

## Nonisotopic substrate for assaying both human zinc and NAD<sup>+</sup>-dependent histone deacetylases

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### Abstract

Histone deacetylases (HDACs) are involved in the regulation of transcription and their inhibitors are a promising class of new anticancer drugs. We have previously reported Boc(Ac)Lys-AMC, also termed MAL, as a fluorescent substrate for HDACs. Now we present a modification of MAL called Z-MAL that is characterized by an increased rate of conversion by histone deacetylases of classes I and II and the recently discovered sirtuins (histone deacetylases class III). MAL and Z-MAL are the first nonradioactive substrates for class III enzymes. The new substrate Z-MAL allows for shorter assay times in inhibitor screening and is applicable to diverse sources of deacetylase activity even with completely different catalytic mechanisms. Interestingly, MAL shows some relative preference toward class II, indicating that subtype selectivity in small-molecule HDAC substrates might be obtained.

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Histone deacetylases (HDACs)<sup>1</sup> are enzymes involved in the posttranslational modifications of histones [1] and other proteins such as p53 [2]. They are divided into three classes in humans [3]. Classes I and II, which one could also call the classical HDACs, consist of zinc-dependent amidohydrolases while class III, the so-called sirtuins, depend on NAD<sup>+</sup> as a cofactor that takes up the acetate to form *O*-acetyl-ADP-ribose [4]. HDACs deacetylate side chains of lysine residues in histone and nonhistone substrates which modifies structural features and hence biological activities of these proteins. In the case of histones this usually leads to transcriptional repression. Aberrant gene silencing by recruited HDAC activity has been linked to the pathogenesis of malignant diseases [5]. Inhibitors of histone deacetylases lead to protein hyperacetylation via intrinsic acetyltransferase activity. This in turn usually causes a relief of tran-

scriptional repression which may also induce reversal of the malignant phenotype [6], and several HDAC inhibitors are already in clinical trials for the treatment of cancer [7]. While HDACs therefore are clearly a target for drug design the link between sirtuin activity and lifespan increase [8] makes it questionable whether the same is true for class III. Nevertheless, potent new sirtuin inhibitors would be very interesting at least for mechanistic studies. Therefore, assays for the measurement of histone deacetylase activity are important tools for the unraveling of the function of these important regulators of key cellular processes and aid in the discovery of new potential therapeutic agents.

Traditional *in vitro* assays monitor the release of tritiated acetic acid from radiolabeled histones [9] or histone peptide fragments [10,11] via extraction and scintillation counting. A high-throughput version using a biotinylated peptide and scintillation proximity is available also [12]. We have introduced fluorescent octapeptides [13] and a small lysine derivative termed MAL [14] as nonisotopic substrates for HDACs. Especially the latter allows for a convenient nonradioactive

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<sup>1</sup> Abbreviations used: HDACs, histone deacetylases; DMSO, dimethyl sulfoxide; TSA, trichostatin A.

screening as we recently have developed a homogeneous method for the determination of its enzymatic conversion that may be amenable to high-throughput screening, the so-called HDASH procedure [15]. One of the drawbacks of our substrate MAL is a limited rate of conversion by HeLa nuclear extract which is a standard source of human HDAC activity [16]. Here, we report an improved substrate with better acceptance by HeLa deacetylase activity. We also present for the first time a nonpeptidic substrate for sirtuin (class III histone deacetylase) activity that has the additional advantage of being nonradioactive.

## Materials and methods

### Chemicals

The fluorescent substrate MAL (**1**) was synthesized from  $\alpha$ -BOC- $\epsilon$ -acetyl lysine (Calbiochem) and 7-amino-4-methylcoumarin (Sigma) as outlined elsewhere [14,17]. Recently, it has also become commercially available (Calbiochem; No. 382155). Trichostatin A (TSA) (**3**) was purchased from Sigma. SW55 (**4**) was synthesized as described in the literature [18]. The sirtuin inhibitor sirtinol (**5**) was purchased from Alexis.

