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A non-isotopic assay for histone deacetylase activity

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ABSTRACT

Inhibitors of histone deacetylase (HD) bear great potential as new drugs due to their ability to modulate transcription and to induce apoptosis or differentiation in cancer cells. To study the activity of HD and the effect of potential inhibitors *in vitro* so far only radioactive assays have existed. For the search of new inhibitors and for the use in HD identification and purification we established a simple, non-radioactive assay that allows screening of large numbers of compounds. The assay is based on an aminocoumarin derivative of an Ω-acetylated lysine as enzyme substrate.

Chromatin structure has an important influence on key nuclear processes such as DNA replication, transcription, DNA repair and rearrangements during differentiation. Chromatin structure and the binding of proteins to DNA can be modulated by reversible acetylation of ε-amino groups of conserved lysine residues in the N-terminal tails of core histones. This modification is established and maintained by histone acetyltransferases and histone deacetylases (HDs). The latter have been recently identified as conserved homologs of transcriptional regulators and nucleolar phosphoproteins (1–2). Experimental evidence suggests that gene regulatory nuclear proteins involved in the control of proliferation and differentiation exert their function by recruitment of histone acetyltransferases or deacetylases (3–4). Increasing evidence has accumulated for the potential of HD inhibitors as new drugs for transcription therapy and cancer chemoprevention (5–8). Therefore, compounds which affect histone acetylation are attractive emerging targets for medicinal chemistry.

The screening for new potent and simple inhibitors of HD is hampered by the enzyme substrate used in the inhibition assay. Either the natural substrate, acetylated histones (9), or synthetic peptides which consist of 8 (10) or 24 (1) amino acids, which are derived from the N-termini of certain core histone species, are used. In either case the substrate is labeled with [^{3}H]acetic acid and the liberation of tritiated acid is measured by scintillation counting. Thus radioactivity is always involved, leading to problems with exposure of laboratory personnel to, and disposal of, radioactive waste. The tritiated histones are gained by a procedure involving pretreatment of chickens with phenylhydrazine over a prolonged period of time, and the sacrifice of the animals cannot be avoided. The degree of acetylation of the prelabeled core histones changes within different preparations and it is difficult to standardize the substrate properties. For synthesis of the oligopeptides solid-phase-technology is required and post-labeling HPLC-chromatography is necessary. There are also assays that rely on immunoblotting of hyperacetylated histones, but they are functional tools rather than assays to measure enzyme activity in a biochemical sense, e.g. (11). Thus we were looking for an easily obtainable and inexpensive substrate for HD which would allow for a convenient non-radioactive *in vitro* assay. In this paper we report on the first fluorescence-based HD activity assay. Such a simple assay that replaces the conventional assay using prelabeled histones or histone peptides is highly desirable with respect to high-throughput screening for possible HD inhibitors. It could also be used to monitor HD activity in protein identification and purification.

Starting from commercial α-N-Boc-Ω-acetyllysine and 7-amino-4-methylcoumarin we have prepared the fluorescent amide MAL (Fig. 1) as a potential substrate for HD using published methodology (12) that involves phosphoryl chloride in pyridine as the activating agent.

The coumarin MAL can be quantitated using a reversed-phase HPLC-system with a fluorescence detector with a limit of detection of 0.9 ng/ml. The quantification is linear from 5 to 300 ng/ml. For the enyzme incubations we used a partially purified rat liver HD which was obtained by chromatography of crude cellular extract on Q-Sepharose. We could observe a clearly time-dependent consumption of the starting material throughout incubation with the enzyme (Fig. 2A). A non-enzymatic cleavage was not observed and a specific reaction was also suggested by the fact that the decrease of the substrate could be completely suppressed by 330 nM trichostatin A (still >95 % at 100 nM) or 80 μM of the structurally unrelated cyclic tetrapeptide inhibitor HC-toxin. Both are potent and specific inhibitors of HDs *in vivo* and *in vitro* (13,14). Proof was given by analysis of the aqueous layer following workup with acidic buffer and ethyl acetate. HPLC-chromatography showed unreacted substrate which was not completely extracted and a second fluorescent material. Analysis of the whole incubation mixture after stopping the reaction with acetonitrile gave the same result (Fig. 2B). We have compared the affinities of rat liver HD to fluorescence labeled lysine and native histones. Dilution of rat liver HD showed that the sensitivities of both assays were nearly identical (duplicate measurements,

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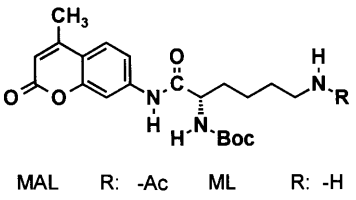

