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Profiling used in lead optimization and drug discovery

Screening and profiling assays for HDACs and sirtuins

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Epigenetic factors are enzymes or proteins that confer, remove or recognize covalent modifications to chromatin DNA or proteins. They can be divided into three broad groups, commonly referred to as the 'writers', 'erasers' and 'readers'. The HDACs and sirtuins, which remove acetyl groups from the ε-amino of protein lysine residues, fall into the 'eraser' category. Due to their important effects on gene expression and involvement in various disease states, these enzymes have been the subjects of many assay development efforts in recent years. Commonly used techniques include mass spectrometry, antibody-based methods and protease-coupled assays with fluorogenic peptide substrates. Recent advances include the development of synthetic substrates for the assay of various newly discovered non-acetyl deacylation activities among the sirtuins.

Introduction

Histone deacetylases (HDACs) are a class of enzymes that remove acetyl groups from ε-N-acetyl lysine amino acids on histones and on non-histone proteins, such as transcription factors (p53, E2F1) and cytoplasmic proteins (Hsp90, α-tubulin). Deacetylation of histones leads to condensed chromatin and repressed gene transcription. Consequently, the HDACs are considered important epigenetic factors of the 'eraser' type, as opposed to epigenetic 'writers' (transferases) or 'readers' (binding proteins).

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Human HDACs are divided, both phylogenetically and in terms of reaction mechanism, into two major groups (Figs. 1 and 2). The first group, Zn(II)-dependent enzymes whose members catalyze the hydrolysis of the lysine ε-acetamide bond, comprises the class I (human HDACs 1–3 and 8), class IIa (HDACs 4–7 and 9), class IIb (HDACs 6 and 10) and class IV (HDAC11) enzymes [1]. The second group is the sirtuins, human SIRTs 1–7 [2,3], also known as class III HDACs, whose members deacetylate lysines in a reaction that employs oxidized nicotinamide adenine dinucleotide (NAD^+) as a cosubstrate (Fig. 2) [4–6]. The nicotinamide moiety is removed from NAD^+ and the acetyl from the lysine is transferred to the ribose from which the nicotinamide was eliminated. The three resulting products are the deacetylated lysine, nicotinamide and *O*-acetyl-ADP-ribose. It should be noted that nicotinamide is a sirtuin inhibitor [7] and that *O*-acetyl-ADP-ribose has signaling functions [8–10].

The use of biochemical HDAC and SIRT assays has become quite widespread in the past decade, particularly following FDA approval of Merck's pan-HDAC inhibitor Vorinostat (SAHA) [11] for cutaneous manifestation in cutaneous T cell lymphoma in 2006, and the subsequent approval of the prodrug Romidepsin for the same indication in 2009 [12]. The discovery of SIRT1 activators with antiaging activities in yeast, mammalian cells, worms and flies have also greatly stimulated research to find both inhibitors and activators of SIRTs [13–18].

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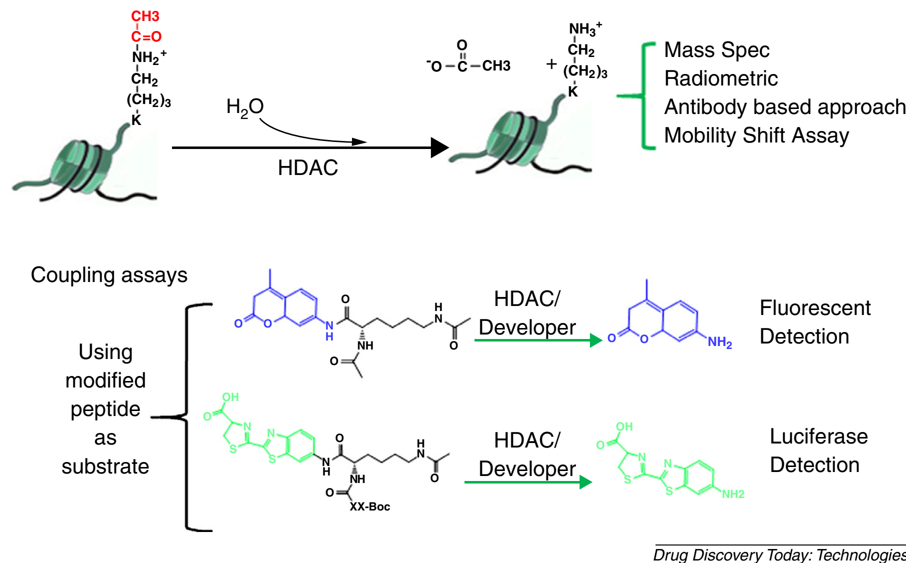

Figure 1. Upper Panel: The hydrolytic deacetylation reaction catalyzed by classes I, II and IV HDACs is shown, along with a list of methods for directly detecting one of the products of the reaction. As denoted by the nucleosome depicted as the substrate and product of the reaction, these assay methods may be applied to natural, unmodified protein substrates. Lower Panel: The structure of two synthetic HDAC substrates used for protease-coupled assays are shown. In each case the moiety required for signal generation is linked to the carboxyl of the acetyllysine that is the target for deacetylation by the HDAC. After deacetylation by the HDAC, these signaling groups, 7-amino-4-methylcoumarin (AMC; Fluorescent Detection) and luciferin (Luciferase Detection), become susceptible to cleavage by the trypsin in the 'Developer' reagent.