### Synthesis

(*S*)-[5-Acetylamino-1-(4-methyl-2-oxo-2*H*-chromen-7-ylcarbonyl)-pentyl]-carbamic acid benzyl ester (**2**), also termed Z-MAL, was synthesized from *N*- $\alpha$ -benzyloxycarbonyl-*N*- $\epsilon$ -acetyl-lysine (0.39 g, 1.21 mmol; prepared according to the literature [19]), 7-amino-4-methylcoumarin (0.21 g, 1.21 mmol), and POCl<sub>3</sub> (0.25 mL). *N*- $\alpha$ -Benzyloxycarbonyl-*N*- $\epsilon$ -acetyl-lysine and equivalent amounts of 7-amino-4-methylcoumarin were dissolved in dry pyridine (7 mL) at  $-15^{\circ}\text{C}$ . Phosphoryl chloride (0.25 mL) was then added dropwise via a syringe with vigorous stirring, resulting in an orange-red solution. After stirring for 1 h at  $-15^{\circ}\text{C}$ , the mixture was then poured into the 10-fold volume of ice/H<sub>2</sub>O and extracted three times with 50 mL of ethyl acetate. The combined organic phase was washed consecutively with H<sub>2</sub>O, 2 M HCl, H<sub>2</sub>O, 5% NaHCO<sub>3</sub>, H<sub>2</sub>O, and saturated brine (50 mL each). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and the solvent was evaporated. The resulting product was chromatographed with ethyl acetate/methanol (20:1). Yield: 0.25 g (43%), mp  $158^{\circ}\text{C}$ . IR: 3311, 2939, 2862, 1684, 1621, 1524. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>):  $\delta$  10.49 (s, 1H), 7.81 (t, 1H, <sup>3</sup>*J* = 5.47 Hz), 7.75 (d, 1H, <sup>4</sup>*J* = 1.75 Hz), 7.70 (d, 1H, <sup>3</sup>*J* = 8.60 Hz), 7.65 (d, 1H, <sup>3</sup>*J* = 7.62), 7.47 (dd, <sup>3</sup>*J* = 8.79 Hz, <sup>4</sup>*J* = 1.96 Hz), 7.32 (s, 5H), 6.25 (s, 1H), 5.01 (s, 2H), 4.12–4.09 (m, 1H), 2.99–2.96 (m, 2H), 2.38 (s, 3H), 1.75 (s, 3H), 1.68–1.27 (m, 6H). <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>):  $\delta$  171.68, 168.59, 159.74, 155.89, 153.36, 152.84,

141.98, 136.70, 128.14 (2 C), 127.62, 127.55 (2 C), 125.75, 115.05, 114.88 112.13, 105.06, 65.43, 55.54, 38.27, 31.32, 28.88, 23.17, 22.65, 18.05. MS (EI): *m/z* 371 (55), 271 (70). Anal. (C<sub>26</sub>H<sub>29</sub>N<sub>3</sub>O<sub>6</sub>) C, H, N.

### Apparatus

A Shimadzu RF 535 was used as fluorescence detector for HPLC. A Lichrosorb RP 18–5  $\mu\text{m}$  (125  $\times$  3 mm; Knauer) column with a guard column of the same material (5  $\times$  3 mm; Knauer) and a Luna 5  $\mu\text{m}$  Phenyl-Hexyl (250  $\times$  4 mm; Phenomenex) column were used for the HPLC analysis of the incubation mixture and the extracted substrate solutions. A BMG Polarstar plate reader was used to quantify the fluorescence in the homogeneous assay. Black BMG micoplates were used for the plate reader based assays.

### Enzymes

Rat liver HDAC was purified with ammonium sulfate precipitation and chromatography on Q-Sepharose with an increasing gradient of sodium chloride according to published procedures [15,20]. Such preparations are commercially available (Calbiochem, Alexis) but some contain tris-buffer which is not compatible with homogenous assay. As the purified rat liver preparation does not contain sirtuin activity (data not shown), a crude extract was used for the sirtuin assay. For this preparation, 15 g of rat liver was cut into small pieces and homogenized in 70 mL of lysis buffer (42 mM Na<sub>2</sub>HPO<sub>4</sub>, 8 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5, 0.25 mM sucrose, 25 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, and Complete protease inhibitors (Roche)) and centrifuged at 20,000g. The supernatant was used as a source for sirtuin deacetylase activity.

