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Improved fluorogenic histone deacetylase assay for high-throughput-screening applications

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Abstract

Histone deacetylases (HDACs) are key targets for chemotherapeutic intervention in malignant diseases. In this paper, a highly sensitive, nonisotopic, homogenous assay for high-throughput screening of HDAC inhibitors is presented. The assay is based on a new fluorogenic peptidic substrate of HDACs comprising an ε-acetylated lysyl moiety and an adjacent 4-methylcoumarin-7-amide moiety at the C terminus of the peptide chain. Upon deacetylation of the acetylated lysyl moiety, molecules are recognized as substrates by trypsin, which releases highly fluorescent 7-amino-4-methylcoumarin molecules in a subsequent step of the assay. The fluorescence increase is directly proportional to the amount of deacetylated substrate molecules, i.e., HDAC activity. Validation of an improved version of the assay revealed (i) a significantly lower enzyme consumption, (ii) an increased screening window coefficient, (iii) a good tolerance toward organic solvents, and (iv) a good suitability for a whole range of different HDAC-like enzymes. The novel assay thus will expedite studies of HDAC-like enzymes and in vitro screening for drug discovery.

Keywords: Fluorogenic substrates; High-throughput screening assay; Histone deacetylase; Acetylpolyamine amidohydrolase

Histone deacetylases (HDACs)[1] are important enzymes for the transcriptional regulation of gene expression in eukaryotic cells. The increasing level of research activity surrounding HDACs witnessed over the past decade has been driven by the abilities of HDAC inhibitors to block angiogenesis and cell cycling and to promote apoptosis and differentiation [1–3]. By targeting these key components of tumor proliferation, HDAC inhibitors have the potential to occupy a major position in the fast-moving cytostatic market [4–9]. The discovery of novel HDAC inhibitors, however, requires assay systems well suited to high-throughput screening (HTS) applications. Unfortunately, most assays available so far are costly in terms of time, labor, and/or radioactive waste (see [10] for review). Currently, there is only one type of nonisotopic, homogenous HDAC assay [10] that has been used in the context of HTS. The assay is based on peptidic substrates that contain an ε-acetylated lysine residue followed by a 4-methylcoumarin-7-amide (MCA) moiety at their carboxytermini. The assay is a two-step enzymatic reaction (Fig. 1). In the first reaction catalyzed by histone deacetylases, acetate is released from ε-acetylated lysine moieties. In the second reaction, the deacetylated peptides are recognized as substrates by trypsin, which cleaves only after deacetylation and then after lysine residues. The assay is highly sensitive and does not demand the consumption of expensive material such as histones. However, since the peptidic substrates used so far are only poor substrates to trypsin, relatively high concentrations of

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[1] *Abbreviations used:* AFU, arbitrary fluorescence units; AMC, 7-amino-4-methylcoumarin; DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane; DMSO, dimethyl sulfoxide; HDAC, histone deacetylase; HTS, high-throughput screening; MCA, 4-methylcoumarin-7-amide; MeCN, acetonitril; NMM, 4-methylmorpholine; NMP, 1-methyl-2-pyrrolidon; RT, room temperature; SAHA, suberoylanilide hydroxamic acid; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TPX, trapoxin; TSA, trichostatin A; TFA, trifluoroacetic acid.

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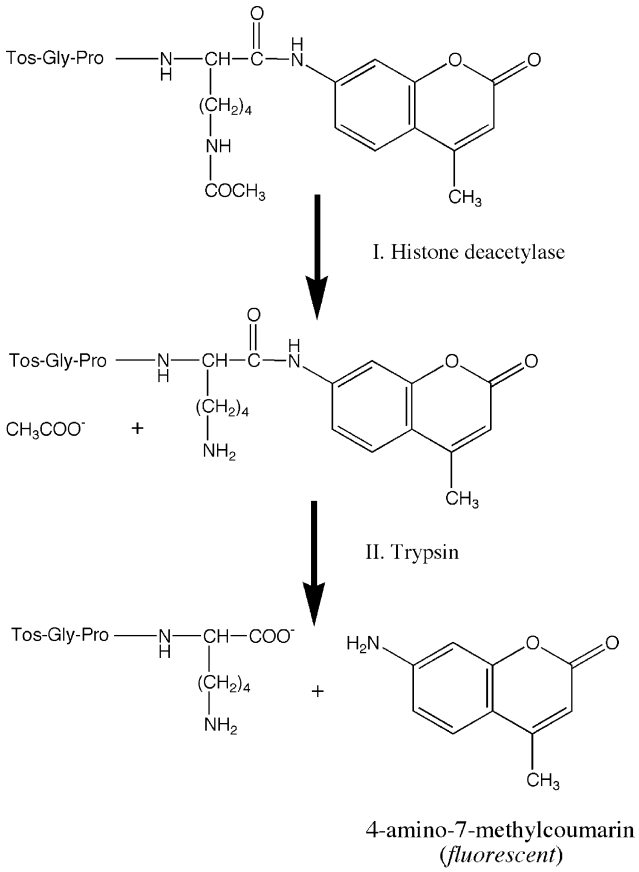

