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Profiling technologies for the identification and characterization of small-molecule histone deacetylase inhibitors

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Abstract

Histone deacetylases (HDACs) are promising drug targets for treating cancer, neurologic, inflammatory and metabolic diseases. Four small molecule inhibitors of HDACs have gained regulatory approval for treating lymphomas and multiple myelomas. Highly sensitive in vitro and cell-based profiling technologies have been developed to discover HDAC inhibitors (HDACi) and characterize their inhibitory potency, target-binding specificity and kinetics. In particular, proteomic profiling can define the specificity of an inhibitor at a single residue resolution. Chemoproteomic profiling can determine the potency, specificity and binding kinetics of an inhibitor on a specific HDAC complex in cell extracts. As inhibitors with new chemical scaffolds are of particular interest to improve HDAC isoform-specificity and pharmaceutical properties, effective profiling technologies will continue to have important utility. Here we briefly review recent developments of HDAC inhibitor profiling technologies and discuss distinct features of various technologies.

Introduction

Histone deacetylases (HDACs) catalyze the removal of the acetyl group from acetyl-lysine residues in diverse protein substrates. Among the 18 mammalian HDACs, 11 enzymes (HDACs 1–11), which are related to yeast Rpd3 (reduced potassium dependency-3) for class I isoforms (HDACs 1–3 and 8), and Hda1 (histone deacetylase 1) for other HDACs, depend on an active site Zn^{2+} ion for their catalytic activity, while the other seven (SIRTs 1–7), related to the yeast silent information regulator (Sir2), use nicotinamide adenine dinucleotide (NAD) as a cofactor for deacetylation (1, 2). Small molecule inhibitors of HDACs (HDACi) were identified from microbial metabolites (e.g., trichostatin A and trapoxin) (3). A chemical biology approach using trapoxin as a probe resulted in the identification of human HDAC1 (4). Trichostatin A (TSA), through its hydroxamic acid functional group (warhead), coordinates the active-site Zn2+ ion in a bidentate fashion using its hydroxamate hydroxyl and carbonyl oxygen atoms (5). Analogs of HDAC inhibitors with

functional groups similar to and distinct from natural products have been synthesized (6, 7). Thus far, three hydroxamic acid analogs (vorinostat, belinostat and panobinostat) and a cyclic peptide (romidepsin) have gained FDA approval for treating cutaneous T-cell lymphomas, peripheral T-cell lymphomas, and multiple myelomas. These FDA-approved compounds exhibit potent inhibitory activities against several HDAC isoforms (8–10). Class I enzymes HDACs 1–3 are highly expressed in cancer cells in various forms of solid tumors and are thus considered more relevant anticancer targets (11–15). Additionally, HDACs 1–3 are implicated in the pathobiology of various neurologic and neurodegenerative conditions (16–19). Therefore, there are intense interests in discovering inhibitors specific to HDACs 1–3 (20–22).

High throughput profiling technologies have been developed and refined to identify and characterize HDAC inhibitors. Here we review briefly recent developments of these technologies.

Cell-based primary and biochemical secondary assays for discovering new HDAC inhibitors

Histone acetylation is generally associated with transcriptional activation, indicating that HDAC inhibition will lead to increased transcription. This concept can be capitalized for discovering new HDACi. The adenovirus major late promoter (Ad-MLP) is a prototypical eukaryotic gene promoter consisting of well-characterized elements including a TATA box, a typical initiator site (INR) and a downstream element (DE). Ad-MLP has played an important role in elucidating the mechanisms of eukaryotic transcription (23). We found recently that Ad-MLP is strongly activated in response to HDAC inhibition using pan inhibitors such as vorinostat or class I HDAC inhibitor entinostat (MS-275, a benzamide analog) (22). Ad-MLP activation by HDACi was observed in transient transfection assays using a luciferase reporter under the control of Ad-MLP (Ad-MLP-Luc) or in stably transfected cells with the chromosomally integrated Ad-MLP-Luc. The HCT116 colorectal cancer cells stably transfected with Ad-MLP-Luc were used for screening new HDACi. Using this platform, a high throughput screen (HTS) campaign was conducted against a drug discovery library with >620,000 compounds (22). Hit compounds were validated using HDAC1 enzymatic activity assays. Known inhibitors such as vorinostat and a number of benzamides were identified as hits in this HTS. Among hits with novel chemical scaffolds, a benzoylhydrazide (UF010) was discovered. The benzoylhydrazide HDACi are selective for class I HDACs 1–3, with the highest selectivity for HDAC3 (22), providing a novel chemotype for synthesizing isoform-selective HDACi. This technology is robust and economic to implement. It is a useful platform for further discovery of new HDACi.