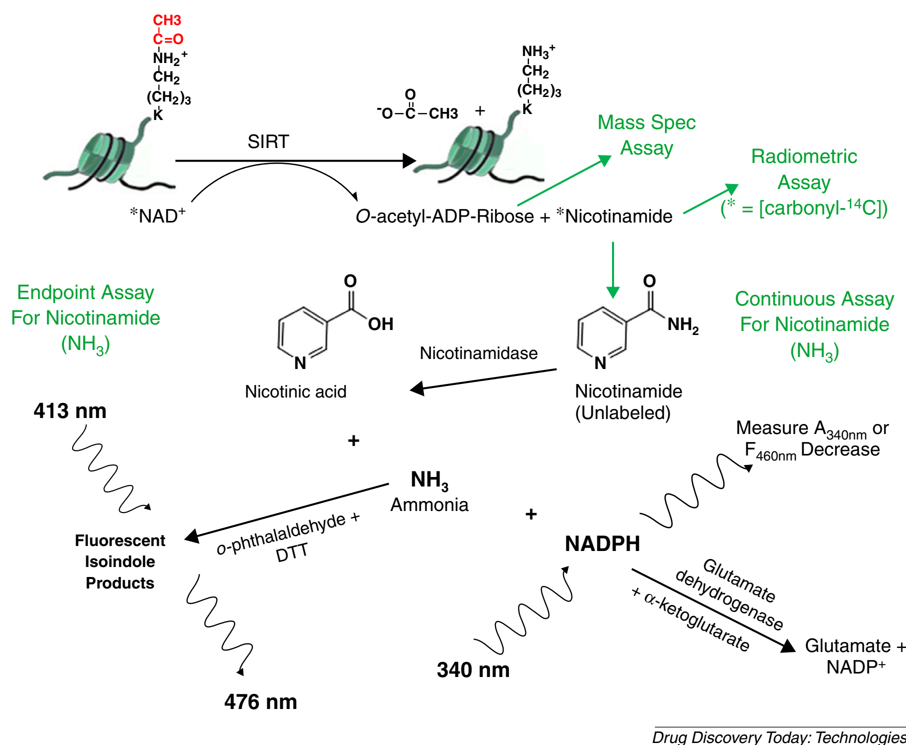

Figure 2. The sirtuin-catalyzed deacetylation reaction is depicted. The acetyl group from the acetylated lysine side-chain is transferred to the co-substrate NAD^+, which breaks down to form *O*-acetyl-ADP-ribose and nicotinamide. Among the HDACs, these two products are unique to the sirtuins (class III HDACs). Beneath the SIRT reaction diagram, several methods are depicted for detection of either *O*-acetyl-ADP-ribose (e.g. mass spectrometry) or nicotinamide (e.g. nicotinamidase cleavage of nicotinamide to nicotinic acid and ammonia. The ammonia generated in the nicotinamidase reaction is then detected by coupling to either enzymatic (glutamate dehydrogenase) or chemical (*o*-phthalaldehyde/DTT) reactions to produce a fluorescent product (NADPH or isoindoles).

Sources of HDAC and sirtuin activity for *in vitro* assays have included intact cultured cells [13,19,20], recombinant enzymes [4,21], crude cell [22], nuclear [23] and tissue [24] extracts and immunoprecipitates [25]. HDAC8 [26], and SIRTs 1 [7], 2 [27], 3 [28] and 5 [29] have good activity when expressed as recombinants in *Escherichia coli*. However, the remaining HDACs have generally required expression in the insect cell/baculovirus system to produce recombinant enzymes with reasonable levels of activity [21].

The class IIa HDACs (4, 5, 7 & 9) have very low deacetylase activities compared to the rest of the HDACs [30–32], leading to the suggestion that their catalytic domains function more like acetyllysine-binding readers than deacetylases [32]. Moreover, it was widely suspected that the deacetylation activities detected in recombinant insect cell-expressed or immunoprecipitated class IIa preparations may have been due to contamination with class I HDACs [30,31]. This proved a serious impediment to the discovery of selective inhibitors for these enzymes, until specific trifluoroacetylated class IIa substrates were developed, first by Lahm and co-workers [30] and later by James Bradner's lab [32]. This topic will be discussed in more detail below.

In general, all class I/II/IV HDAC activity assay development must be based on measuring the rates of one of three things: (1) appearance of free acetate, (2) disappearance of the acetylated substrate or (3) appearance of the deacetylated product. The traditional approaches used in histone acetyltransferase (HAT) assays, such as MS, radioisotope detection and anti-acetylation antibodies are used, but particularly for compound screening and profiling applications, peptide-based fluorescence assays are far more popular. These methods can also be applied to the sirtuins (SIRTs or class III HDACs), but because these enzymes use NAD^+ as a cosubstrate, and produce nicotinamide and *O*-acetyl-ADP-ribose, additional assay options are available.