Fig. 1. Principle of the histone deacetylase assay. The assay comprises two steps. In step I the ε-acetylated lysyl moieties of the peptidic substrate is deacetylated. In step II deacetylated substrate molecules are cleaved by trypsin which at the same time releases fluorescent 7-amino-4-methylcoumarin. Fluorescence measurement is done at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 460$ nm.

the latter are required to drive the (second) reaction to completion within a short period of time. Furthermore, mainly due to the high viscosity of the aqueous solutions pipetted in the course of the assay, relatively large pipetting errors can occur, impairing the value of the assay in the context of automation and, in particular, in high-throughput screening. In addition, the tolerance of organic solvents that are usually used to dissolve drug candidates prior to assays has not yet been explored.

Here we present an improved version of the assay comprising (i) a new substrate that is well compatible with trypsin digestion and (ii) a different composition of assay buffers that facilitates accurate pipetting. The new assay is characterized in terms of screening window coefficient [11], tolerance of organic solvents, and suitability for monitoring the activity of a broad range of enzymes including HDACs and related bacterial enzymes. Proof-of-concept experiments with well-known inhibitors of HDAC indicate that the new assay is well suited to high-throughput screening applications to identify novel HDAC inhibitors.

Material and methods

Synthesis of fluorogenic substrate Tos-Gly-Pro-Lys-(Ac)MCA

Boc-Lys(Ac)MCA (0.11 mmol; Bachem, Switzerland) was treated with 1 ml of 50% TFA in DCM for 1 h at RT and dried by evaporation using an excess of hexane to form an azeotrope. The dry product was dissolved in 0.2 ml DMF; 0.2 ml 0.6 M Tos-Gly-Pro-OH (Bachem) in DMF, 0.2 ml 0.5 M TBTU (Bachem), and 36 μl *N*-methylmorpholine (NMM; Bachem) were added and the reaction mixture was incubated for 1 h at RT. 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. The product Tos-Gly-Pro-Lys(Ac)MCA was dried and produced 50 mg of a white powder (68% yield). LC-MS (ESI): calculated for $C_{32}H_{39}N_5O_8S$ (M + H), 654.8; found, 655.5. The substrate was dissolved in DMSO and diluted with HDP buffer (15 mM Tris–HCl, pH 8.1, 250 μM EDTA, 250 mM NaCl, 0.1% PEG8000) to give a 300 μM solution containing 1.0% DMSO.

Standard HDAC 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). If not otherwise stated, reactions were carried out in 96-well microplates. Rat liver enzyme (Calbiochem, Bad Soden, Germany; 89 U/ml, 4.7 mg/ml) was diluted 1:6 with HDP buffer. Recombinant human HDAC 8 (Biomol, Hamburg, Germany; 0.6 U/μl) was diluted 1:4 in HDP buffer. For standard HDAC assays, 60 μl of HDP buffer was mixed with 10 μl of diluted enzyme solution at 30 °C. The HDAC reaction was started by adding 50 μl substrate solution in HDP buffer followed by incubation at 30 °C for the time indicated. The reaction was stopped by adding 80 μl trypsin/TSA stop solution (0.01 mg/ml trypsin in 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 2 μM TSA, 30% (v/v) isopropanol). Trypsin from porcine pancreas (Type IX-S, 13,000–20,000 BAEE units/mg; Sigma, Taufkirchen, Germany) was used. After a 20-min incubation period at 30 °C, the release of AMC was monitored by measuring the fluorescence at 460 nm ($\lambda_{ex} = 390$ nm). Fluorescence intensity was calibrated using free AMC. For standard time course experiments, a substrate concentration of 125 μM was used in the initial 120-μl HDAC reaction. K_m and V_{max} values were determined using different substrate concentrations. 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 HDAC inhibitors or organic solvents