HeLa nuclear extract was purchased from Geneka (200  $\mu\text{g}/40 \mu\text{L}$ ; catalogue No. 100200) and from Upstate (50  $\mu\text{g}/25 \mu\text{L}$ ; catalogue No. 12-309). The recombinant sirtuin hSIRT1 was purchased from Biomol (3.5 U/ $\mu\text{L}$ ; catalogue No. SE-239).

Recombinant HDAC1 [11] and HDAC6 [21] were prepared as full-length FLAG-tagged proteins as outlined elsewhere [22,23].

### Reactivity assay with rat liver enzyme

For the reactivity with the HPLC assay [24] compounds **1** and **2** were used as substrates and an internal standard, 7-hydroxycoumarin, was always included for quantitation purposes. The HPLC assay was performed on the Lichrosorb column with acetonitrile/water (40/60 v/v) as mobile phase at a flow rate of 0.6 mL/min. Excitation wavelength was 330 nm and emission wavelength 390 nm. Retention times were 3.65 min (**1**), 5.40 min (**2**), and 2.15 min (7-hydroxycoumarin). Results

are taken from at least duplicate determinations. To determine the resulting metabolites, we used a Luna 5- $\mu$ m Phenyl-Hexyl column with acetonitrile/water/trifluoroacetic acid (55/45/0.01 v/v/v) as mobile phase at a flow rate of 0.5 mL/min. Retention times were 8.24 min for **1**, 4.01 min for the metabolite of **1**, 9.66 min for **2**, 3.99 min for the metabolite of **2**, and 7.66 min for 7-hydroxycoumarin. Stock solutions of compounds **1** and **2** were made at 12.6 mM in DMSO and were further diluted with enzyme buffer (1.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 18.6 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.9, 0.25 mM EDTA, 10 mM NaCl, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol). A stock solution was prepared using an aliquot of 10  $\mu$ L of the substrate (**1** or **2**), 15  $\mu$ L of a solution of the standard 7-hydroxycoumarin in DMSO (6.3 mg/mL), and enzyme buffer to a total volume of 1 mL. A 10- $\mu$ L amount of the substrate/standard stock solution was added to a mixture of 100  $\mu$ L of rat enzyme preparation (at 4 °C) and 10  $\mu$ L of buffer. After 15 min at 4 °C, the mixture was then incubated at 37 °C for the desired length of time. After this time, the reaction was stopped by the addition of 72  $\mu$ L of 1 M HCl/0.4 M sodium acetate and 800  $\mu$ L ethyl acetate. After it was centrifuged (10,000 rpm, 5 min), an aliquot of 200  $\mu$ L of the upper phase was taken and the solvent removed by a stream of nitrogen. The residue was dissolved in 600  $\mu$ L of chromatography eluent, and 20  $\mu$ L was injected via autosampler onto the HPLC system. The amount of remaining substrate is calculated relative to the substrate control without enzyme (each as quotient of the peak area of the substrate divided by the peak area of the internal standard). For the determination of the metabolites, the incubation was stopped by addition of 1000  $\mu$ L of acetonitrile instead of 72  $\mu$ L of 1 M HCl/0.4 M sodium acetate. The mixture was then centrifuged (10,000 rpm, 5 min) and the supernatant was taken off the enzyme pellet and 20  $\mu$ L were injected via autosampler into the HPLC system.

#### *Reactivity assay with HeLa nuclear extract*

The same procedure as above was run using commercial HeLa nuclear extracts instead of rat liver enzyme preparation. The extracts were diluted with enzyme buffer (1:10). Instead of 100  $\mu$ L enzyme preparation, 45  $\mu$ L (Geneka) or 60  $\mu$ L (Upstate) of the dilution were taken; 5  $\mu$ L of buffer or inhibitor solution and 10  $\mu$ L of substrate/standard solution were added. The mixture was incubated for 15 min at 4 °C and then for 3 h (Geneka) or 90 min (Upstate) at 37 °C and consecutively treated as described above.