Mass spectrometry (MS)

MS is used more for proteomic analysis rather than as a tool for HDAC screening. However, it is playing important roles in the HDAC inhibitor discovery process. One great example is the process that Bantscheff and coworkers have used to analyze the interactions of HDAC inhibitors with protein targets from cell extracts [33]. In this study, a pan-HDAC inhibitor was conjugated with sepharose beads as a probe matrix which could bind and pull down all HDACs, plus any interacting proteins or complexes which were bound to them. Then, in parallel experiments, a specific drug of interest, at a range of concentrations, was incubated with cell extracts and the probe matrix. If the free drug competed with the bead-bound probe for binding to a particular HDAC, both it and its interacting partners would be knocked off the bead with a concentration dependence characterized by a particular IC50. Remaining bead-bound proteins were trypsinized and tagged with a distinct isobaric tandem mass tag before being pooled, analyzed and quantified by LC–MS/MS. Using this approach, 16 HDAC inhibitors were analyzed and the authors reported their distinct patterns of selectivity within the class I and class IIb HDACs and their associated complexes. A notable finding was that some inhibitors thought to be relatively nonspecific exhibit selectivity in the context of the native HDAC complexes. For example, aminobenzamide inhibitors displayed selectivity for the HDAC3-NcoR complex. Employing an adaptation of their approach for high-throughput screening, the authors identified the anti-inflammatory drug bufexamac as a class IIb (HDAC6, HDAC10) inhibitor.

MS has also been used in a more traditional HTS approach to identify HDAC inhibitors by screening of individual recombinant enzymes. Gurard-Levin *et al.* screened over 100,000 compounds against HDAC8 by using the SAMDI technique (combination of self-assembled monolayers (SAMs) with matrix assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS). The enzyme reactions were carried out in regular 384-well plates with the substrate Ac-GR-K(Ac)-FGC-NH_2, and the final reaction mixtures were transferred to the array plate that to allow the substrate/product peptides to be immobilized to the maleimide-terminated monolayers. After washing and matrix (trihydroxyacetophenone, 50 mg mL^{-1} in acetone) treatment, the plate was analyzed with an Applied Biosystems 4800 MALDI TOF/TOF spectrometer, which takes approximately 26 min to process 384 spots. The authors also screened a portion of this library (4500 compounds) with the *Fluor de Lys* (FdL) HDAC8 assay kit (Enzo Life Sciences, NY) (a protease coupled fluorescent assay discussed below) and compared the hit rate to that of the SAMDI assay. Noting the higher hit rate in the FdL assay, the authors opined that the fluorescent approach had a potential for a higher false positive rate relative to the label-free approach of MS, speculating that this might be due to the presence of the fluorophore in the FdL substrate (Ac-RH-K(Ac)-K(Ac)-AMC). Although Gurard-Levin and colleagues [34] did not present sufficient information to assess this possibility, a simpler explanation for the higher hit rate in the FdL assay than in SAMDI may lie in the assay concentrations of the two substrates relative to their respective K_m's ([SAMDI Substrate] = 10 μM, K_m not reported [34]; [FdL-HDAC8] not specified [34], K_m = 1.5 mM [35]). Issues arising from the use of fluorophore labeled substrates and trypsin will be discussed in more detail in the section on protease coupled assays.

Assay by the release of radiolabeled acetate

Shortly after the discovery that histones are acetylated on the ε-amino function of lysine residues [36–38], Inoue and

Fujimoto discovered a histone deacetylase activity in calf thymus [24]. They described an assay in which either a 'biologically acetylated' (nuclei incubated with [^{14}C] acetate) or a chemically acetylated ([^{14}C] acetic anhydride treatment) calf thymus histone preparation was used as substrate. The enzymatically released [^{14}C] acetic acid was separated for scintillation counting by acidifying the deacetylase reaction and extracting the labeled acetic acid into an ethyl acetate phase. Subsequently, histones from immature chicken erythrocytes labeled with [^{3}H] acetate became the most commonly used substrate [39] and the same extraction technique was later applied with a radiolabeled, chemically acetylated peptide [40].

Subsequent refinements of the acetate release assay eliminated the need for the ethyl acetate extraction. For example, a kit introduced by Upstate (Millipore) used streptavidin beads to separate the biotinylated, radiolabeled peptide substrate from the released, labeled acetate. With a similar substrate, Nare *et al.* used streptavidin-coated scintillation proximity beads to follow the *decrease* in signal due to deacetylation [41]. Nevertheless, radioisotope-based HDAC assays require the synthesis of ^{14}C or ^{3}H-labeled substrate and either a cumbersome separation process or acceptance of the poor signal to noise ratio of a decreasing signal assay. Currently, therefore, the most widely used HDAC assays are non-radioactive, with fluorescence as the leading mode of detection.

Antibody-based assays

In the past couple of decades numerous antibodies that recognize lysine-acetylated peptides and proteins with varying degrees of specificity have been developed and applied to detecting deacetylation activities in various formats, such as ELISA, Western Blot, etc. [42]. CisBio (Codolet, France) has also developed a few HTS-amenable deacetylase assays by using a HTRF (homogeneous time resolved fluorescence). In this assay format, a signal is generated through fluorescent resonance energy transfer between a donor and an acceptor molecule when they are in close proximity to each other. This assay format has been widely used for targets such as GPCRs, Kinases, protein–protein interaction, and recently adapted for epigenetic targets [43]. For detecting deacetylation with HTRF, the peptide substrate is labeled with biotin, which can bind with the streptavidin-XL665 conjugate (acceptor). The anti-acetyl antibody is conjugated with Europium-cryptate, which acts as the fluorescence donor. The assay is a two-step process, with the deacetylation reaction as step one, and the addition of the two conjugates for quantitation as step two. Therefore the assay is homogeneous with the simple add and read detection approach that is preferred for HTS. Cisbio has developed two peptide substrates, one which has been used with SIRTs 1 and 3, and another for the class I/II HDACs 1, 2 and 6.