For standard HDAC inhibition assays, inhibitor diluted in 60 µl of HDP buffer was mixed with 10 µl of diluted enzyme and the solution was preincubated at RT for 5 min. Alternatively, organic solvent replacing the inhibitor was used as indicated. The HDAC reaction was started by adding 50 µl (final concentration: 125 µM) substrate solution followed by 60 min of incubation at 30 °C. Hence, the standard 120-µl HDAC reaction contained 0.4% (v/v) DMSO. The reaction was stopped by adding 80 µl trypsin/TSA stop solution. After a 20-min incubation period at 30 °C, the release of AMC was monitored by measuring the fluorescence at 460 nm ($\lambda_{ex} = 390$ nm) with the help of a robotic workstation (CyBi-Screen-Machine, CyBio AG, Germany) including a Polarstar fluorescence reader (BMG, Germany). Fluorescence intensity was calibrated using AMC. IC_{50} measurements were carried out with final inhibitor concentrations between 10^{-4} and 500 nM (TSA, SAHA) or between 10^{-4} and 10^{4} µM (MS-275). Again, the AMC signals were recorded against a blank with buffer and substrate but without the enzyme. All experiments were carried out in triplicate.

Statistical analysis of screening assay quality (Z′-factor)

The screening window coefficient (Z'-factor) was obtained as described [11], analyzing 10 negative control reactions (no inhibitor) and 10 positive control reactions (no HDAC). Briefly, the Z'-factor was calculated from mean values (μ_{c+}, μ_{c-}) and standard deviations (σ_{c+}, σ_{c-}) of positive and negative controls, respectively:

$$Z'_i = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}.$$

Results and discussion

The purpose of this work has been to improve and validate the two-step HDAC assay method [10] for high-throughput screening applications. Briefly, in the first step of the assay, HDAC releases the acetate moiety from ε-acetylated lysine residue of a substrate peptide. In the second step, the deacetylated peptides containing now unprotected lysine residues are recognized by trypsin and are subsequently cleaved to release AMC (Fig. 1). Structure–function analysis of histone deacetylase enzymes had revealed that the recognition of ε-acetylated lysine residues by class I and II HDACs is rather insensitive to changes in sequence context, due to the absence of a specific globular domain which is present only in Sir2 proteins [12]. Indeed, K_m values for tripeptidic substrates of different sequence are of the same order and very much resembled those for histones, the natural substrates [10]. However, deacetylated versions of the substrate peptides used so far were rather poor substrates of trypsin ($K_m \geqslant 594$ µM), demanding high concentrations of the latter within the second step of the assay and thus leading to a high consumption of the enzyme. We thus synthesized Tos-Gly-Pro-Lys-(Ac)MCA as a novel HDAC substrate, since the deacetylated version of this peptide is known to be a good substrate of trypsin [13]. For trypsin characterized under assay conditions we measured a K_m of 7.37 µM and a V_{max} of 14.4 µmol min^{-1} mg^{-1}. Consequently, trypsin concentrations in the second step of the assay could be decreased. Pipetting accuracy, e.g., related to viscosity, has a major influence on the screening window coefficient [11], i.e., the reliable identification of hits in a combinatorial library, in particular, when using robotic workstations such as the CyBi-Screen-Machine (CyBio AG, Germany). We therefore modified the composition and thus the viscosity of two solutions used in the assay: (i) in the original HDAC assay buffer 10% (v/v) glycerol was replaced by 0.1% (w/v) PEG8000 resulting in a modified reaction buffer (HDP buffer: 15 mM Tris–HCl, pH 8.1, 250 µM EDTA, 250 mM NaCl, 0.1% (w/v) PEG8000); (ii) 30% (v/v) isopropanol was added to the trypsin stop solution which now contained a 1000-fold lower trypsin concentration (0.01 mg/ml trypsin in 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 2 µM HDAC-inhibitor TSA, 30% (v/v) isopropanol). In principle, HDAC assays were carried out as described [10]. As with previous substrates the fluorescence enhancement approached 30-fold relative to both, the concomitant unhydrolyzed substrate and the substrate with an acetylated lysine residue (data not shown). As shown in Fig. 2A, the acetylated version Tos-Gly-Pro-Lys-(Ac)MCA of the substrate is not cleaved by trypsin, whereas the deacetylated substrate Tos-Gly-Pro-Lys-MCA is fully cleaved within 1 min under assay conditions, using trypsin concentrations reduced by 1000-fold as compared to those of the previous protocol [10]. Next, time-dependent cleavage experiments with rat liver HDAC, recombinant HDAC 8, and a bacterial HDAC-like amidohydrolase from *Bordetella/Alcaligenes* strain *FB188* were carried out (Fig. 2B). In this case, aliquots of the HDAC reaction were taken at different time points and the deacetylation reaction was stopped by the addition of trypsin/TSA stop solution. After a 20-min incubation, the AMC release was monitored by measuring the fluorescence at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 460$ nm using a BMG Polarstar microplate reader (gain: 73). As a result these experiments revealed that the new substrate is well suited to monitor HDAC enzymatic activity over time. However, HDAC 8 cleaved the substrate less efficiently than rat liver HDAC, which consists primarily of HDAC 1 activity. This finding is supported by the fact that neither Ac-Arg-Gly-Lys(Ac)-

