VIP Microarrays

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Fluorous-Based Small-Molecule Microarrays for the Discovery of Histone Deacetylase Inhibitors**

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Reversible acetylation plays a key role in defining chromatin states and in regulating transcription from genomic DNA differentially across distinct tissues.[1–3] Histone deacetylases (HDACs) function in this process by catalyzing the hydrolysis of *N*-acetyl groups on lysine residues found in the N-terminal tails of histone proteins.[4] This process mediates cell differentiation, correlates with epigenetic inheritance, and is deregulated in human disease, among others.[1–3,5]

Identifying novel HDAC inhibitors is an increasingly active area of research.[6–10] Trapoxin, which is a naturally occurring HDAC inhibitor, was instrumental in the original discovery of HDAC1.[4] Suberoylaniline hydroxamic acid (SAHA/vorinostat), which inhibits multiple members of the HDAC family of enzymes, has been approved recently for the treatment of cutaneous T-cell lymphoma.[11–13] Tubacin, which is the first homologue-selective inhibitor (having selectivity for HDAC6), has illuminated the function of HDAC6 and tubulin acetylation.[14–19]

Biochemical, enzyme-activity assays involving fluorescent readouts are frequently used to identify new HDAC inhibitors. However, this approach requires expensive reagents and equipment, and can be difficult to perform in a high-throughput manner. Small-molecule microarrays (SMMs) provide an attractive alternative for high-throughput identification of HDAC inhibitors. Currently, there are no reported uses of SMMs to identify new HDAC inhibitors, including ones having selectivity for specific members of the HDAC family. Traditional SMMs use various chemistries to attach compounds covalently.[20–25] Many of these approaches either take advantage of latent functionalities that result in heterogeneous molecular display on the surface, or require synthetic modification of compounds to obtain homogeneous display. Fluorous tags are versatile tagging groups for chemical-library synthesis and can facilitate noncovalent immobilization on fluorinated glass surfaces.[26–31,37] A previous report from Pohl and co-workers demonstrated fluorous microarrays as a powerful screening tool for carbohydrate-binding proteins.[26] Herein, we demonstrate that fluorous-based SMMs enable screening for HDAC inhibitors by allowing controlled molecular display of inhibitory functionality, low uniform background signals, and excellent signal-to-noise ratios.

SMMs were evaluated as a tool for identifying HDAC binders or inhibitors by using a three-part validation (Figure 1). Quantitative fluorescence data were collected from probed arrays and used to generate a list of positives. Non-fluorous tagged equivalents of the compounds were then tested in a fluorescence-based biochemical activity assay with the same set of enzymes to determine enzymatic inhibition. Furthermore, thermodynamic and kinetic binding data were collected for non-fluorous-tagged compounds binding to one of the HDACs by using surface plasmon resonance (SPR)

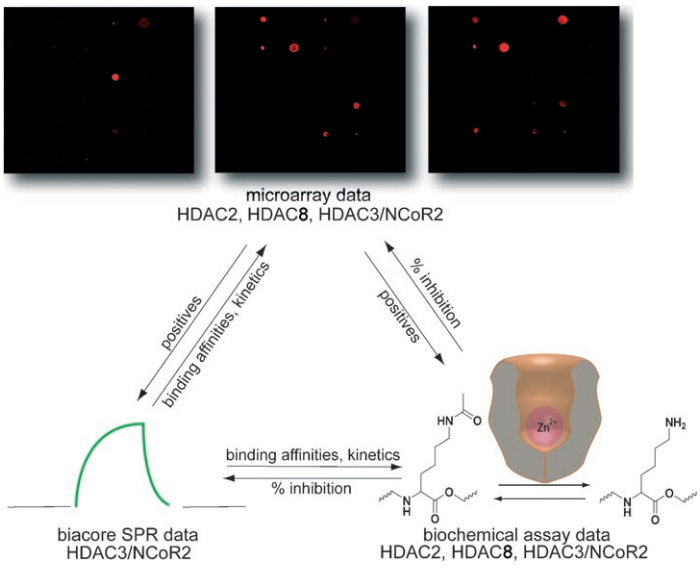

Figure 1. Experimental approach to validating the use of fluorous-based SMMs for HDAC inhibitor discovery.