Protease-coupled assays

The homogeneous, protease-coupled assay with fluorophore conjugated substrate has been the workhorse for the past decade for both HDAC and SIRT drug discovery activities (Fig. 1). In this assay format, the substrate is conjugated with a fluorophore, most commonly via an amide bond to the carboxyl function of the target acetyllysine residue itself. After an HDAC or sirtuin reaction, a developer solution containing trypsin and an inhibitor (trichostatin A for HDACs, nicotinamide for sirtuins) is added to stop the reaction and digest the deacetylated product, releasing a free fluorophore which, at the appropriate Ex/Em wavelengths has increased fluorescence relative to the amide form. Since trypsin, and other lysyl peptidases such as EndoLysC, cut on the C-terminal side of lysine, but not acetyllysine, the signal is proportional to deacetylation. The reaction is simple to perform and HTS friendly. Biomol's *Fluor de Lys*™ (FdL) assay kits (Enzo Life Sciences, NY) have been the most popular product line in this format [7,13,19,44–47]. The first generation fluorophore used in such assays was typically AMC (7-amino-4-methylcoumarin) (Ex/Em: 360 nm/460 nm). Later versions of the assay incorporated fluorophores such as rhodamine green (FdL Green, Enzo Life Sciences, NY; Ex/Em: 485 nm/528 nm), whose higher wavelength excitation and emission helps to avoid interference by quenching or fluorescence from compounds absorbing and emitting in the near UV and blue.

Other variants of these assays, while still relying on trypsin cleavage to specifically release a signaling moiety C-terminal to the deacetyled lysine, have replaced fluorescence with another mode of detection. Examples include the *Color de Lys*™ substrate (Enzo Life Sciences, NY; *p*-nitroaniline, absorbance 405 nm) *Chemilum de Lys*™ (Enzo Life Sciences, NY; undisclosed chemiluminescent group, emission 520 nm) and the HDAC-Glo™/SIRT-Glo™ assays [48] (Promega, Madison, WI; luciferin/luciferase luminescence; Fig. 2). The two luminescence assays are far more sensitive than the equivalent fluorescence assays. However, additional components are needed to produce the signals, an 'Enhancer' for *Chemilum* and luciferase/ATP for the Glo assay. These may require secondary screening to check for compound interference with the assay. Occasionally it has been suggested that inhibition of the trypsin coupling step common to all of these assays could be the source of false hits or require secondary screening [34,49]. However, for the fluorescent screening assays, compound inhibition of trypsin need be of little concern, since the trypsin can be used at concentrations of ~150–700 μM, well above the typical range of compound screening concentrations (~10–100 μM).

The original FdL substrate comprises a single lysine, linked as an amide to AMC and acetylated at both the α- and ε-amino positions (Ac-Lys(Ac)-AMC). It was deliberately designed as a simpler variant of the 'MAL' substrate

(Boc-Lys(Ac)-AMC), which Hoffman *et al.*, using an assay readout based on HPLC separation of the acetylated and deacetylated forms rather than trypsin cleavage, had shown to function as an HDAC substrate [50]. Both of these substrates are cell permeable and have been used in cell-based assays [13,51].

While the single lysine substrates perform well with an enzyme source, such as HeLa nuclear extract, rich in native deacetylase complexes, their activity with various recombinant enzymes, for example, HDAC8 and sirtuins other than SIRT1, is relatively poor. Substrates with somewhat improved activity were identified from a series of short peptides with the structure Ac-$X_{(3\text{-}4)}$-K(Ac)-AMC and based on native acetylation sites in histones H3, H4, p53 and other proteins [13,15]. The most widely applicable of these, Ac-RHK-K(Ac)-AMC ('*Fluor de Lys* SIRT1', Biomol/Enzo) is based on p53 sequence terminating in Lys382(Ac).

Substrates with the fluorophore linked to the K(Ac) carboxyl, are straightforward to design and use in trypsin-coupled assays. Note that for the Ac-RHK-K(Ac)-AMC peptide, although trypsin can cut after 'R_1' and after K_3 in this substrate, only the cut following the deacetylated K_4 produces a signal, that is, free AMC. In fluorogenic peptide substrates that include sequence on the carboxy side of the target K(Ac) these deacetylation-independent cleavage sites must be removed. For example, a group at Sirtris based a SIRT1 screening assay on the substrate Ac-EE-K(biotin)-GQSTSSHS-K(Ac)-(Nle)-STEG-K(TAMRA)-EE-NH_2 and the deacetylation-dependent fluorescence polarization decrease occurring after trypsin cleavage and the addition of streptavidin [16]. This peptide actually derives from sequence surrounding p53 Lys382(Ac), but all arginine and lysine residues, other than those with their ε-aminos blocked with acetyl, biotin or TAMRA, have been replaced with serine. Marcotte *et al.* had earlier reported a similar SIRT1/SIRT2 substrate in which the fluorescence quenching group QSY-7 takes the place of biotin and TAMRA fluorescence is increased after deacetylation and trypsin cleavage [52]. An alternative strategy is to replace all lysines, other than the acetylated target, with arginines and use the lysine-specific protease EndoLysC rather than trypsin.