#### *Reactivity assay with a sirtuin*

For the determination of the reactivity with hSIRT1, a different enzyme buffer (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl<sub>2</sub>) was used for

all dilutions and incubations. The substrate solutions were prepared as described above. A NAD<sup>+</sup> stock solution was prepared in water (6 mM); 5  $\mu$ L of the substrate stock solution was mixed with 2, 2.5, or 5  $\mu$ L of hSIRT1, 5  $\mu$ L of the NAD<sup>+</sup> solution, and enzyme incubation buffer to a total volume of 60  $\mu$ L. The incubation at 37 °C was stopped after 4 or 8 h by the addition of 36  $\mu$ L of 1 M HCl/0.4 M sodium acetate, extracted with 400  $\mu$ L of ethyl acetate, and consecutively treated as mentioned above. For the determination of the IC<sub>50</sub> of sirtinol (**5**), inhibitor solutions of 60, 20, 6, 2, and 0.6 mM were prepared in DMSO; 5  $\mu$ L of the substrate/internal standard stock solution was added to a mixture of 2.5  $\mu$ L of hSIRT1, 5  $\mu$ L of the NAD<sup>+</sup> stock solution, 1  $\mu$ L of each inhibitor solution, and 46.5  $\mu$ L of the enzyme incubation buffer. This mixture was incubated for 16 h at 37 °C. The reaction was stopped and analyzed as described above.

For the sirtuin assay with the crude rat liver preparation, 5  $\mu$ L of the stock solution of NAD<sup>+</sup> (24 mM in water) or 5  $\mu$ L of buffer and 10  $\mu$ L of a stock solution of SW55 (**4**) (240  $\mu$ M in buffer) were mixed with 90  $\mu$ L of the enzyme preparation. For the inhibitor testing, a stock solution of sirtinol was prepared in DMSO (24 mM); 5  $\mu$ L of the inhibitor solution or 5  $\mu$ L of DMSO as vehicle were added to the enzyme. The reaction was started by the addition of 10  $\mu$ L of the stock solution of substrate/internal standard. The incubation at 37 °C was stopped after 2, 4, or 16 h by the addition of 72  $\mu$ L of 1 M HCl/0.4 M sodium acetate. The mixture was then extracted with 800  $\mu$ L of ethyl acetate and analyzed on the HPLC system as described above.

#### *Homogeneous HDAC activity assay*

For the homogeneous assay [15], stock solutions of **1** and **2** of 12.6 mM in DMSO were prepared. Aliquots of 10  $\mu$ L of these solutions were diluted in enzyme buffer (1.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 18.6 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.9, 0.25 mM EDTA, 10 mM NaCl, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol) to a total volume of 1 mL and used as substrate solutions. For inhibitor testing, the stock solution of TSA (**3**, 3.3 mM in DMSO) was diluted to 3.3, 1.1, 0.33, 0.11, and 0.033  $\mu$ M in enzyme buffer; 50  $\mu$ L of the rat liver enzyme was mixed with 10  $\mu$ L of the buffer as blank, with 5  $\mu$ L of the substrate solution and 5  $\mu$ L of the buffer as negative control and with 5  $\mu$ L of the substrate solution and 5  $\mu$ L of the positive control solution (3.3  $\mu$ M TSA) as a positive control. Samples were prepared by mixing 50  $\mu$ L of the enzyme solution with 5  $\mu$ L of each inhibitor solution and 5  $\mu$ L of the substrate solution. These mixtures were then incubated for 90 min at 37 °C and the reaction was stopped by the addition of 190  $\mu$ L of a mixture of TSA solution (3.3  $\mu$ M in enzyme buffer), borate buffer

(0.1 mM boric acid, adjusted with 1 M NaOH to pH 9.5), and a solution of naphthalene-2,3-dicarboxaldehyde (16 mM in methanol) in a ratio of 5/180/5 (v/v/v). The fluorescence was measured at 330/390 nm. The amount of remaining substrate in the positive control with TSA is calculated relative to the negative control without inhibitor to give the 100% value. Partial conversion is related to that value.