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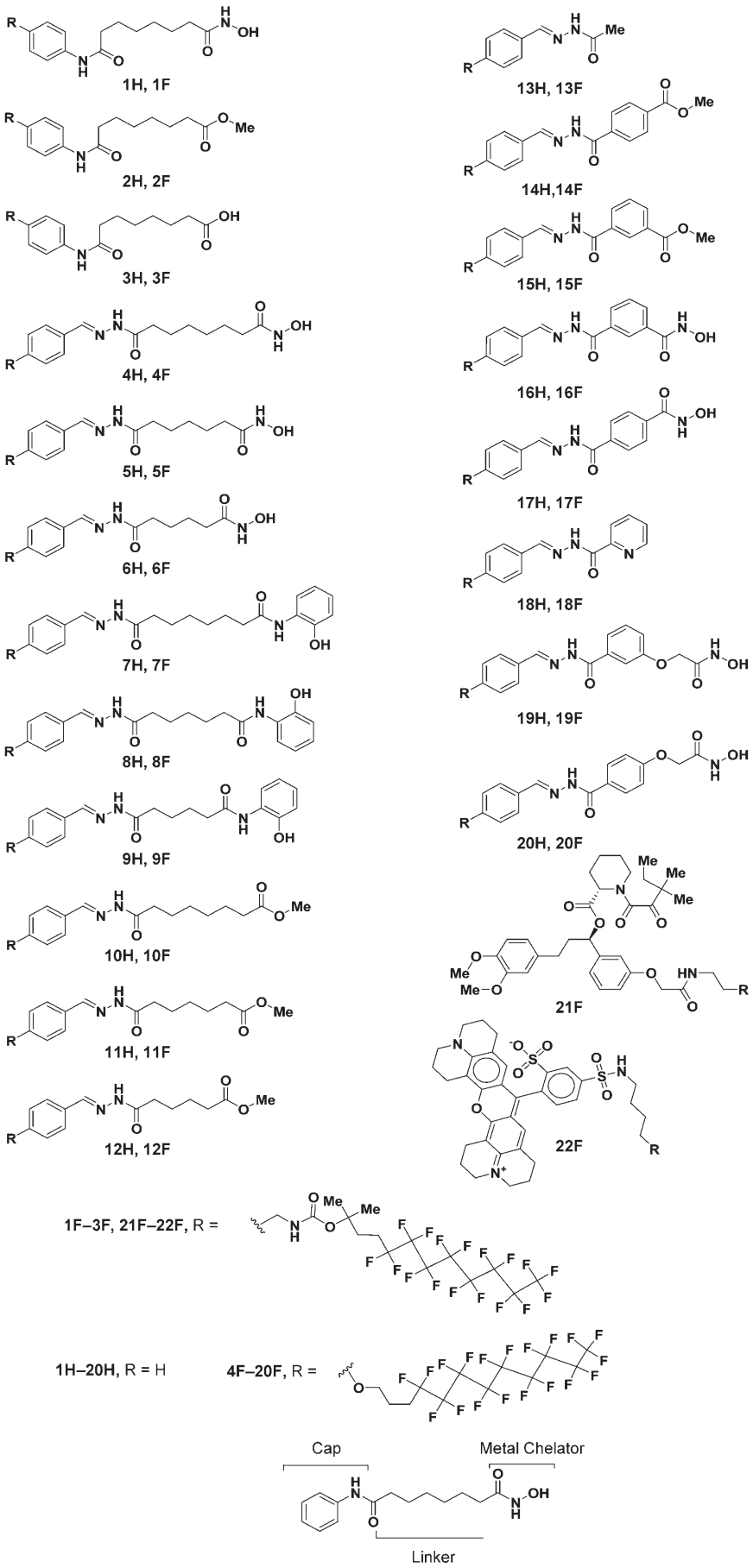
methods.[32,33] Finally, SMM binding data, biochemical activity data, and SPR data were compared to assess the accuracy of fluorous microarrays in identifying HDAC inhibitors.