Early attempts to determine the intrinsic deacetylase activity of the class IIa HDACs (HDACs 4, 5, 7 & 9), whether with an acetylated fluorogenic substrate [44] or by radiolabeled acetate release [53] were characterized by controversy and contradictory results. Some workers suggested that the apparent activity in preparations from human cells were due to associated class I HDACs, in particular HDAC3 [54,55], raising the possibility that even the reduced activity reported for class IIa enzymes expressed exogenously in insect cells [56] might be the result of contaminating endogenous HDACs [30,31].

The subsequent development of trifluoroacetylated fluorogenic substrates for the class IIa enzymes helped shed new light on this question [30,32]. Working with *E. coli*-expressed recombinant human HDAC4, Lahm and colleagues showed that its extremely low (but measurable) deacetylase activity was attributable to an active site His residue found in all class IIa enzymes, as opposed to the transition state-stabilizing Tyr residue found in the homologous position in class I enzymes. By screening fluorogenic substrates with alternative acyl groups on the lysine side chain, they found that the inherently less stable amide formed by a trifluoroacetyl group, produced a single-lysine substrate (Boc-K(TFA)-AMC) with which HDACs 4, 5 and 7 had high activity, but with which HDACs 1, 3 (class I) and 6 (class IIb) showed relatively low rates of deacylation. Using acetylated or trifluoroacetylated forms of a tripeptide substrate (Boc-LGK(Ac or TFA)-AMC), Bradner *et al.* compared the activities for HDACs 1–9 [32]. Similar to the Lahm *et al.* results [30], the class IIa enzymes only showed activity with the TFA peptide [32]. However, Bradner and colleagues also found that HDAC8 (class I), an enzyme omitted from the Lahm *et al.* analysis, was only active with the TFA substrate [32].

The Lahm *et al.* study [30] surveyed HDACs 1–3 and 4–7, whereas HDACs 1–9 were tested by Bradner *et al.* [32]. We have filled in some of the gaps in their analyses by assaying HDACs 1–11 with both Boc-K(TFA)-AMC and Boc-LGK(Ac or TFA)-AMC and comparing those activities with those obtained with the p53K382Ac substrates Ac-RHK-K(Ac)-AMC (HDACs 1–7, 9–11) or Ac-RHK(Ac)-K(Ac)-AMC (HDAC8) (Fig. 3). Note that HDAC8 has little activity with Ac-RHK-K(Ac)-AMC, while, to our knowledge, Ac-RHK(Ac)-K(Ac)-AMC is the best *acetylated* substrate of this type known for HDAC8. As Lahm and Bradner indicated in their papers, the substrates Ac-LGK(TFA)-AMC and Boc-K(TFA)-AMC are the preferred class IIa substrates, with Ac-LGK(TFA)-AMC the more active of the two. As observed by the Bradner group, HDAC8 activity is high with Boc-LGK(TFA)-AMC, but also with the Lahm TFA substrate, Boc-K(TFA)-AMC. HDAC8 activity with both of these two class IIa substrates was thousands of fold higher than with the p53 acetylated substrate and Ac-LGK(Ac)-AMC (data not shown). For class I HDACs 1–3, comparison of the p53 peptide, Ac-RHK-K(Ac)-AMC and Ac-LGK(Ac)-AMC found comparable activity. However, for the two class IIb enzymes, results were mixed, with HDAC6 having generally low activity, but best with the p53 acetylated substrate and HDAC10 displaying a preference for the Bradner class IIa substrate, Ac-LGK(TFA)-AMC.

Based on these substrate preference profiles, we typically will assay the class IIa HDACs with either of the two TFA substrates. However, our standard practice is still to assay the remaining HDACs with acetylated p53(379-382) peptide substrates, using Ac-RHK(Ac)-K(Ac)-AMC for HDAC8, and Ac-RHK-K(Ac)-AMC for all others.

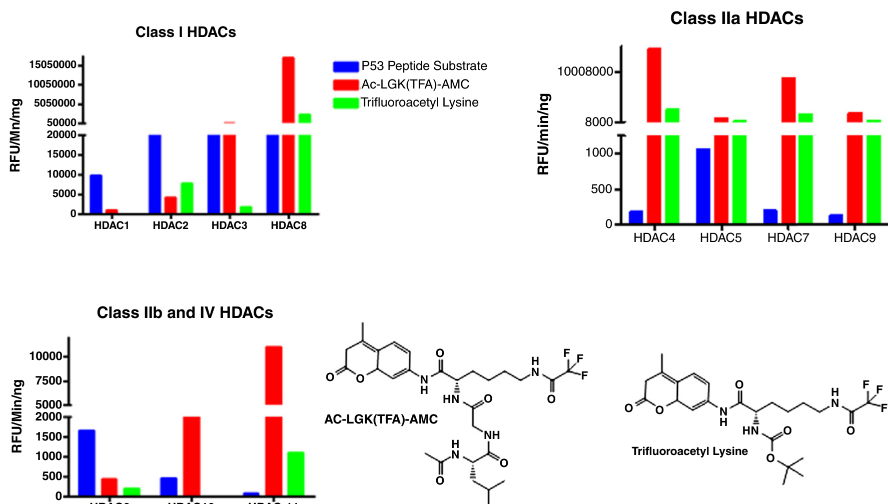

Figure 3. The relative deacylation efficiency of the class I, class IIa/b and class IV HDACs with three synthetic, fluorogenic substrates is shown. One of these, the 'p53 Peptide Substrate' (Ac-RHK-K(Ac)-AMC), is acetylated on the lysine side chain of the residue forming an amide bond with 7-amino-4-methylcoumarin (AMC). The other two substrates (structures), Ac-LGK(TFA)-AMC and 'Trifluoroacetyl Lysine' (Boc-Lys(TFA)-AMC), are conjugated to the AMC in the same way as the p53 Peptide Substrate, but each bears a trifluoroacetyl (TFA) group on the lysine side chain rather than an acetyl.

