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On the mechanism of action of small molecule therapeutics targeting histone acetylation modifiers and binders

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Members of the zinc-dependent histone deacetylase (HDAC) family of proteins are attractive drug targets for the potential treatment of various oncologic as well as non-oncologic diseases which have been linked to misregulated HDAC activities, and thus, altered transcriptional outcomes. Consequently, the development of HDAC inhibitors for epigenetic therapy has spurred a lot of scientific and medical interest reflected by regulatory approval of two HDAC inhibitors for the treatment of cutaneous T-cell lymphoma (CTCL). Likewise, the discovery of selective and potent small molecule inhibitors of the bromo and extra terminal domain (BET) proteins has drawn a lot of attention which has led to a rapidly growing understanding of the use of these compounds in a variety of disease models and in the clinic. For example, aberrant genetic alterations involving *BRD3* and *BRD4* are oncogenic drivers in NUT midline carcinoma, and pharmacological targeting of these fusion proteins is currently under clinical investigation. BET inhibitors target the tandem bromodomains of BET proteins, which are thought to be the mediator of the majority of cellular BET functions. Through binding to acetylated lysines of histones, BET proteins recruit other transcriptional co-regulators leading to transcriptional modulation.

The aim of this study was to apply a quantitative proteomics-based approach to characterize the modes of action of various HDAC inhibitors and a recently discovered selective and potent BET inhibitor in the context of cellular protein complexes. Target profiles were assessed by pursuing a strategy that employed two affinity enrichment methods as well as a newly developed assay for the unbiased assessment of chromatin targeting activities of small molecule compounds:

1. Chemoproteomics-based experiments conducted with immobilized analogs of HDAC and BET inhibitors provided insights into compound potency and selectivity.
2. Protein-protein interactions assessed by immunoaffinity purification of endogenous proteins and their interactors aided in deconvolving compound-targeted protein complexes.
3. Chromatin targeting activities of small molecule compounds were further evaluated in an assay that uses isolated chromatin as the affinity matrix and enables selectivity profiling in a completely unbiased manner.

All experiments were performed using non-denaturing conditions in settings that reproduce *in vivo* protein-protein and protein-drug interactions more accurately than using purified recombinant proteins or fragments thereof. The key new insights on the mechanism of action of small molecule therapeutics targeting histone acetylation modifiers and binders are:

- The in-depth analysis of the BET protein interaction network revealed a crucial molecular link between BET proteins and protein complexes that had previously been implicated in the pathogenesis of mixed-linage leukemia, suggesting that BET inhibitors may be efficacious in treatment of mixed-lineage leukemia.
- Potency and selectivity profiling of a recently reported BET inhibitor elucidated its selectivity against other chromatin-bound proteins in nuclear enriched fractions.
- Extensive chemoproteomics profiling of the widely used phosphoinositide-3-kinase tool inhibitor LY294002 and its lipid kinase-inactive analog LY303511, demonstrated their activity as domain-selective BET inhibitors, thus establishing a new bromodomain pharmacophore selective for BET proteins.

- Confirmation of BET inhibitor selectivity using an unbiased quantitative mass spectrometry-based assay to assess the chromatin target profile of small molecule compounds.
- The in-depth analysis of HDAC interaction networks led to the discovery of a novel HDAC1/2 protein complex termed MiDAC complex that is assembled during mitosis.
- Novel potential off-targets of approved HDAC drugs were discovered.
- Evidence was obtained for thus far unknown complex selectivity of aminobenzamide HDAC inhibitors, e.g. inability to target HDAC1/2- and Sin3-containing transcriptional repressor complexes.
- Evidence was obtained for isoform selectivity of HDAC inhibitors that were previously perceived as unselective when assayed by using recombinant proteins.

In general, this study underscored how orthogonal affinity-proteomics methods contribute to the identification of high-confidence protein interactors, and aid in analyzing compound-targeted proteins and dissecting protein complexes.