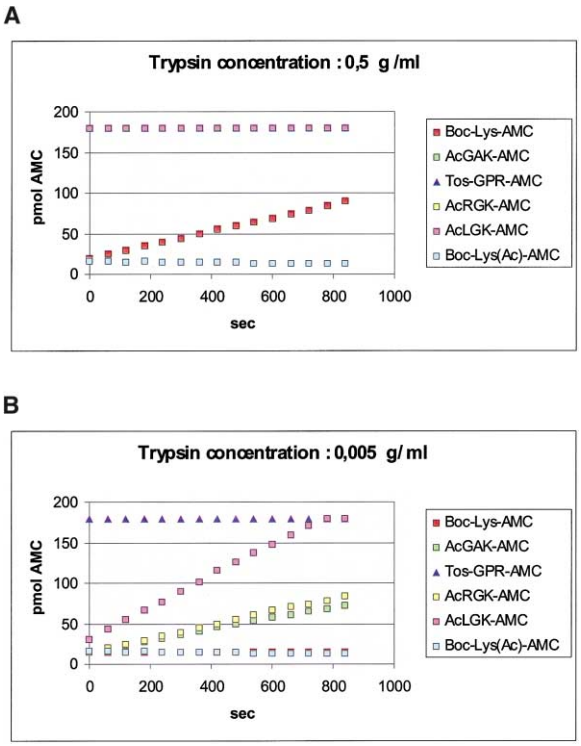

Figure 3. Time-Dependent Cleavage of Various Substrates by Trypsin

As substrates, **(1)** Boc-Lys-MCA, **(2)** Ac-Gly-Ala-Lys-MCA, **(3)** Tos-Gly-Pro-Arg-MCA, **(4)** Ac-Arg-Gly-Lys-MCA, **(5)** Ac-Leu-Gly-Lys(Ac)-MCA, and **(6)** Boc-Lys(Ac)-MCA were used. Trypsin cleavage was carried out at two different trypsin concentrations; (A) 0.5 μg/ml and (B) 0.005 μg/ml. Cleavage was monitored by measuring the fluorescence of 7-amino-4-methylcoumarin released during the reaction. Note that substrates AcLGK-AMC, AcGAK-AMC, AcRGK-AMC, and Tos-GPR-AMC show exactly the same cleavage characteristics at a trypsin concentration of 0.5 μg/ml (A).

In general, 100 μl of HDAC reaction mixture (containing the substrate) was combined with 100 μl of trypsin/TSA solution. Experiments with low volume cavity 1536-well microplates proved that reliable signals were also obtained with 5 μl HDAC reaction mixture and 5 μl trypsin/TSA solution (data not shown).

Next, the substrates with ε-acetylated lysine residues were used to monitor the enzymatic activity of rat liver HDAC over time (Figure 4). In each case, aliquots of the HDAC reaction were taken at different time points, and the deacetylation reaction was stopped by the addition of trypsin/TSA solution. After a 20 min incubation, the AMC release was monitored by measuring the fluorescence at λ_{ex} = 390 nm and λ_{em} = 460 nm using a BMG Polarstar microplate reader (gain, 73). All substrates were deacetylated similarly, as indicated by comparable kinetics of AMC release. In addition, preliminary data on Boc-Lys(Ac)MCA indicate that at least this substance is also a suitable substrate to a recently discovered bacterial enzyme with similarities to acetylpolyamine amidohydrolases (C. Hildmann and A.S., unpublished data).

To characterize the substrates in more detail, K_M and V_{max} were determined for rat liver-derived HDAC and for