Biochemical profiling of HDACs

The amino acid sequence and protein structure of the catalytic domain of diverse HDACs are conserved from bacteria to humans (1, 5, 24). Amino acid substitutions during evolution result in different HDAC isoforms that display distinct substrate specificity and function. Biochemical profiling of HDAC isoforms using diverse small molecule HDACi (chemical phylogenetic analysis) can reveal surprising differences between closely related isoforms

within a phylogenetic class (8). For example, biochemical profiling indicates that HDAC8 seems to be more closely related to the class IIa HDACs, while HDAC6 appears to catalytically resemble HDACs 1–3 (class I) (8). It has been shown that the class IIa HDACs cannot deacetylate acetyllysine substrates (25–28). Nonetheless, biochemical assays show that the class IIa HDACs are potent binder of acetyllysine, providing compelling evidence that the catalytic domain of these HDACs is a potential acetyllysine "reader" (8). HTS biochemical profiling also allows for rapid establishment of the structure-function relationship (SAR) for structurally diverse HDACi. The availability of HDAC substrates that enable sensitive detection and highly active enzymes is key to implementing robust and efficient HTS biochemical profiling. Fluorogenic and luminescent HDAC activity assays have been successfully used for HTS biochemical profiling (8, 22, 29).

Chemoproteomic profiling for characterizing HDACs

HDAC inhibitors coupled to solid matrixes have been used to identify specific targets (4, 30–32). Taunton et al. linked the microbially derived cyclotetrapeptide HDACi trapoxin to agarose matrix and this affinity medium was used to identify the human HDAC1 (4). Using an active site-directed chemical probe in conjunction with photo-crosslinking for profiling HDACs in cell culture, one can isolate direct targets as well as closely associated proteins, enabling the identification of components of an HDAC complex (31, 33). Salisbury and Cravatt synthesized an HDAC active site-directed chemical probe, SAHA-BPyne, in which vorinostat (SAHA) is linked to benzophenone and alkyne moieties (31). SAHA-BPyne can be UV activated for crosslinking to HDAC target and proteins in close proximities. Biotin-azide is then attached to the probe through click chemistry to enable affinity purification. This affinity profiling technology identified HDAC1, HDAC2, HDAC6 and their associated proteins (31).

Bantscheff et al. devised a chemoproteomic competition-binding assay that can profile HDACi target in the context of specific protein complexes (30). In this assay, cell extract, rather than purified individual HDACs, is incubated with an HDACi probe immobilized to solid matrix. A solvent control or an HDACi over a range of concentrations is then added to compete against immobilized HDACi affinity probe for specific binding sites on a HDAC complex. Proteins that remain bound to the immobilized affinity probe are then captured and digested with trypsin. The resulting peptides are subject to tagging with a distinct isobaric tandem mass tag (TMT). The tagged peptides are quantified using liquid chromatography (LC) coupled with tandem mass spectrometry analysis (MS/MS). The authors labeled each peptide with six distinct TMT tags, corresponding to control and five different concentrations of a competing "free" HDACi, resulting in six reporter signals in MS/MS spectra. Increased concentration of the free HDACi will intensify the competition for binding to a specific target against HDACi immobilized in matrix. Thus the MS signal intensities of peptides derived from a target protein relative to the vehicle control will decrease. An inhibition curve can be generated for all identified proteins for determining IC_{50}. Strikingly, Bantscheff et al. could define an HDAC complex through congruent inhibition curves for components of the complex (30). This chemoproteomic profiling technology enables accurate classification of HDACi and cellular HDAC complexes. Interestingly, benzamides specific to class I HDACs were found to be inactive to inhibit

HDAC1/2-containing SIN3 complex (30), possibly due to inability to bind to the active site of the SIN3 complex (34). This chemoproteomic profiling technology has also been used to determine binding kinetics of HDACi to HDACs and corresponding complexes. Consistent with findings that benzamides display slow-on/slow-off target-binding kinetics (35, 36), benzamide analogs (e.g., entinostat) were shown to slowly bind to HDAC1, HDAC2 and their complexes. Intriguingly, they quickly bind to HDAC3 or the HDAC3-containing NCOR complex with a relatively shorter residence time compared to their very long residence times on HDAC1 and HDAC2 (34).