In a small screen for SIRT1 inhibitors using the p53 peptide substrate Ac-RHK-K(Ac)-AMC, resveratrol and a number of related plant polyphenols were found to stimulate SIRT1 deacetylation activity [13]. A high-throughput screen of SIRT1 at Sirtris (now a GSK company), conducted with a fluorescence polarization readout and the substrate Ac-EE-K(biotin)-GQSTSSHS-K(Ac)-(Nle)-STEG-K(TAMRA)-EE-NH_2, yielded a series of synthetic Sirtuin Activating Compounds (STACs), which, like resveratrol [13], acted to lower the peptide substrate K_m [16]. Given that the anti-aging effects of resveratrol were dependent on SIRT1's homologs in several eukaryotic model systems (yeast [13], nematodes [18], fruit flies [18]) and given SIRT1's role in metabolic regulation and possible mediation of the beneficial health effects of calorie restriction (see reviews [57,58]), these discoveries naturally elicited a great deal of excitement. However, although resveratrol's and other STACs' kinetic effects on these substrates' deacetylation were confirmed by radiolabeled [59,60] and HPLC [61] assays, it was also shown that removal of the AMC fluorophore from the +1 position (relative to K(Ac)) of the FdL substrate [59,60] or of the TAMRA fluorophore from the +6 position of the Sirtris substrate [61] eliminated the activating effects.

Curiously though, there is a large and still growing list of reports showing that resveratrol and other compounds, identified as SIRT1 activators in *in vitro* assays with fluorophore-labeled substrates, have cellular effects that are eliminated when SIRT1 is knocked down or inhibited (see [62] and references therein). Of course, the studies demonstrating substrate fluorophore-dependence for activation [59–61] suggested that such cellular (and organismal) effects might result from indirect activation of SIRT1. The mechanism of resveratrol's SIRT1-dependent stimulation of mitochondrial biogenesis has been an area of particular dispute, with the case for direct activation [62] contending with indirect explanations based on AMPK activation, possibly mediated by inhibition of cAMP phosphodiesterases and a consequent increase in the cellular level of SIRT1's cosubstrate NAD^+ [63–65]. However, recent reports have considerably strengthened the argument for direct SIRT1 activation by an allosteric mechanism [66–68]. Natural, aromatic amino acids (Trp/Phe/Tyr; W/F/Y) in the K(Ac) + 1 and/or K(Ac) + 6 positions were found to support SIRT1 activation by resveratrol and other STACs, as assayed with unmodified peptide substrates. Moreover, these activation-supporting motifs and their combined form, K(Ac)-(W/F/Y)-X_5-(W/F/Y), occur naturally >650 times in the human acetylome, including in activation-competent sequences from the known SIRT1 substrates FOXO3a and PGC-1α. Indirect explanations for SIRT1 activator effects were rendered less plausible by the finding that a resveratrol-insensitive SIRT1 mutant (human E230K; mouse E222K) which, absent an activator, is indistinguishable from wild-type in its NAD^+ and acetylated substrate kinetics, does not restore STACs-induced increases in mitochondrial biogenesis to SIRT1 KO fibroblasts, whereas wild-type SIRT1 does. Thus, at least in the context of SIRT1 and its activators, the placement of the bulky, aromatic fluorophores on these particular peptide substrates may have served as mimics of the positioning of aromatic amino acid side chains relative to targeted acetyllysines in SIRT1's

protein substrates. Finally, the recent publication of SIRT1 crystal structures, one in complex with resveratrol [67], the other in complex with a synthetic STAC [68], have made it clear that there is indeed an allosteric regulatory domain in the N-terminal region of SIRT1 that binds STACs. Moreover, the E230 residue appears to play a crucial role in the activation mechanism [67,68]. With respect to sirtuin assays, the overall upshot of the story may be that, contrary to earlier critiques [59–61] of fluorophore-labeled peptide substrates as prone to ‘artifacts’ in the detection of SIRT1 activator hits, certain fluorophore-labeled peptide substrates may actually be desirable to employ in screening for activators, since they may be serving as conveniently assayable proxies for native SIRT1 substrate motifs.

SIRT5, one of the three mitochondrial sirtuins [69], appears to be a lysine deacetylase [70,71], but also a much more active lysine demalonylase/desuccinylase [72,73]. Fluorogenic, succinylated substrates for trypsin-coupled assays, Ac-K(Succinyl)-AMC and $(Ac\text{-}K(Succinyl))_2$-RG [74] are commercially available (Enzo Life Sciences, NY). It is interesting to note that although most other recombinant HDACs (1, 4–11) and SIRTs (1–4, 6, 7) have no detectable desuccinylase activity with Ac-K(Succinyl)-AMC and although SIRT5 is >100-fold more active than the two class I HDACs that do have measurable activity (HDAC2, HDAC3/NCOR1 complex), approximately half of the activity with this substrate in a HeLa total cell extract is inhibitable with trichostatin A, a class I/class II HDAC inhibitor [74]. These results, along with the recent discovery of succinylation/malonylation on histone lysines [75] would suggest that histone lysine succinylation may be a dynamically regulated epigenetic modification, albeit one whose function is yet to be determined. Similarly, SIRT6 [76–78], SIRT2 and other sirtuins [77] have recently been shown to harbour defatty-acylation activities. These can be assayed by protease-coupled assays employing, for example, a substrate with the structure XXX-K(Myristoyl)-AMC [78].