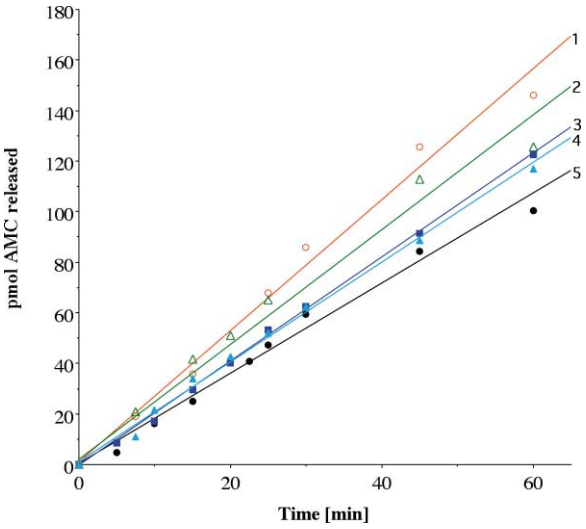

Figure 4. Time-Dependent Activity of Rat Liver HDAC

As substrates, **(1)** Ac-Gly-Ala-Lys(Ac)-MCA, **(2)** Ac-Leu-Gly-Lys(Ac)-MCA, **(3)** Boc-Lys(Ac)-MCA, **(4)** Ac-Gly-Gly-Lys(Ac)-MCA and **(5)** Ac-Arg-Gly-Lys(Ac)-MCA were used.

recombinant human HDAC 8 (Table 1). A number of deacetylation reactions were carried out at substrate concentrations between 0.1 and 500 μM. Endpoint AMC fluorescence was monitored for deacetylation times between 5 and 60 min. The initial velocity was calculated using the first 10 min of the deacetylation reactions. A Hanes plot was prepared from the kinetic data to calculate K_M and V_{max} values (Table 1). With rat liver HDAC, Boc-Lys(Ac)-MCA revealed a K_M of 3.5 μM, whereas all tripeptide substrates yielded K_M values between 26 and 43 μM, which more strongly resembles the K_M value for histones (20 μM) [30], the natural substrate. With recombinant human HDAC 8 and Ac-Arg-Gly-Lys(Ac)-MCA or Boc-Lys(Ac)-MCA as substrates, K_M values of ≥500 μM were obtained. This finding is supported by the fact that a H4-derived peptide is also not a good substrate for the enzyme [38]. Interestingly, HDAC 8 contains a stretch of mainly acidic amino acids which is not present in HDAC 1–3 and which may be responsible for a more demanding substrate specificity.

To test the feasibility of the assay for the identification of HDAC inhibitors, the assay was carried out in the presence of TSA, a known potent inhibitor of HDACs. Inhibitor concentrations between 10^{-4} nM and 10^{4} nM

Table 1. K_M and V_{max} Values for Selected HDAC Substrates

	Rat Liver Histone Deacetylase*	
Peptide Sequence	K_M [μM]	V_{max} [pmol s^{-1} mg^{-1}]
Boc-Lys(Ac)MCA	3.7 ± 1.7	4.41 ± 0.10
Ac-Arg-Gly-Lys(Ac)MCA	27.5 ± 4.9	3.61 ± 0.29
Ac-Gly-Gly-Lys(Ac)MCA	32.6 ± 3.2	4.27 ± 0.26
Ac-Leu-Gly-Lys(Ac)MCA	44.2 ± 1.0	5.26 ± 0.28
Ac-Gly-Ala-Lys(Ac)MCA	35.2 ± 3.4	5.59 ± 0.74

*Rat liver HDAC (Calbiochem, Bad Soden, Germany), 89 U/ml, 4.7 mg/ml.