#### Reactivity assay with the FLAG-HDAC1 and FLAG-HDAC6

The immunoprecipitation of the enzymes from the cell lysates was performed as described elsewhere [22]. M2 agarose (Sigma) was used at 25  $\mu$ L/mL lysate. The immunoprecipitated enzymes were washed in enzyme buffer and used as enzyme source in the homogeneous assay; 2.5  $\mu$ L of the agarose beads were resuspended in 52.5  $\mu$ L and mixed with 5  $\mu$ L of the buffer as blank and with 5  $\mu$ L of the substrate solution for the reactivity assay. As a control, 55  $\mu$ L of enzyme buffer was mixed with 5  $\mu$ L of the substrate solution. These mixtures were then incubated for 3 h at 37 °C. The reaction was stopped and analyzed as described above. The amount of remaining substrate in the enzyme-containing samples was calculated relative to the amount of substrate in the control samples.

## Results

The fluorescent substrate MAL (**1**) is readily deacetylated by rat liver but not so well by HeLa nuclear extract histone deacetylases. The benzyloxycarbonyl (Z) modification Z-MAL (**2**) (see Fig. 1) was also shown to be accepted as a substrate for histone deacetylases. We incubated both substrates with rat liver enzyme preparation as a source of histone deacetylases of classes I and II (immunoblotting data not shown) and were able to observe a time-dependent conversion of both substrates. The Z analogue of MAL is more quickly deacetylated than MAL (see Fig. 2). After 1 h of incubation at 37 °C, more than 65% of Z-MAL but only 38% of MAL is deacetylated. To test whether Z-MAL is able to replace MAL in the homogeneous assay, we measured the  $IC_{50}$

value of trichostatin A (**3**, see Fig. 3) using the homogeneous histone deacetylase assay HDASH [15] with **1** and **2** as substrates (see Fig. 4). The  $IC_{50}$  was  $6.04 \pm 0.67$  nM for **1** as substrate and  $10.1 \pm 1.5$  nM for **2** as substrate. We then investigated whether the newly discovered HDAC substrate **2** can be used in assaying human histone deacetylase activity which converted MAL rather slowly. HeLa nuclear extract serves as a source for different histone deacetylases of classes I and II (immunoblotting data not shown). We were able to observe a significant difference in conversion (see Fig. 5) with **2** being deacetylated in both instances to a much larger extent in the same time frame. We then wanted to analyze the reactivity of the substrates toward purified HDAC subtypes. We prepared FLAG-tagged HDAC1 as a member of class I [11] and FLAG-tagged HDAC6 as a class II enzyme (subclass IIb) [21]. As shown in Fig. 6, **2** is converted to a larger extent by HDAC1 than is **1** in the same time frame. For HDAC6, the difference between the conversion rates of **1** and **2** is not statistically significant.

So far no experiments with nonpeptidic substrates have been performed with class III histone deacetylases (sirtuins). After incubation of **1** and **2** for 8 h at 37 °C with 17.5 U of hSIRT1 as a recombinant member of the sirtuins, we observed a deacetylation of about 90% of **2** but only about 50% of **1** (see Fig. 7A). With only 7 U of the enzyme and 4 h at 37 °C, we still see a significant conversion of **2** but not of **1**. To check

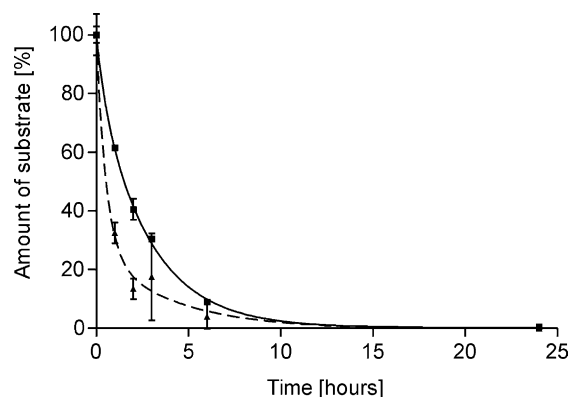


Fig. 2. Time-dependent reaction of **1** (■) and **2** (▲) with rat liver enzyme ( $n = 2$ ,  $\pm$ SD).