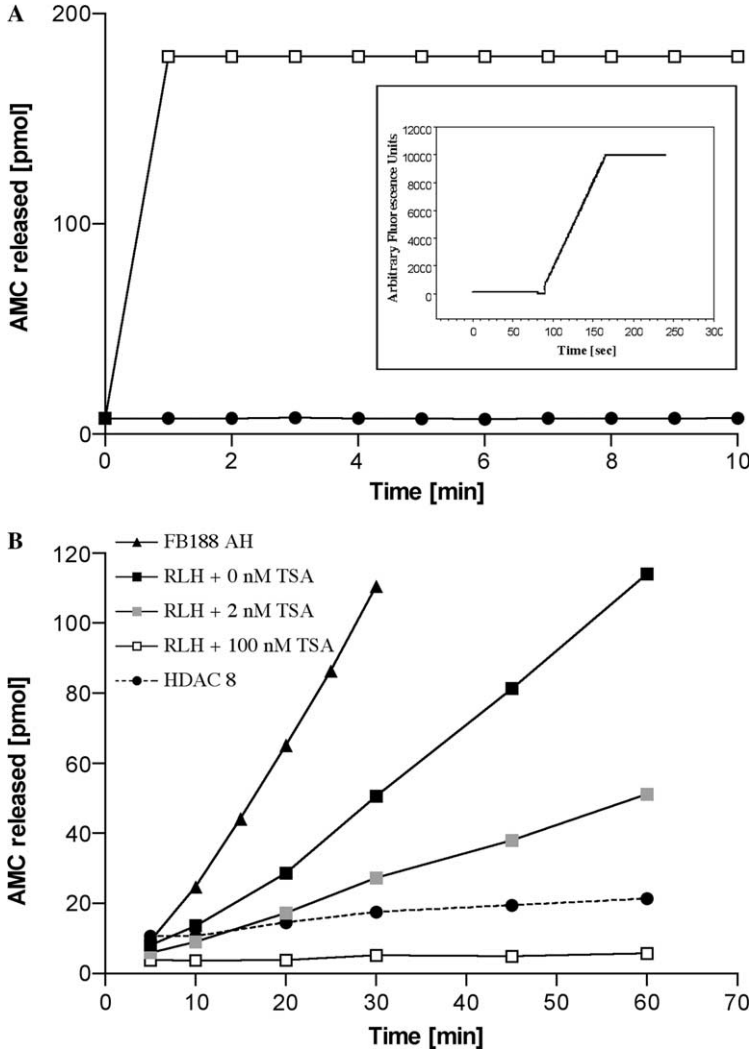

Fig. 2. Time course of AMC release from different substrates. (A) Trypsin digest of Tos-Gly-Pro-Lys(Ac)MCA (closed circles) and Tos-Gly-Pro-Lys-MCA (open squares). A substrate concentration of 300 μM was used. In the inset the first minute of cleavage of the latter substrate is depicted. Here, the final trypsin concentration is 0.05 μg/ml. Measurements were done in a standard fluorimeter (Hitachi F-4500, Colora, Hannover, Germany). (B) Standard two-step HDAC assay with rat liver HDAC (RLH) with 0, 2, and 100 nM TSA. Two-step HDAC assay with recombinant HDAC 8 (Biomol, Hamburg, Germany) and with bacterial FB188 amidohydrolase [15]. All assays except that for the latter enzyme were performed with 125 μM Tos-Gly-Pro-Lys(Ac)MCA as a substrate; for FB188 amidohydrolase, 125 μM Boc-Lys(Ac)MCA was used.