Microarrays were printed with a set of 20 fluorous-tagged molecules anticipated to be a mix of active and inactive inhibitors (Scheme 1). Compounds **1F** to **3F** are fluorous-tagged SAHA analogues that serve as controls. The other 17 compounds are part of a collection of candidate HDAC inhibitors with varied linkers, metal chelators, and affinities.[34] Dimethylsulfoxide (DMSO) and a fluorous-tagged compound known to bind FKBP12 were printed as negative controls.[21] We probed the arrays with purified His-tag fusions of HDAC2, HDAC3/NCoR2 peptide complex (HDAC3/NCoR2), and HDAC8 (we have determined that we are able to assess the biochemical activity of these zinc-dependent enzymes accurately). Arrays were then incubated with an Alexa-647-labeled anti-His antibody to detect HDAC binding.

Fluorescence imaging revealed nearly identical profiles for HDAC2 and HDAC3/NCoR2, whereas HDAC8 displayed significant differences (Figure 2). Fluorescence intensity at 635 nm was measured for each printed compound feature and averaged over at least 30 replicates. Compounds displaying greater than twofold signal above background (established with DMSO controls) were classified as positives (Figure 2). Compound **1F**, a fluorous SAHA analogue, displayed almost 10-fold signal over background with HDAC3/NCoR2 and 12-fold over background with HDAC2. The low-potency free acid and methyl ester analogues of SAHA (**2F** and **3F**) showed significantly lower signal in these profiles. Eight other compounds in these two profiles also displayed fluorescence above the twofold threshold. Free SAHA was also used in a competition assay with HDAC3/NCoR2, which markedly changed the array profile (see the Supporting Information). SAHA is known to be a weak inhibitor of HDAC8, correlating with the observed weak signal of **1F** in the profile. **11F** is among the three com-



Scheme 1. Small molecules tested on microarrays, in biochemical activity assays, and with SPR assays.