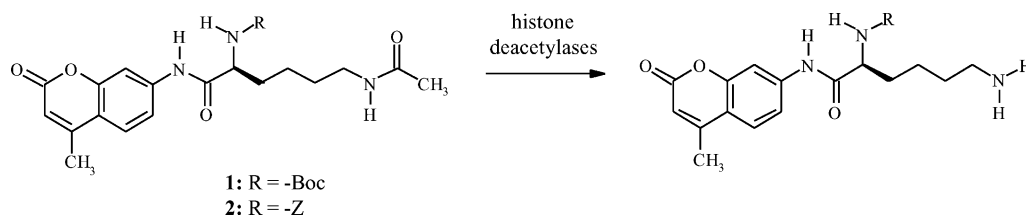


Fig. 1. Deacetylation of MAL (**1**) and Z-MAL (**2**) by histone deacetylases.

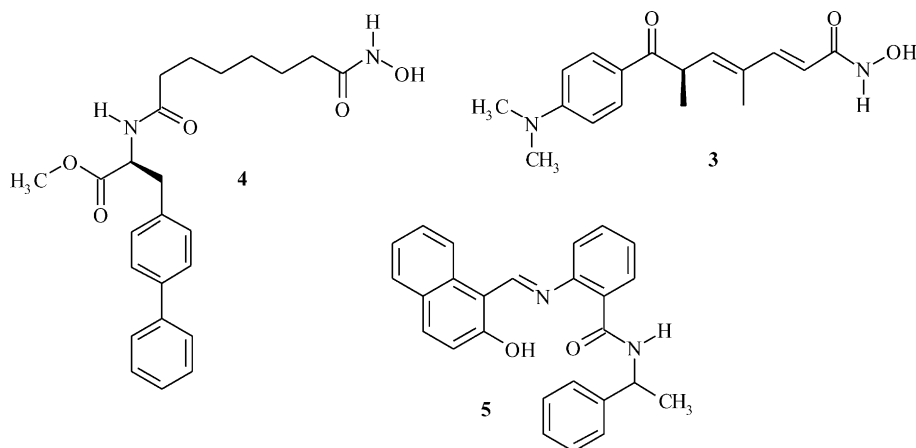
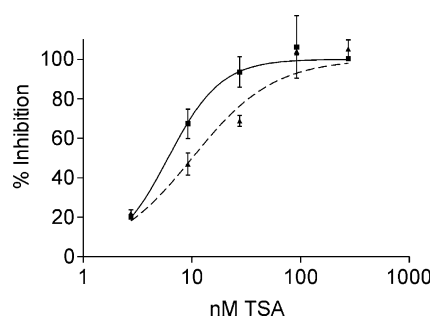
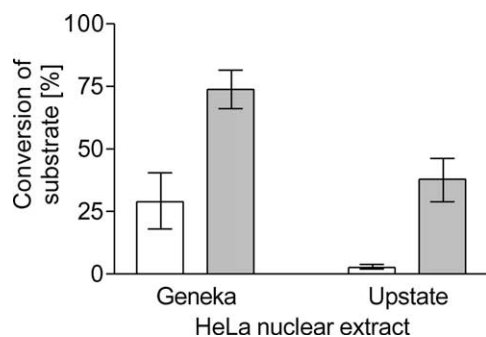
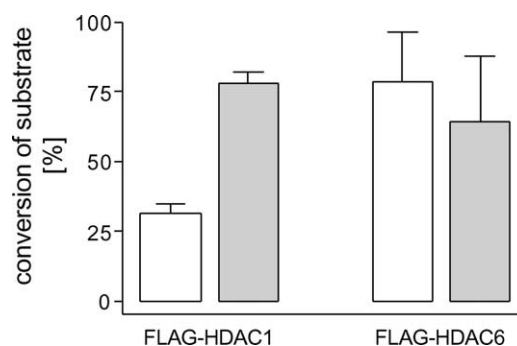


Fig. 3. Specific inhibitors of different histone deacetylases.