Figure 1. Fluoresence-labeled lysines. MAL, N-(4-methyl-7-coumarinyl)-N-α-(*tert*.-butyloxy-carbonyl)-N-Ω-acetyllysinamide. ML, N-(4-methyl-7-coumarinyl)-N-α-(*tert*.-butyloxy-carbonyl)-lysinamide. Boc-lysines were coupled with equimolar amounts of 7-amino-4-methylcoumarin in dry pyridine (6 ml/mmol) using phosphoryl chloride (0.25 ml/mmol). After standard workup amides were isolated using flash column chromatography (Merck silica 60, ethyl acetate/methanol 10:1 for MAL; ethyl acetate/hexane 1:1 for Ω-Fmoc-compound). Purity and identity were assured by IR, MS, ^{1}H and ^{13}C-NMR and elemental analyses. 400 mg of the Ω-Fmoc-derivative were deprotected using 2 g of 20% (v/v) dry piperidine in dry DMF. The crude amine ML was characterized by NMR and MS and was sufficiently pure for HPLC comparison with incubation product.

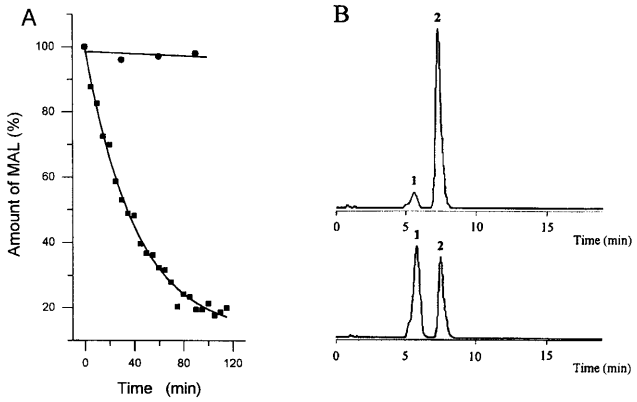

Figure 2. Time-dependent reaction of MAL with rat liver HD. The high speed supernatant of rat liver extract was subjected to chromatography on Q-Sepharose using a linear gradient of NaCl (0–0.5 M) for elution. Fractions of 5 ml were collected and assayed for HD activity, active fractions (fractions 40–50) were pooled and used as enzyme source. (**A**) The fluorescent substrate (10 µl of a solution of 14.7 µg/ml H_2O) was incubated in 24 parallel reactions with rat liver HD (100 µl, 37°C). Every 5 min, one reaction was stopped (72 µl of 1 M HCl/0.4 M sodium acetate, 800 µl of ethyl acetate) and centrifuged. An ethyl acetate aliquot of 600 µl was taken and the solvent was removed by a stream of nitrogen. The amount of remaining substrate was determined after redissolving in 600 µl of eluent by HPLC (LiChrosorb RP-18, 5 µm, 150 × 4 mm, Knauer, acetonitrile/water 40:60) and fluorescence detection ($\lambda_{Exc.}$ = 330 nm, $\lambda_{Em.}$ = 395 nm) and is shown in % (-■-). Reaction was fully suppressed by adding trichostatin A (Wako) at a final concentration of 330 nM (-●-). (**B**) Analysis of the whole incubation mixture after stopping the reaction with acetonitrile at 5 (upper chromatogram) and 30 (lower chromatogram) min, respectively, shows decreasing MAL (t_{ret} = 6.99 min, 2) and increasing formation of ML (t_{ret} = 5.54 min, 1; flow rate = 1.2 ml/min; Multospher 100 RP-18, 250 × 4 mm, Phenomenex, acetonitrile/water 40:60, 0.01% trifluoroacetic acid).

results not shown) and we have determined K_M values of 0.68 µM/l for the synthetic substrate and 20 µM/l for tritiated histones.

The identity of the postulated deacetylation product ML was ensured by its synthesis. Ω-Fmoc-α-Boc-lysine was coupled with 7-amino-4-methylcoumarin applying the phosphoryl chloride method used for the synthesis of the substrate. The putative product was obtained by piperidine mediated deprotection. HPLC analysis confirmed the identity of the synthetic material with the product of the incubation of MAL with HD. Additionally, synthetic and enzymatic product gave identical MS-spectra and identical daughter-ion spectra in MS-MS of m/z = 404 using HPLC-MS (result not shown).

We have succeeded in finding a simple fluorescent substrate for HD which can be easily obtained from commercial starting materials and may possibly be commercially available itself soon. It can be quantitated in low nanomolar concentrations by HPLC analysis and fluorescence detection which allows for the determination of the inhibitory ability of putative inhibitors of HD with IC_{50} values from the nanomolar to the micromolar range. This process could be subjected to automation and modified for high-throughput-screening. This assay should facilitate the search for new inhibitors of HD which will be an important task in the light of recent results that link malignant disease with aberrant chromatin acetylation. A rapid and simple assay for screening of deacetylase inhibitors should therefore be a valuable tool in drug discovery as well as in basic research in the histone acetylation field.

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