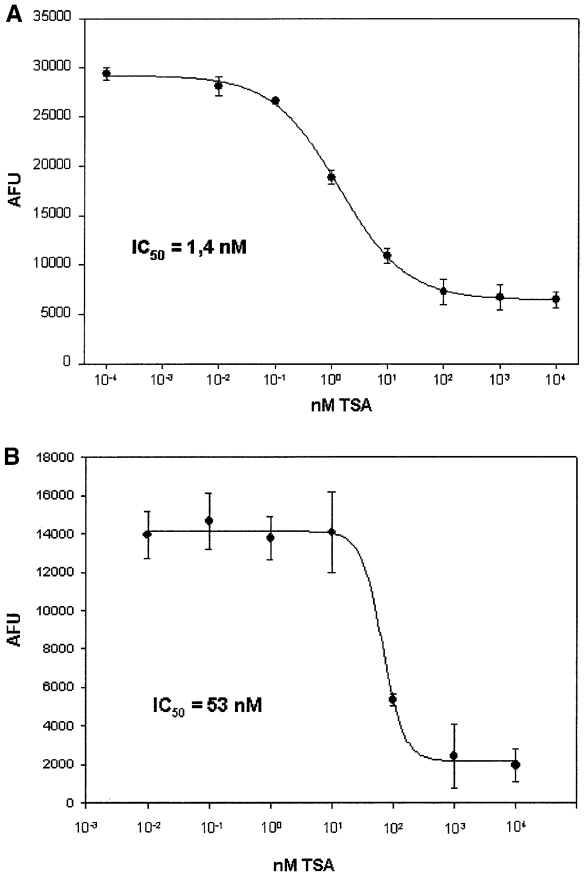

Figure 5. IC_{50} Measurements for Trichostatin A Using Rat Liver HDAC (A) and Recombinant Human HDAC 8 (B)

TSA were used. All assays were performed with Ac-Arg-Gly-Lys(Ac)-MCA as a substrate at a final concentration of 250 μM. The semilogarithmic plots (Figure 5) analyzed with the help of the Graph Pad Prism software afforded an IC_{50} value of 1.4 nM for rat liver HDAC, which is in the range of the IC_{50} value of 2.6 nM reported recently for the same enzyme and Boc-Lys(Ac)-MCA as the substrate [33]. For recombinant human HDAC 8, the analysis of the semilogarithmic plot yielded an IC_{50} value of 53 nM. The only IC_{50} value reported so far for this enzyme is 3 nM, as measured for the deacetylation of radioactively-labeled histones [39]. The differences may be caused by the usage of different substrates and assay types.

Discussion

Fluorescence-based biological assays have been widely used to monitor reactions [40], in particular, in the context of high-throughput screening for inhibitors of pharmaceutically interesting target enzymes. Suitable assay formats, however, have not yet been developed for all important targets. For HDACs, a particularly promising class of new tumor targets, the most frequently used assay type is still isotopic and nonhomogeneous in nature [21]. Only recently, the first type of fluorescence-based assay for HDACs was developed [30]. This assay, however, only solved the problem halfway. Since substrate and product differ chemically in one acetyl moiety, but not significantly in their fluorescence properties, this assay type absolutely requires separation steps prior to fluorescence detection and is therefore intrinsically nonhomogeneous.

Homogeneous fluorogenic assays are, however, highly desirable, e.g., assays in which the quenched (nonfluorescent) fluorophore is enzymatically released as a then strongly fluorescent moiety in the scope of the enzymatic reaction. A recent example of this type of fluorogenic assay is the liberation of AMC by penicillin G acylase [41].

The novel HDAC assay presented herein combines the specificity of the deacetylation reaction with the advantages of a homogeneous fluorogenic assay in a two-step process. The HDAC substrates exhibit both an ε-acetylated lysine residue within a histone-like sequence context and an adjacent essentially nonfluorescent 4-methyl-coumarine-7-amide residue. Following deacetylation of the lysine residue, the product is recognized by trypsin, which releases 7-amino-4-methylcoumarin, a well-studied fluorophore detectable by standard fluorescence measurements. Since acetylated HDAC substrates are not substrates for trypsin, AMC fluorescence is correlated to deactetylase activity.

In our studies, four synthetic peptides, as well as the commercially available Boc-Lys(Ac)-MCA, proved to be well suited as substrates for the two-step assay. All substrates presented here were recognized with significant signal-to-noise ratio by rat liver HDAC. In addition, Ac-Arg-Gly-Lys(Ac)-MCA was also shown to be a suitable substrate for recombinant human HDAC 8. Surprisingly, all substrates, including Boc-Lys(Ac)-MCA, which lacks extensive mimicry of the natural histone substrates, show similar properties as far as K_M and V_{max} values are concerned. Thus, it seems that the recognition of ε-acetylated lysine residues by HDACs is rather insensitive to changes in sequence context. As a result, it should be possible to generate peptidic HDAC substrates of the type described here that are optimized with respect to the second assay reaction, i.e., trypsin cleavage. This could be desirable since current substrates are still not the most suitable substrates for trypsin and thus require not only rather large amounts of trypsin, but also an inhibitory amount of trichostatin A to be added at the same time. This is particularly true for the commercially available Boc-Lys(Ac)-MCA, which requires by far the highest trypsin concentrations as well as long incubation times. Preliminary experiments with Tos-Gly-Pro-Lys(Ac)-MCA, a derivative of a known good substrate of trypsin [42], indicate that it also works as a good substrate for rat liver HDAC, and in addition, only requires trypsin concentrations 100-fold lower as compared to the standard conditions described herein.