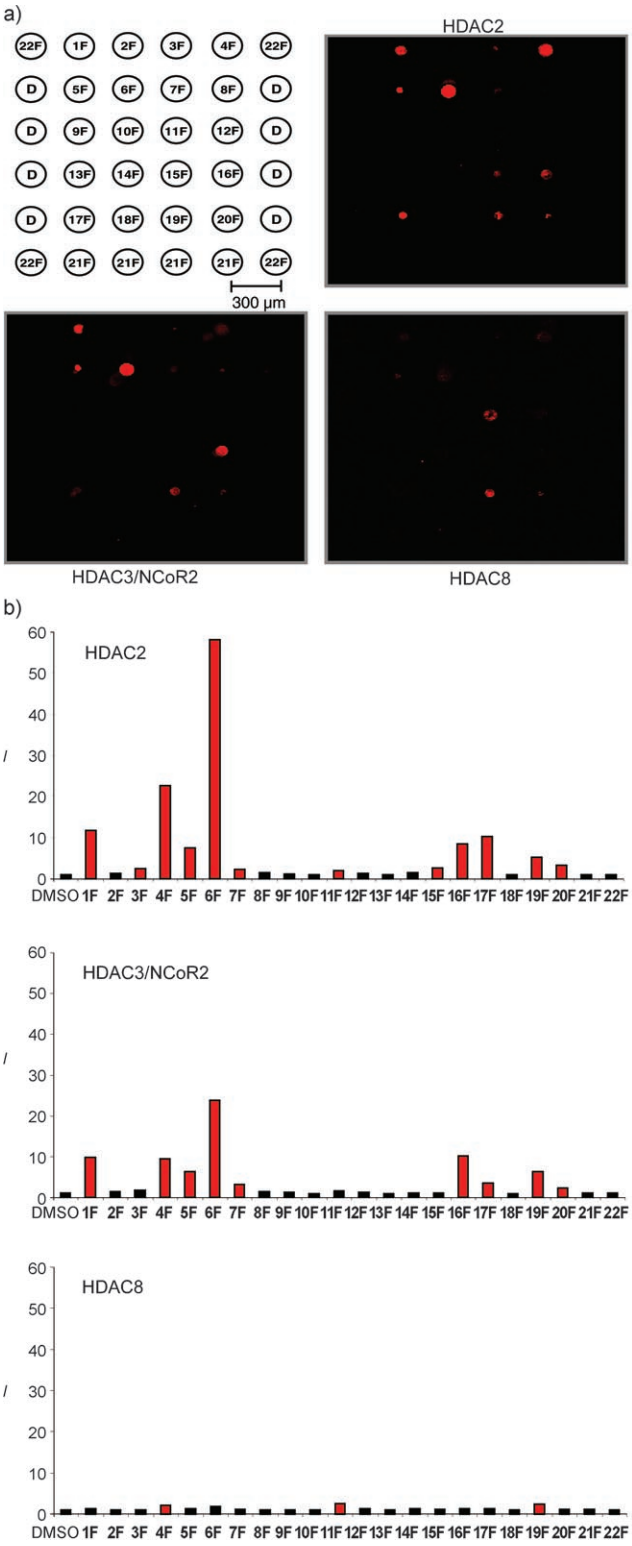


Figure 2. SMM data for HDAC2, HDAC3/NCoR2, and HDAC8: a) The arrays were probed with protein followed by an Alexa-647-labeled anti-pentaHis antibody. b) The histograms represent fold signal intensities with respect to the background signal, established from features containing DMSO only (D in array key). Values are averages of at least 30 replicates. Red bars indicate intensities that are more than twofold greater than the background signal and classify as positives.

pounds that showed significant signal over background in the HDAC8 profile.

Non-fluorous analogues of each compound (**1H**–**20H**) were then assessed for enzymatic inhibition by an established biochemical activity assay (Figure 3).[35] Ten compounds for HDAC2 and nine compounds for HDAC3/NCoR2 demonstrated 10% inhibition or greater at 333 nM. As anticipated, only compounds with metal-chelating elements, such as hydroxamates and *ortho*-hydroxy anilides proved to be effective inhibitors of these enzymes. Results from biochemical activity assays and SMM assays were congruent, with eight of ten inhibitors (80%) for HDAC2 and eight of nine (89%) for HDAC3/NCoR2 also classifying as positives on the SMMs. Compound **16H**, which demonstrated no inhibitory activity at 333 nM but whose analogue **16F** was classified as a positive, showed considerable inhibitory activity at 3.33 μM (data not shown). For HDAC8, only four compounds showed greater than 20% inhibition, with six weaker inhibitors falling between 10–20% inhibition. Unexpectedly, three of these weaker inhibitors were methyl ester analogues. Fifty percent of the strongest inhibitors (2/4) of HDAC8 also classified as positives on the SMMs, showing good agreement between the data sets.

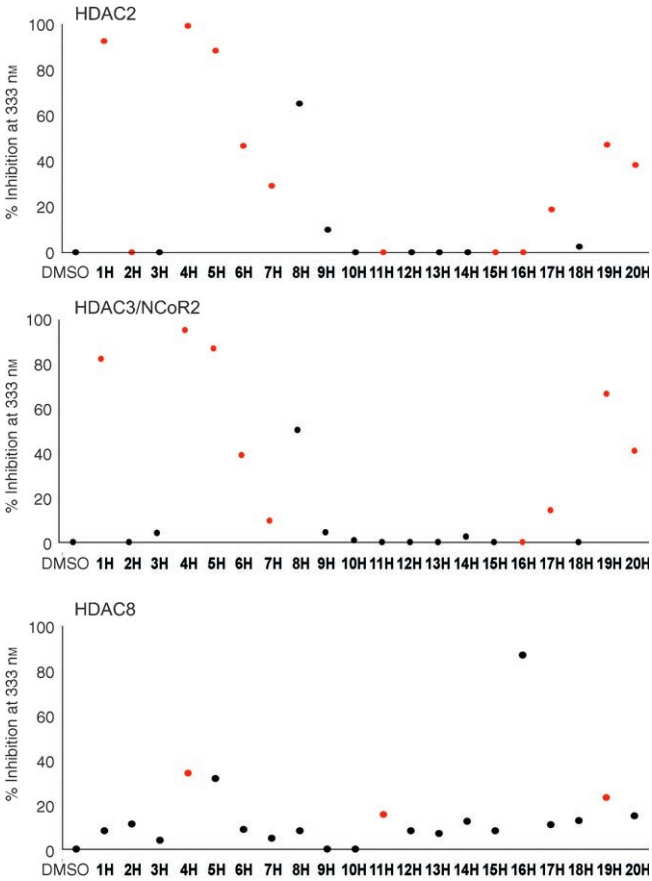


Figure 3. Biochemical activity assay data for HDAC2, HDAC3/NCoR2 complex, and HDAC8. Elements highlighted in red mark compounds classified as positives on SMMs.