Mobility shift assay (MSA)

MSA was developed by Caliper (a PerkinElmer company) to detect peptides that can be separated by charge ratios, such as the mixtures of phosphorylated and unphosphorylated peptides produced in kinase or phosphatase assays. Interference from fluorescent compounds is minimized due to the electrophoretic separation and direct visualization of the fluorophore-labeled substrate and product [79,80].

For HDAC assays, Caliper is offering three peptide substrates, the histone H3-based FITC-(AHA)-TGG-K(Ac)-APR-COOH, H4-based FITC-(AHA)-LGKGGA-K(Ac)-COOH and the p53-based FITC-(AHA)-TSPQPK-K(Ac)-NH_2. Fan *et al.* have also developed three 9-fluorenylmethoxycarbonyl (Fmoc)-labeled peptides, derived from p53, Fmoc–K-K(Ac)-NH_2, Fmoc–K-K(Ac)-L-NH_2, and Fmoc–RHK-K(Ac)-NH_2, for SIRT 1 and 2 assays [81]. The same group has also demonstrated that SIRT1 MSA assays can also be performed with non-peptide substrates [82].

The HDAC or SIRT reactions can be performed in a 384 well format, with only a small portion of the reaction mixture required to perform each electrophoretic separation and detection by the microfluidic chip-based instrument. The data is analyzed ratiometrically using the signals from the separated acetylated peptide substrate and the deacetylated product. The technique can be used both for end point screening assays for and time-dependent assays for kinetic studies, on- and off-rate determinations, K_i determinations, etc. The system is, however, hard to adapt for assays with larger peptides or whole protein substrates.

HDAC inhibitor binding assays

Several assays for class I/II HDAC inhibitor binding have been based on the displacement of fluorophore-labeled analogues of non-specific, hydroxamate HDAC inhibitors [49,83–86]. The principle of these assays is similar to that of the MS technique employed in the chemoproteomic study of Bantscheff *et al.* described in the Mass Spectrometry (MS) section [33], except that a signal is generated by a change in the fluorescence properties of the probe due to its displacement from the HDAC, rather than by the intensity of the MS signal from the HDAC bound to the bead-conjugated probe. Depending on the particular probe and enzyme combination, various types of fluorescence signal generation have been employed, including quenching of the intrinsic tryptophan fluorescence of the bacterial HDAC homolog, HDAH by the bound probe [83], quenching of the probe’s fluorescence when bound to HDAC8 [85], increased fluorescence polarization from the HDAC-bound probe [49] and dual increases in fluorescence polarization and lifetime from the bound probe [84]. While certain of these assays may be limited to [83] or only demonstrated with a particular HDAC [85], a recently developed TR-FRET (Time Resolved-Fluorescence Resonance Energy Transfer) assay has been successfully applied to six different recombinant class I and class IIb enzymes (HDACs 1–3, 6, 8 & 10) [86]. In this system, a europium (Eu) labeled donor reagent (Eu-anti-GST-tag or biotin-anti-His-tag plus Eu-streptavidin) is bound to the GST or His-tag on the recombinant HDAC thus allowing TR-FRET to the acceptor, one of two HDAC ‘tracers’, undescribed but each presumably consisting of a hydroxamate pan-HDAC inhibitor moiety conjugated to allophycocyanin [86]. Although such an assay does not measure deacetylation activity, it can offer certain advantages over the standard protease-coupled fluorescent assay, including the relative insensitivity of TR-FRET to interference from fluorescent test compounds, the ability to assay low activity HDACs at low enzyme concentration and the straightforward determination of slow binding inhibitor kinetics.

SIRT-specific assays

Sirtuins use oxidized nicotinamide adenine dinucleotide (NAD^+) as a cosubstrate. Since the deacetylation reaction produces nicotinamide (NAM) and *O*-acetyl-ADP-ribose (OAcAR) in equal proportions to the deacetylated lysine substrate, some sirtuin-specific assays have been based on the detection of these products (Fig. 2). For example, McDonagh *et al.* have described a microplate filtration assay in which NAD^+, radiolabeled on the NAM moiety ([carbonyl-^{14}C]NAD^+) is used as the SIRT substrate [87]. Following the SIRT reaction, unreacted NAD^+ is captured by filtration through a boronic acid resin, which does not bind the released [carbonyl-^{14}C]NAM, thus allowing its quantitation by scintillation counting. Two coupled assays for non-radioactive quantitation of the released NAM have been described [66,88]. In each of these assays NAM is hydrolyzed by a nicotinamidase to nicotinic acid and ammonia and a subsequent reaction with the ammonia is used to generate the signal (Fig. 2). Smith and colleagues developed a continuous coupled assay in which the ammonia from the nicotinamidase step plus added NADPH are consumed in a second enzymatic reaction, the reductive amination of α-ketoglutarate catalyzed by glutamate dehydrogenase [88]. While Smith *et al.* monitored the decrease of the NADPH absorbance at 340 nm [88], it would also be possible to follow the reaction by measuring the NADPH fluorescence emission at 460 nm. In the Hubbard *et al.* assay the SIRT1-generated NAM is continuously hydrolyzed by the yeast nicotinamidase Pnc1p, but then the ammonia is quantitated in an endpoint fluorescence measurement (Ex. 420 nm/Em. 460 nm) after derivatization with *o*-phthalaldehyde and DTT [66] (optimum ammonia-specific wavelengths: Ex. 413 nm/Em. 476 nm) [89]. Hubbard *et al.* also describe a SIRT1 assay based on quantitation of OAcAR with the RapidFire High-Throughput Mass Spectrometry System (Agilent, Wakefield, MA) [66]. A sensitive fluorescence assay for quantitation of the remaining NAD^+ in a SIRT reaction has been described [90]. However, its need for acetophenone, strong base, formic acid and a 110 °C incubation step in order to generate the fluorescent NAD^+ adduct, makes its HTS-friendliness somewhat dubious.