Fig. 4. IC<sub>50</sub> determination of trichostatin A with **1** (■) and **2** (▲) in the homogeneous assay ( $n = 2$ ,  $\pm$ SD). The rates of conversion without inhibitor were 54% for **1** and 77% for **2**.Fig. 5. Conversion of **1** (open bars) and **2** (filled bars) with HeLa nuclear extract ( $n = 2$  or  $3$ ,  $\pm$ SD).

whether the deacetylation of **2** is indeed catalyzed by sirtuins and not by a contamination with histone deacetylases of classes I or II, we added 5  $\mu$ M of the class I and II specific inhibitor trichostatin A (**3**). We could not detect any inhibition of the deacetylation of **2** (data not shown). We then determined the IC<sub>50</sub> of the specific sirtuin inhibitor sirtinol (**5**) (see Fig. 3) [25] with hSIRT1 and were able to observe an IC<sub>50</sub> value of 60  $\mu$ M, which is comparable to reported data in the literature (reported IC<sub>50</sub> values of 68  $\mu$ M for the re-

Fig. 6. Conversion of **1** (open bars) and **2** (filled bars) with recombinant histone deacetylases ( $n = 3$ – $6$ ,  $\pm$ SD).

combinant yeast sir2p and of 38  $\mu$ M for the recombinant human hGST-SIRT2) [25] (see Fig. 7B).<sup>2</sup> For the unspecific sirtuin inhibitor nicotinamide [26], we were able to observe an inhibition of the conversion of **2** at 30  $\mu$ M (21% inhibition) and 100  $\mu$ M (44% inhibition) (data not shown). We were not able to detect sirtuin activity in our chromatographically purified rat liver enzyme (data not shown). Therefore, we used a crude extract from rat liver and were able to detect NAD<sup>+</sup>-dependent deacetylase activity (see Fig. 8). The histone deacetylases of classes I and II have to be inhibited in that crude extract to detect the sirtuin activity. To accomplish this task, we used the specific inhibitor of classes I and II, SW55 (**4**) [18] (see Fig. 3). The NAD<sup>+</sup>-dependent deacetylase activity from this source can be inhibited by sirtinol (**5**). The high variability of the data can be explained with the very insoluble sirtinol, which precipitates easily in buffer- and protein-containing solutions and with the crude enzyme preparation.

<sup>2</sup> Footnote added in proof: We have recently observed that *E. coli* expressed recombinant Sirt2 appears to precipitate in vitro in the presence of sirtinol. Some of this precipitation might have contributed to the inhibition of the enzyme by sirtinol observed in vitro.

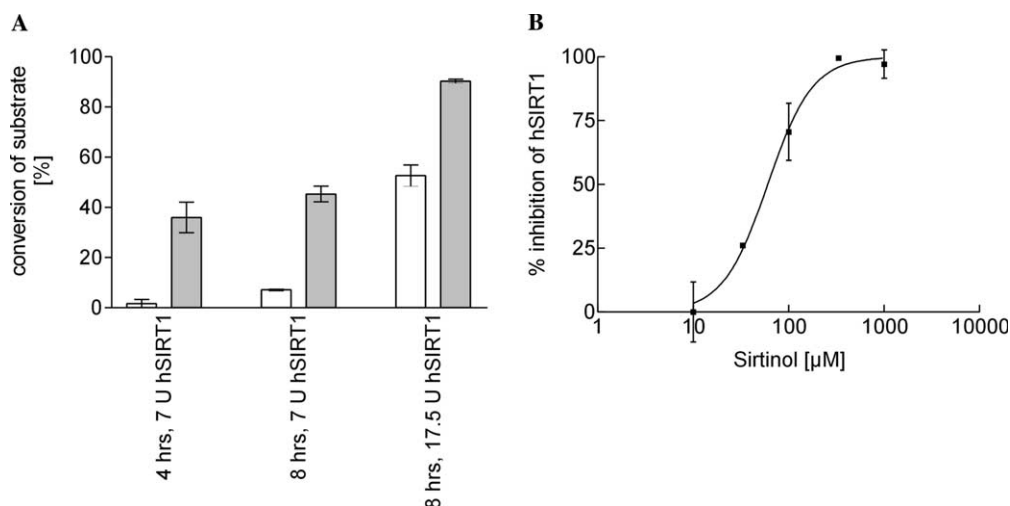


Fig. 7. (A) Conversion of **1** (open bars) and **2** (filled bars) with recombinant hSIRT1 ( $n = 2$ ,  $\pm$ SD). (B)  $IC_{50}$  determination of sirtinol with **2** and hSIRT1 ( $n = 2$ ,  $\pm$ SD). The rate of conversion without inhibitor was 44%.