Concerning the substrate specificity in particular of HDACs, relatively little is known and hence predictions about the general usability of the new assay type are more speculative. Some studies suggest that only Sir2 proteins, in contrast to class I and class II HDACs, have a specific globular domain that possibly is responsible for the recognition of the acetyl-lysine-containing substrate. The absence of a corresponding domain in class I and class II enzymes suggests that other proteins may

functionally substitute for this domain in vivo. Thus, class I and class II HDACs may be relatively unspecific in the absence of such cofactors [43]. We would therefore conclude that our assay may very well be suited not only for class I enzymes, as demonstrated for rat liver HDAC and human HDAC 8, but also for class II enzymes and even other classes of HDAC-like enzymes. This view is supported by the fact that the activity of a recently discovered enzyme which belongs to the group of bacterial acetylpolyamine amidohydrolases can also be monitored using the new assay.

The main limitation of our assay is its inability to permit continuous monitoring of enzyme activity, as is always the case with endpoint assays. However, in the case of the substrates reported here, continuous monitoring of HDAC activity could, in principle, be achieved by replacing trypsin cleavage and subsequent standard fluorescence analysis by a measurement mode that images sensitively the chemical environment of the fluorophor. It remains to be shown whether techniques such as fluorescence intensity distribution analysis (FIDA) [44] or burst-integrated fluorescence lifetime measurements (BIFL) [45] could be suitable tools in this context.

The assay described here is well suited in the context of high-throughput screening for HDAC inhibitors, even in small reaction volumes. Potent inhibitors of trypsin, however, could impair assay results, e.g., in blocking the AMC release from deacetylated substrates. There are at least two possible precautions that have to be taken. First, very high trypsin concentrations could be used to overcompensate inhibitor concentrations in order to leave sufficient active trypsin for cleavage of deacetylated substrate molecules. Second, a parallel test of trypsin inhibition should be mandatory in order to exclude inhibition of the discriminator enzyme trypsin; this is not necessarily a disadvantage since identified deacetylase inhibitors are also optimized in specificity as a result of such a procedure.

Significance

Fluorogenic substrates of HDAC were prepared by chemical synthesis. These basically nonfluorescent substrates featured: (1) a short peptide sequence derived from histone H4; (2) an acetylated lysyl moiety; and (3) a subsequently following MCA moiety. Deacetylation of acetylated lysyl moieties by HDAC yielded molecules that only then were efficient substrates for trypsin. Cleavage by trypsin resulted in the release of AMC and, thereby, a fluorescence increase as a function of deacetylated molecules availability, i.e., the extent of HDAC activity. The assay demonstrated an ~30-fold enhancement of fluorescence monitoring reactions with rat liver HDAC and recombinant human HDAC 8. The substrate specificity of rat liver HDAC was examined in greater detail. In regard to the deacetylation reaction, Boc-Lys(Ac)-MCA is a slightly more favorable substrate as compared to the tripeptidic substrates Ac-Arg-Gly-Lys(Ac)-MCA, Ac-Gly-Gly-Lys(Ac)-MCA, Ac-Leu-Gly-Lys(Ac)-MCA, and Ac-Gly-Ala-Lys(Ac)-MCA. The latter, however, have the advantage of being digested by trypsin significantly faster as compared to Boc-Lys(Ac)-MCA. As a consequence, the consumption of trypsin is much lower when using tripeptidic substrates. The feasibility of using the aforementioned probes for in vitro inhibition assays in microplates was demonstrated by measurement of IC_{50} values for TSA, a known HDAC inhibitor. IC_{50} values of 1.4 nM determined with the rat liver enzyme and 53 nM measured for recombinant human HDAC 8 were in good agreement with previously published data. The described method is thus a simple and sensitive homogeneous assay to characterize modulators of HDAC activity and is applicable to in vitro high-throughput screenings for HDAC inhibitors.