Proteomic profiling for characterizing HDACs

Acetylated histones and non-histone proteins are HDAC substrates. Proteomic technologies have been used to identify acetylated proteins (acetylome) (37–41). These high throughput studies have revealed diverse acetylated proteins ranging from histones, transcription factors and many proteins involved in regulating cellular metabolism. The wide distributions of acetylated proteins portrait complex roles of posttranslational acetylation in cell biology. Although substrate specificity of different HDAC isoforms has been sporadically reported, proteomic profiling combined with genetic and chemical biology approaches has the potential to catalog specific targets of individual deacetylases. Choudhary et al. (38) investigated impact of pan inhibitor vorinostat and class I HDACi entinostat on acetylome using a quantitative proteomic method (SILAC, or stable isotope labeling with amino acids in cell culture) by quantifying relative abundance of acetylated peptides in HDACi-treated and control untreated cells. This study shows that, as expected, both vorinostat and entinostat induced histone acetylation, while vorinostat, but not entinostat, increased acetylation of cytoplasmic substrates. Interestingly, both HDACi largely did not affect acetylation of mitochondrial proteins, which presumably are regulated by SIRTs (38). Surprisingly, both inhibitors affected only a small fraction (~10%) of cellular acetylome. Overall, this study reveals a clear landscape of protein acetylation and highlights the power of proteomic profiling in conjunction with HDACi chemical biology.

In a recent study, Scholz et al. profiled the inhibitory patterns for 19 HDACi with different specificity for all 18 human deacetylases. Proteomic profiles of affected acetylation sites for most inhibitors provide a rather accurate portrait of its chemotype and inhibitory specificity. For example, the benzamide analogs increased the acetylation levels for a similar subset of proteins, most of which are involved in nuclear functions such as chromatin-based processes. Likewise, the hydroxamic acid inhibitors affected a cohesive subgroup of substrates. Consistent with an early study (38), these HDACi affected acetylation of only a small subset of overall acetylated proteins (~6% of the acetylome). Surprisingly, most of the HDACi commonly used as research tools did not seem to affect the acetylation of H2B N-terminal sites (42), while the benzoylhydrazide inhibitor UF010 specific for class I HDACs was shown to inhibit H2B deacetylation (22), suggesting that inhibitors in different chemical class may have common as well as distinct HDAC targets. Notably, the SIRT1 and SIRT2 inhibitor tenovin-6 (43) affected almost exclusively acetylation of mitochondrial substrates (43), while the classical SIRT inhibitor nicotinamide inhibited deacetylation of proteins in the nuclear, cytoplasmic and mitochondrial compartments (42).

Proteomics-based profiling can resolve difference at the level of individual acetylation sites, which is not matched by other technologies. Reagents for capturing acetylated peptides with high affinity are critical for increased coverage of acetylated sites (38, 42). Whereas quantitative proteomic profiling can provide a high-resolution view of HDACi specificities, comprehensive profiling is expensive and requires a high-level of relevant expertise. Current acetylation profiling studies are limited to a few cell lines. Expanded studies on other systems will reveal whether HDACi exhibit different specificity in a cell-type and biological context-dependent manner.

Conclusions

HDACs regulate protein acetylation that impacts many aspects of cell biology. Diverse HDAC inhibitors have been used as chemical probes that have revealed critical insights into the structure and function of HDACs and their complexes. High-throughput profiling technologies have significantly advanced our understanding of pharmacology of HDACi as well as HDAC biology. Nevertheless, many outstanding questions remain. For example, current HDACi exert a surprisingly limited impact on cellular acetylome (38, 42). One possibility is that the access of an inhibitor to the catalytic core of some specific HDAC complexes may be limited. Alternatively, current inhibitors might be ineffective to inhibit the vast majority of cellular HDAC activities. Clearly, new inhibitors of distinct chemotype and target specificity will continue to play important roles in probing structure and function of HDACs. The development and application of powerful profiling technologies will not only enrich our knowledge of HDACs but will also enable the discovery of potent and safe HDACi for broadly applicable clinical applications.

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