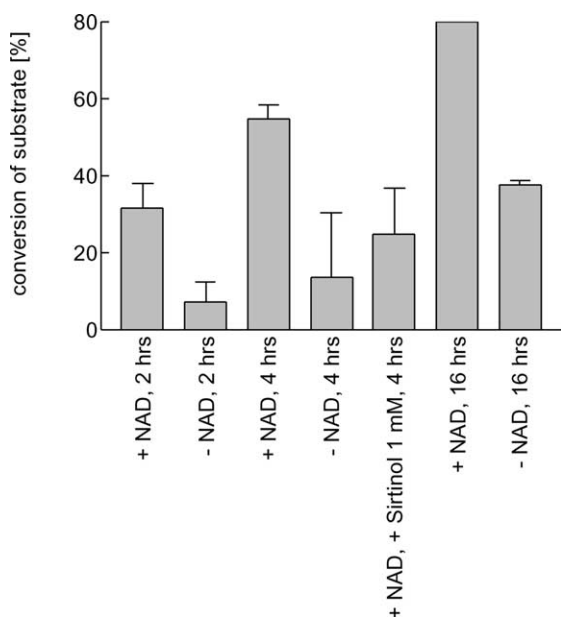


Fig. 8. Conversion of **2** with crude rat liver preparation under specific inhibition of classes I and II HDAC (all samples contain 20  $\mu$ M of the class I and II inhibitor SW55) with and without  $NAD^+$  and with and without the specific sirtuin inhibitor sirtinol ( $n = 2$ ,  $\pm$ SD). Differences between 4 h  $-NAD$  and 4 h  $+NAD$  + inhibitor sirtinol are not significant.

## Discussion

The new fluorescent histone deacetylase substrate Z-MAL can be considered a valuable improvement of the established fluorescent substrate MAL. We were able to show that Z-MAL is accepted by histone deacetylases of all human classes known so far. The deacetylation of Z-MAL by mixtures of various classes of deacetylases and by examples of purified recombinant

subtypes from all three classes is more rapid and, with the exception of FLAG-HDAC6, occurs to a larger extent than the deacetylation of MAL. Thus, it has a broader range of application and shows a greater sensitivity toward samples with low enzymatic activity. On the other hand, the observed class preference of MAL indicates that it may serve as a starting point for the development of subtype-selective small-molecule substrates. The conversion of Z-MAL to its metabolite can be inhibited by specific inhibitors with concentrations in the same range as the published data. This substrate Z-MAL may be valuable for rapid nonisotopic screening of HDAC activity and its inhibition. It can replace MAL in the homogeneous histone deacetylase assay that was published by our group previously and should lead to decreased assay time in high-throughput screening. Especially valuable is the conversion of this new substrate by human and rat liver  $NAD^+$ -dependent class III histone deacetylases (sirtuins) that show a mechanism of catalysis completely different from those of classes I and II. Existing deacetylation assays rely on the determination of released acetate, which despite its usage is not very suitable for the sirtuins. As the cleaved acetyl group is transferred and *O*-acetyl-ADP-ribose is formed only part of the radioactivity will be liberated as [ $^3H$ ]acetic acid that is extracted and counted in the standard protocols. This is the first report of a non-peptidic substrate for this enzyme class which can be quantitated directly and should be very valuable in the search for new inhibitors and in the elucidation of the cellular functions of this poorly understood class of deacetylases. The proof of principle that  $NAD^+$ -dependent class histone deacetylase activity can be selectively detected and inhibited in readily available extracts that contain class I and II deacetylases is a very valuable extension of the scope of potential applications.

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