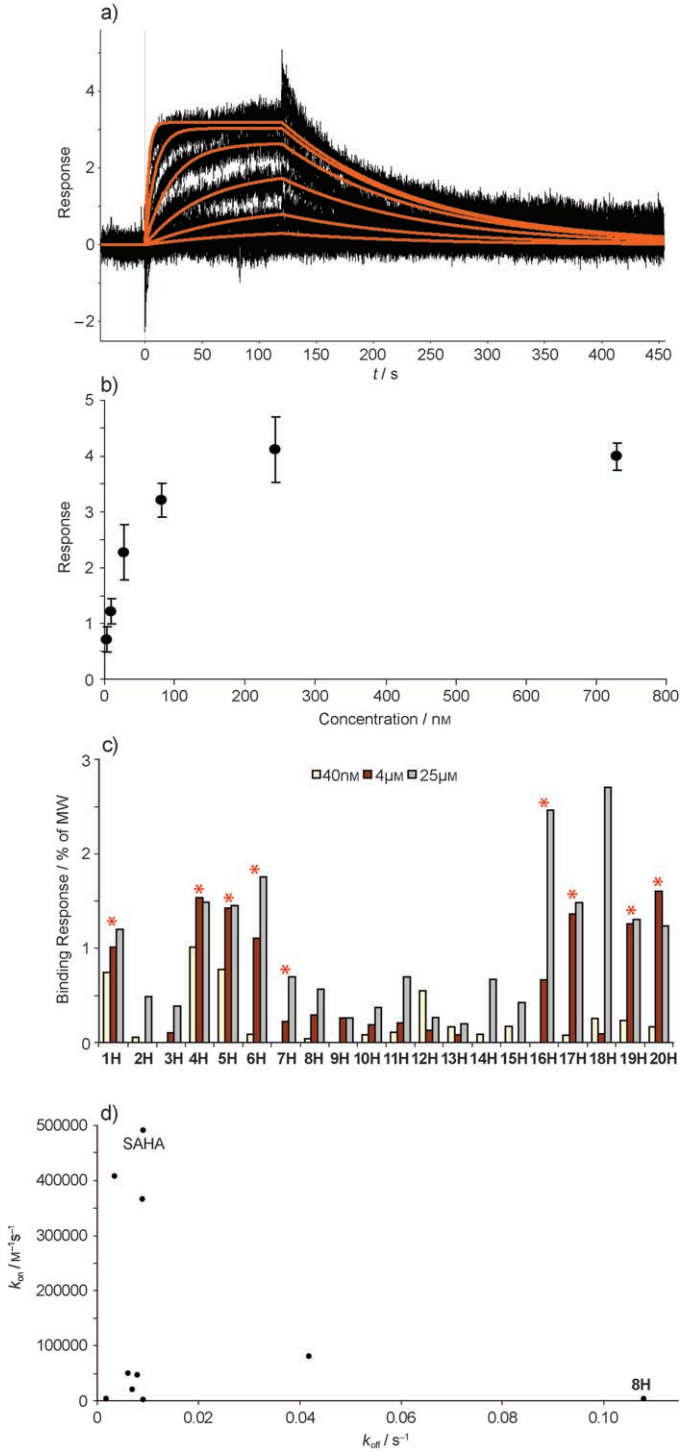


Figure 4. Compounds **1H**-**20H** were tested for binding to HDAC3/NCoR2 by using SPR methods: a) SAHA was characterized ($n=3$) by measuring binding in a dilution series (3 nM to 729 nM). Thermodynamic and kinetic analyses of these curves yielded binding constants: $k_{on}=4.9\times10^{5}\ M^{-1}s^{-1}$, $k_{off}=9.18\times10^{-3}\ s^{-1}$, $K_D=22$ nM. b) Plot of concentration versus response from SAHA dilution series used to calculate the equilibrium dissociation constant. c) Plot showing compound affinities at three concentrations. Red asterisks indicate compounds scored as positives in the SMM experiments. d) Plot of k_{on} versus k_{off} for compounds with measurable kinetics from the SPR ranking assay.