MCA nor Boc-Lys(Ac)-MCA [10] nor a H4-derived peptide are good substrates for HDAC 8. This difference in enzymatic activity may be due to structural differences, i.e., a stretch of mainly acidic amino acids in HDAC 8 which is not present in HDAC 1-3 and which may be responsible for a more demanding substrate specificity.

In addition, time-dependent cleavage experiments in the presence of standard HDAC inhibitor TSA showed that the improved assay is also well suited to identify HDAC inhibitors in a concentration-dependent manner (Fig. 2B). Experiments with low-volume cavity 1536-well microplates demonstrated that reliable signals were also obtained with 6 μl HDAC reaction mixture and 4 μl trypsin/TSA stop solution (data not shown). Finally, experiments were carried out with rat liver HDAC to determine the assay signal dependence on increasing concentrations of HDAC in the first step of the reaction. It turned out that the assay signal is linear at least over the range between 1.2 and 12 U/μl HDAC in the first step of the reaction.

To characterize the novel substrate in more detail, K_m and V_{max} values were determined for rat liver-derived HDAC (Table 1). A number of deacetylation reactions were carried out at substrate concentrations between 10 and 400 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, Tos-Gly-Pro-Lys(Ac)-MCA revealed a K_m of 69 ± 12 μM, whereas previous

Table 1
K_m and V_{max} values for selected HDAC substrates

Peptide sequence	Rat liver histone deacetylase[a]		FB188 amidohydrolase	
	K_m (μM)	V_{max} (pmol s^{-1} mg^{-1})	K_m (μM)	V_{max} (pmol s^{-1} mg^{-1})
Tos-Gly-Pro-Lys(Ac)MCA	68.6 ± 11.9	6.1 ± 0.29	⩾ 290	n.d.
Boc-Lys(Ac)MCA[b]	3.7 ± 1.7	4.41 ± 0.10	127 ± 24	175 ± 20
Ac-Arg-Gly-Lys(Ac)MCA[b]	27.5 ± 4.9	3.61 ± 0.29	n.d.	n.d.
Ac-Gly-Gly-Lys(Ac)MCA[b]	32.6 ± 3.2	4.27 ± 0.26	n.d.	n.d.
Ac-Leu-Gly-Lys(Ac)MCA[b]	44.2 ± 1.0	5.26 ± 0.28	n.d.	n.d.
Ac-Gly-Ala-Lys(Ac)MCA[b]	35.2 ± 3.4	5.59 ± 0.74	n.d.	n.d.

[a] Rat liver histone deacetylase (Calbiochem, Bad Soden, Germany), 89 U/ml, 4.7 mg/ml.
[b] Data for rat liver histone deacetylase from [10].

tripeptide substrates yielded K_m values between 26 and 43 μM, which altogether resembles the K_m value of 20 μM for histones, the natural substrate, which was measured in a standard radioactive assay [14].

In addition to applications with true eukaryotic HDACs the two-step assay is also well suited to characterize bacterial HDAC-like enzymes such as the recently discovered HDAC-like amidohydrolase from *Bordetella/Alcaligenes FB188* (DSM 11172) [15]. Here, however, Boc-Lys(Ac)MCA proved to be a superior substrate revealing a K_m of 127 ± 24 μM. Surprisingly, this bacterial enzyme is also able to deacetylate [^{3}H]-acetate-prelabeled chicken histones with a K_m of 40 μM [15]. In the same assay, true HDACs of eukaryotic origin revealed K_m values mostly between 30 and 82 μM [16]. Interestingly, FB188 amidohydrolase represents a prototype of enzymes that are not cleaved, i.e., inactivated, by trypsin. In this case, a modification of the original two-step assay may be used that is essentially one step and contains trypsin already from the beginning. In this version the assay also permits continuous monitoring of enzyme activity (data not shown). In conclusion, the novel assay is well suited to different HDACs and even other classes of HDAC-like enzymes such as bacterial amidohydrolases.