Cell-based assays

Traditional antibody-based assays such as the Western blot and enzyme-linked immunosorbent assay (ELISA) have been commonly used to detect endogenous level of acetylated lysine on Histone H3 and H4 in cell lysates [91]. More recently, several assay methods have been modified to allow for high-throughput screening of compounds inhibiting HDACs in cells. For example, AlphaLISA and LANCE assay formats from PerkinElmer have been adapted for screening of compounds against HDACs (PerkinElmer, Inc., Waltham, MA). In the AlphaLISA assay, acceptor beads are conjugated to antibodies against such targets as acetylated histone H3K9. Acetylated histone is then detected by its dual capture by (1) streptavidin donor beads linked to biotin-anti-histone-H3 (C-term) and (2) acceptor beads coupled to the antibody against H3K9Ac. Several acceptor beads for detecting different acetyled histone residues such as H3K27ac and the acetylated p53 residue p53K382Ac are available now from PerkinElmer. As noted in Protease-Coupled Assays section, the fluorogenic single-lysine HDAC substrates, Boc-Lys(Ac)-AMC and Ac-Lys(Ac)-AMC, are cell permeable and have been employed in cell-based assays [13,51]. The latter of these two is the basis of a Biomol kit now offered by Enzo Life Sciences (Farmingdale, NY) [92]. Promega (Madison, WI) has developed a similar cell-based assay in the HDAC-Glo™ format (see Protease-Coupled Assays) that employs a novel cell-permeable luminogenic substrate containing an acetylated lysine peptide, based on a histone H4 sequence, linked to an aminoluciferin [93]. The disadvantage of these fluorogenic and luminogenic assays is that the substrates used are synthetic, and not the endogenous substrates of HDACs and SIRTs in cells.

High content imaging technology has been applied to detection of the cellular effects of HDAC inhibitors. Modulation of histone acetylation by small molecule HDAC inhibitors leads to the activation of transcription of a finite number of genes [94]. It has been reported that HDAC inhibitors induce p21 expression [95]. p21 inhibits CDK1 and CDK2, which mediate the cell cycle progression at phase G1. Thus the expression level of p21 in cells can be used as a surrogate marker for the activity of HDAC inhibitors [96]. PerkinElmer reported that the potent pan-HDAC inhibitor trichostatin A (TSA) inhibits histone deacetylation and induces p21 expression in U2OS wild-type cells using the Operetta® High content Imaging System in a wide field fluorescence mode [97]. TSA treatment led to significantly higher p21 expression in the nuclei of individual cells detected by an anti-p21 primary antibody (Santa Cruz, sc-397) followed by an Alexa-Fluor®555 secondary antibody conjugate. In addition, cellular translocation technologies combined with imaging systems are in use for drug discovery against other molecular targets. Because HDAC functions are dependent on their translocation to the nucleus, those same assay formats should be adaptable for measuring HDAC translocation to allow for identification of selective inhibitors of these enzymes. Studies by Geng *et al.* showed that the class I/II HDAC inhibitor, LBH589, can act by blocking translocation of HDACs from the cytoplasm to the nucleus [98]. This would suggest that assays to measure nuclear translocation of the deacetylating enzymes could provide approaches for discovery of novel inhibitors, particularly when used in conjunction with high-throughput confocal imaging systems. This approach could provide a route to identification of compounds that inhibit via mechanisms distinct from a blockade of HDAC catalytic sites.

Conclusions

After more than a decade of intensive effort, multiple formats have been developed for the assay of HDACs and sirtuins. These include mass spectrometry, radiolabeled acetate release, detection with acetyllysine-specific antibodies, protease-coupling with fluorogenic and other types of peptide substrates, electrophoretic mobility shift, inhibitor binding/displacement and the detection of sirtuin-specific products. Current day HDAC and sirtuin drug development researchers are in the enviable position of being able to choose from amongst these formats an assay type that is particularly well suited to given screening or other applications. For example, the convenience and economy of fluorogenic, peptide-based, protease-coupled assays make them an attractive option for HTS. Alternatively, for smaller scale, cell-based applications, antibody-based assays or novel approaches involving mass spectrometry and/or inhibitor displacement may have unique advantages for the study of HDAC and sirtuin activities in the context of their native multiprotein complexes.

Conflict of interest

Konrad T. Howitz is an employee of Reaction Biology Corporation, which offers HDAC and sirtuin screening services that employ certain assay methods described in the article, specifically protease-coupled assays with fluorogenic peptide substrates.

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