For a few compounds, the data derived from microarray and biochemical activity assays for each of the HDACs did not correlate well. To account for these differences, SPR experiments were conducted with HDAC3/NCoR2 to examine the thermodynamic and kinetic binding behavior of these compounds (Figure 4). SAHA was first rigorously characterized with HDAC3/NCoR2 to establish that the enzyme was competent for binding while displayed on the surface (Figure 4a,b). The empirically determined dissociation constant of 22 nM correlates with previously published IC_{50} values, providing confidence in the assay.[6]

The remaining 19 compounds were then evaluated at three different concentrations to rank their affinities and binding kinetics. The non-fluorous analogues corresponding to positives in the SMM experiments displayed significant binding in an SPR-based ranking assay (Figure 4c).[32–33] Compound **8H** displayed 50% enzymatic inhibition yet its fluorous analogue did not classify as a positive. We note that **8H** also had the fastest relative dissociation rate constant of the compounds tested (Figure 4d). Discrepancies between the different data sets may be explained by an inability of the microarrays to identify enzyme binders with relatively fast dissociation rates.

Previous studies have demonstrated that small-molecule microarrays can be used effectively with whole-cell lysates.[21] To test if fluorous microarrays can be used to detect native HDACs, arrays were incubated with whole-cell lysates from 293-MSR cells. Since HDAC3 is present in 293-MSR cells,[36] arrays were probed with mouse monoclonal anti-HDAC3 antibody mixed with Alexa-647-labeled goat anti-mouse antibody (Figure 5). Six of the seven positives on these

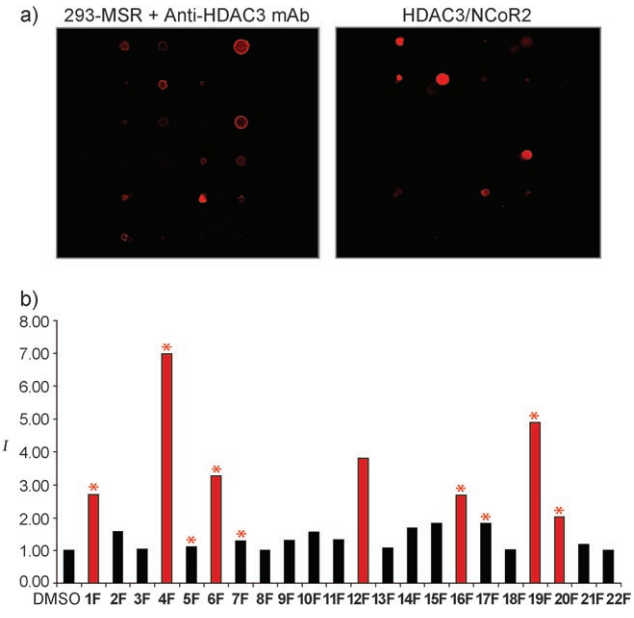


Figure 5. Small-molecule microarray data for 293-MSR cell lysate: a) Images of arrays treated with lysate and purified HDAC3/NCoR2. b) Histogram of fold signal intensities with respect to the background signal for lysate-treated arrays. Red bars indicate positives and asterisks indicate compounds that were positives with purified HDAC3/NCoR2.

arrays also classified as positives with purified HDAC3/NCoR2, showing good agreement.

In conclusion, there is a strong correlation between 1) small molecules that bind HDACs identified from fluorous-based SMMs, 2) inhibitors identified with biochemical activity assays, and 3) binders identified from SPR assays. Fluorous-based SMMs therefore offer a viable method for discovering novel HDAC inhibitors in the future. Profiles generated from these arrays against different HDAC homologs may aid in the discovery of selective inhibitors, which is a particularly important challenge in modern chromatin research.

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