To characterize the screening assay quality with regard to the screening window coefficient (Z'-factor) a statistical evaluation of negative control reactions (no inhibitor) and positive control reactions (no HDAC) was carried out for (i) the original protocol [10] using Boc-Lys(Ac)MCA, (ii) the original protocol using Tos-Gly-Pro-Lys(Ac)MCA and a 1000-fold lower trypsin concentration in the second step of the assay, and (iii) the new protocol described herein which is based on Tos-Gly-Pro-Lys(Ac)MCA, lower trypsin concentrations and improved buffer conditions. Whereas the original assay (i) exhibited a Z'-factor of 0.07 protocols (ii) and (iii) revealed Z'-factors of 0.39 and 0.84. Improvements comprised an increase in the dynamic range (difference between mean values each of negative and positive control reactions) and improvements with regard to standard deviations. Thus, the assay in its present form is classified as an "excellent assay" [11] with regard to (statistical) screening assay quality (screening window coefficient).

Organic solvents such as DMSO, MeCN, or NMP are routinely used to dissolve drug candidates prior to high-throughput screening. Thus, HTS assays ideally should be robust against small concentrations of these solvents. Consequently, the effect of increasing solvent concentrations on the AMC signal was tested for the standard two-step HDAC assay (Fig. 3). While up to 2% (v/v) of DMSO had no drastic effect on the assay signal (<9% of the signal), a concentration as small as 1% (v/v) of NMP already decreased the fluorescence signal by approximately 42%. MeCN held an intermediate position. Thus, the results suggest the use of DMSO as the

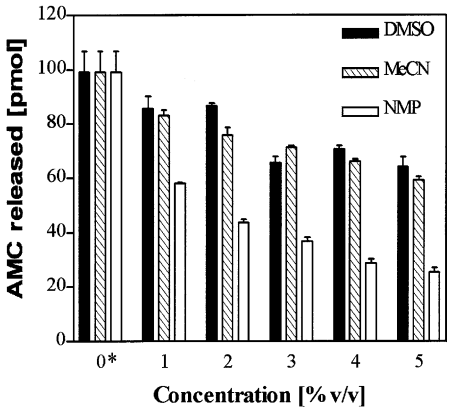

Fig. 3. Influence of organic solvents on AMC release in the two-step HDAC assay. Effect of increasing DMSO, MeCN, and NMP concentrations; 60 μl of HDP buffer containing organic solvent was mixed with 10 μl of diluted enzyme and the solution was preincubated at RT for 5 min. The HDAC reaction was started by adding 50 μl substrate solution (final concentration: 125 μM in 120-μl reaction volume) followed by 60 min of incubation at 30 °C. The reaction was stopped by adding 80 μl trypsin/TSA stop solution. After a 20-min incubation period at 30 °C, the released AMC was monitored by measuring the fluorescence at 460 nm (λ_{ex} = 390 nm). (*Since the substrate solution included 1% (v/v) DMSO, each standard 120-μl HDAC reaction contained 0.4% (v/v) DMSO in addition to the final concentrations of MeCN or NMP. Concentrations given for DMSO are final concentrations except for "0%" which really contained 0.4% (v/v) DMSO originating from the substrate solution.)

superior solvent for compounds tested in the HDAC assay.

To test the feasibility of the new assay with Tos-Gly-Pro-Lys(Ac)MCA as a substrate for the identification and characterization of histone deacetylase inhibitors, the assay was carried out in the presence of various standard inhibitors of histone deacetylases (Fig. 4) and rat liver HDAC. Earlier, we confirmed that the inhibition of trypsin by all three inhibitors is neglectable (data not shown). The semilogarithmic plots analyzed with the help of the Graph Pad Prism software indicated an IC_{50} value of 1.3 nM for the TSA inhibition of rat liver HDAC, which is in the range of the IC_{50} values of 1.4 nM [10] and 2.6 nM [17] reported recently for the same enzyme/inhibitor combination and substrates Ac-Arg-Gly-Lys(Ac)MCA and Boc-Lys(Ac)MCA, respectively. For SAHA an IC_{50} value of 9.6 nM was obtained. In contrast, previous publications reported IC_{50} values between 127 and 259 nM, depending on the assay used [18]. The differences may be caused by the usage of different substrates and assay types. For MS-275 (termed MS-27-275 in the first reports) we measured an IC_{50} value of 8.8 μM which is in the range of the IC_{50} value of 4.8 μM published in a recent report, however, for a different type of enzyme and assay [19].