Experimental Procedures

Synthesis of Fluorogenic Substrates

For **Ac-Arg-Gly-Lys(Ac)-MCA (8)**, H-Gly-2-chlorotrityl resin **(1)** (Novabiochem; 269 mg; 167 μmol of reactive groups) was reacted for 1 hr at room temperature (RT) with 0.5 mmol of Fmoc-Arg(Pbf)-OH **(2)** in N,N-dimethylformamide (DMF) (0.6 M) preactivated with 0.5 mmol TBTU (0.5 M in DMF) and 1.5 mmol NMM. The reaction mixture was filtered, and the coupling step was repeated once. After filtration, the resin-bound dipeptide **(3)** was Fmoc deprotected using 2 × 2 ml 20% piperidine in DMF. Acetylation of the N terminus was carried out by incubation with 2 ml 2.5% (v/v) acetic anhydride and 2.5% (v/v) diisopropylethylamine (DIPEA) in DMF for 30 min at RT. The reaction mixture was filtered, and the acetylation step was repeated once. The resin was subsequently washed with 20 ml each of DMF, dichloromethane (DCM), and ether and finally dried in vacuo. Cleavage of **(1)** was carried out in two subsequent reactions using 4 ml 3% trifluoroacetic acid (TFA) in DCM for 1 hr at RT. Evaporation using an excess of hexane led to Ac-Arg(Pbf)-Gly-OH **(4)**, having a yield of 80% with respect to the H-Gly-2-chlorotrityl resin. The crude product was purified by reversed phase HPLC using a 250 × 10 mm C18 column (Jupiter, Phenomenex, Aschaffenburg, Germany), eluted by methanol and dried in vacuo.

Boc-Lys(Ac)-MCA **(5)** (111.4 mg; Bachem, Switzerland) was treated with 1 ml of 50% TFA in DCM for 1 hr at RT and dried by evaporation using an excess of hexane to form an azeotrope. The product again was purified by reversed phase HPLC using a 250 × 10 mm C18 column (Jupiter, Phenomenex), eluted by methanol, and dried in vacuo to yield pure H-Lys(Ac)-MCA **(6)**. From both purified products **(4)** and **(6)**, 0.1 mmol was separately dissolved in 0.2 ml DMF. Ac-Arg(Pbf)-Gly-OH **(4)** was preactivated with 0.1 mmol TBTU and 0.3 mmol of NMM and reacted with H-Lys(Ac)-MCA **(6)**. After 2 hr at RT, the reaction was complete, as confirmed by LC-MS, and the solvent was evaporated to give Ac-Arg(Pbf)-Gly-Lys(Ac)-MCA **(7)**. The arginine protection group was cleaved within 2 hr in 90% TFA. The solvent was evaporated, and the crude product was purified using semipreparative LC-MS (Waters ZQ) with mass detection as the trigger for fraction collection (Waters/MassLynx). The product Ac-Arg-Gly-Lys(Ac)-MCA **(8)** was dried and produced a white powder in 60% yield. LC-MS (ESI): calculated for $C_{28}H_{40}N_8O_7$ (M+H): 601.31; found: 601.4.

Ac-Arg-Gly-Lys-MCA (9) was prepared in a similar way to **(8)**. Instead of Boc-Lys(Ac)-MCA **(5)**, H-Lys(Z)-MCA **(10)** (118 mg; Bachem, Switzerland) was used to give a final yield of 30%. LC-MS (ESI): calculated for $C_{26}H_{38}N_8O_6$ (M+H): 559.30; found: 559.4.

Ac-Gly-Gly-Lys(Ac)-MCA (11), **Ac-Leu-Gly-Lys(Ac)-MCA (12)**, **Ac-Gly-Ala-Lys(Ac)-MCA (13)**, and their deacetylated counterparts **Ac-Gly-Gly-Lys-MCA (14)**, **Ac-Leu-Gly-Lys-MCA (15)**, and **Ac-Gly-Ala-Lys-MCA (16)** were prepared as described for **(8)** and **(9)** using the appropriate building blocks. In each case, the overall yield was between 30 and 60%. LC-MS (ESI) for **(11)**: calculated for $C_{24}H_{31}N_5O_7$ (M+H): 502.23; found: 502.2. LC-MS (ESI) for **(12)**: calculated for $C_{28}H_{39}N_5O_7$ (M+H): 558.29; found: 558.2. LC-MS (ESI) for **(13)**: calculated for $C_{25}H_{33}N_5O_7$ (M+H): 516.26; found: 516.2. LC-MS (ESI) for **(14)**: calculated for $C_{22}H_{29}N_5O_6$ (M+H): 460.22; found: 460.3. LC-MS (ESI) for **(15)**: calculated for $C_{26}H_{37}N_5O_6$ (M+H): 516.28; found: 516.3. LC-MS (ESI) for **(16)**: calculated for $C_{23}H_{31}N_5O_6$ (M+H): 474.24; found: 474.2.