In summary, the novel HDAC assay combines the specificity of the deacetylation reaction with the advantages of a homogeneous fluorogenic assay in a two-step process. The main limitation, however, is the inability to permit continuous monitoring of enzyme activity as is always the case with endpoint assays. Only occasionally, e.g., in the case of the FB188 amidohydrolase, the HDAC-like enzyme is stable against trypsin digest, permitting a single-step continuous assay.

Altogether, the assay described herein is well suited to measure the enzymatic activity of HDACs and related enzymes, e.g., those belonging to the group of bacterial HDAC-like (acetylpolyamine) amidohydrolases. The assay can be carried out in small reaction volumes. It is robust against small concentrations of DMSO and MeCN. Furthermore, it exhibits a very high screening window coefficient (Z'-factor), in particular when

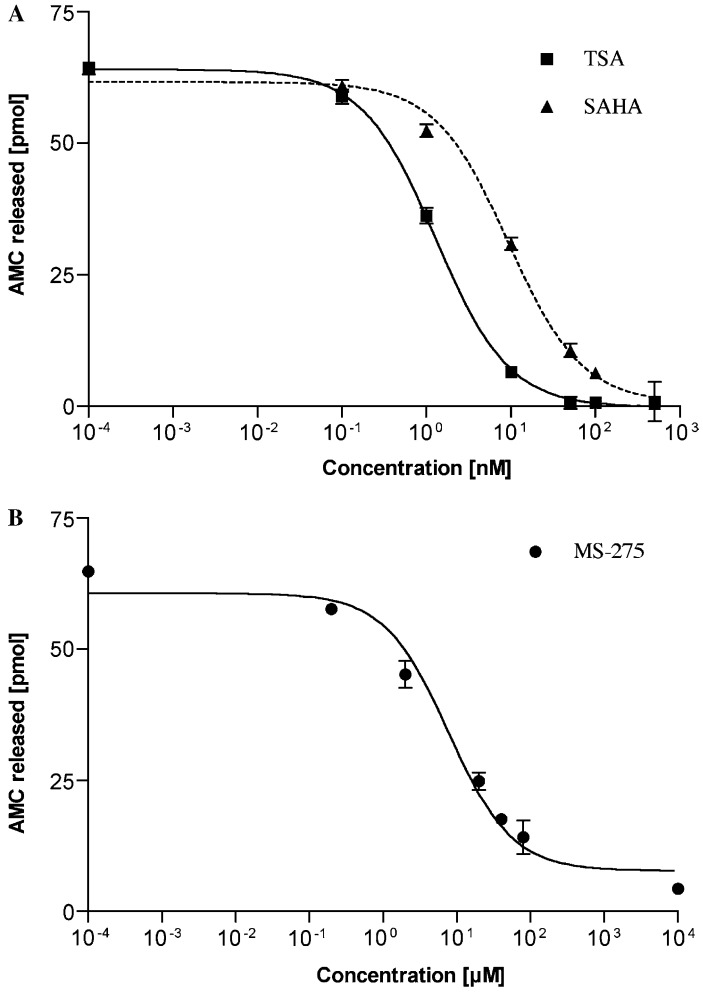

Fig. 4. IC_{50} measurements for different HDAC inhibitors using rat liver HDAC. (A) Trichostatin A (TSA) and SAHA; (B) MS-275. IC_{50} values were determined using the Graph Pad Prism 3.0 software.

performed with the help of robotic workstations. In addition, proof-of-concept experiments indicate that the assay is appropriate to characterize various, chemically unrelated HDAC inhibitors. In conclusion, the new assay is well suited to high-throughput screening efforts in the process of screening combinatorial libraries for new HDAC inhibitors and thus in the development of new anticancer drugs.

Acknowledgments

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Note added in proof

Meanwhile Heltweg et al. published a second type of homogeneous, nonisotopic histone deacetylase assay [20] which has been reviewed recently [21].

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