All substrates were dissolved in DMSO and diluted with HDAC buffer (15 mM Tris-HCl [pH 8.1], 250 μM EDTA, 250 mM NaCl, 10% glycerol) to give 1 mM solutions containing 1.7% DMSO.

Trypsin Cleavage Experiments

Diluted substrate solutions were prepared by diluting 180 pmol of substrate in 100 μl HDAC buffer at 30°C. 100 μl of trypsin solution (10 mg/ml trypsin in 50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 μM TSA) were added, and the mixture was incubated for 14 min at 30°C. The release of AMC was monitored each minute by measuring the fluorescence at 460 nm (λ_{ex} = 390 nm) with the help of a robotic workstation (CyBi-Screen-Machine, CyBio AG, Germany) including a Polarstar fluorescence reader (BMG, Germany). The transfer of microplates from the pipetting station to the fluorescence reader took 20 s. Fluorescence intensity was calibrated using free AMC. The AMC signals were recorded against a blank with buffer and substrate but without the enzyme. All experiments were carried out at least in triplicate.

Standard HDAC Assays

All pipetting and fluorescence detection steps were carried out with the help of a robotic workstation (CyBi-Screen-Machine, CyBio AG) including a Polarstar fluorescence reader (BMG). Rat liver enzyme (Calbiochem, Bad Soden, Germany; 89 U/ml, 4.7 mg/ml) was diluted 1:6 with HDAC buffer. Recombinant human HDAC 8 (Biomol, Hamburg, Germany; 0.6 U/μl) was diluted 1:4 in HDAC buffer. For standard HDAC assays, 60 μl of HDAC buffer was mixed with 10 μl of diluted enzyme solution at 30°C. The HDAC reaction was started by adding 30 μl substrate solution in HDAC buffer followed by 30 min of incubation at 30°C. The reaction was stopped by adding 100 μl trypsin solution (10 mg/ml trypsin in 50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 μM TSA). After a 20 min incubation period at 30°C, the release of AMC was monitored by measuring the fluorescence at 460 nm (λ_{ex} = 390 nm). Fluorescence intensity was calibrated using free AMC. For standard time course experiments, 20 pmol of substrate was used in the initial 100 μl HDAC reaction. K_M and V_{max} values were determined by measuring the fluorescence AMC generated by enzymatic cleavage of 2–50 pmol of substrate. The experimental data were analyzed using a Hanes plot. The AMC signals were recorded against a blank with buffer and substrate but without the enzyme. All experiments were carried out at least in triplicate.

Inhibition Experiments with Trichostatin A

For standard HDAC inhibition assays, inhibitor diluted in 50 μl of HDAC buffer was mixed with 10 μl of diluted enzyme, and the solution was preincubated at 30°C for 5 min. The HDAC reaction was started by adding 40 μl substrate solution in HDAC buffer followed by 30 min of incubation at 30°C. The reaction was stopped by adding 100 μl trypsin solution (10 mg/ml trypsin in 50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 μM TSA). After a 20 min incubation period at 30°C, the release of AMC was monitored by measuring the fluorescence at 460 nm (λ_{ex} = 390 nm) with the help of a robotic workstation (CyBi-Screen-Machine, CyBio AG) including a Polarstar fluorescence reader (BMG). Fluorescence intensity was calibrated using AMC. IC_{50} measurements were carried out with 25 pmol of Ac-Arg-Gly-Lys-MCA (**9**) and inhibitor concentrations between 10^{-4} nM and 10^{4} nM TSA in the initial 100 μl HDAC reaction. Again, the AMC signals were recorded against a blank with buffer and substrate but without the enzyme. All experiments were carried out in triplicate.

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