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Structural Biasing Elements for In-Cell Histone Deacetylase Paralog Selectivity

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Histone deacetylases (HDACs) are the key enzymatic components of protein complexes responsible for deacetylation of histone and nonhistone protein substrates.[1] To date, 11 human HDACs have been cloned and partially characterized.[2] There is growing evidence that the acetylation state of proteins, and thus this HDAC enzyme family, plays an important role in the modulation of a number of biological processes, including transcription,[3] microtubule structure and function,[4] and the cell cycle.[5] Small molecule inhibitors of the HDACs such as trichostatin A (TSA) and trapoxin B (TPX) (Figure 1), while not paralog- or family member-selective, have already played an important role in discovering HDACs[2a] and in elucidating HDAC function.[2a,6] Therefore, the development of paralog-selective small molecule HDAC inhibitors will be a valuable tool for the characterization of HDAC paralog-specific biological functions.[6a] Here we report on the structural origins of in-cell paralog selectivity demonstrated by two 1,3-dioxanes, tubacin **1** and histacin **2** (Figure 1), which were generated by diversity-oriented synthesis[7] and identified in a cytoblot screen.[8]

1 and **2** were originally classified as selective inducers of tubulin acetylation and histone acetylation, respectively, in A549 cells.[8] Recent work by Hubbert and co-workers[9] demonstrating that HDAC6 is a tubulin deacetylase suggested to us that small molecules that selectively induce tubulin acetylation would likely be paralog-selective HDAC6 inhibitors. Extensive biological characterization of **1** has demonstrated that this is indeed the case.[10] It follows that selective inducers of histone acetylation such as **2** are, at the very least, HDAC6-insensitive inhibitors, if not potentially selective inhibitors of another HDAC paralog.

We were interested in identifying the structural basis for the in-cell paralog selectivity demonstrated by **1** and **2**. In particular, we were intrigued by the structural similarity between **1** and an HDAC inhibitor that is currently being evaluated clinically for the treatment of cancer, suberoylanilide hydroxamic acid (SAHA) (Figure 2).[11] In contrast to **1**, treatment of A549 cells with 5 μM SAHA markedly increased both levels of acetylated tubulin and acetylated histone as determined by immunofluorescence microscopy (Figure 3). This result demonstrates that the SAHA substructure alone is insufficient for HDAC6 paralog selectivity in cells and validates the use of 1,3-dioxane diversity as a selectivity generating element.[7]

These findings led us to wonder whether an analogous structural dissection of **2** would yield similar conclusions about the origin of its HDAC selectivity. Thus, pimeloylanilide *o*-aminoanilide (PAOA) **5** was synthesized in four steps from pimelic acid and tested in A549 cells. We were surprised to find that **5** demonstrates the same selectivity as **2** (Figure 4), indicating that in this case the dioxane scaffold is not necessary for selective induction of histone acetylation, but that the *o*-aminoanilide element alone may be sufficient. The selectivity of **5** was confirmed by treating A549 cells with **5** for 5 h, harvesting and lysing the cells, and then analyzing the lysates by Western blot (Figure 5).

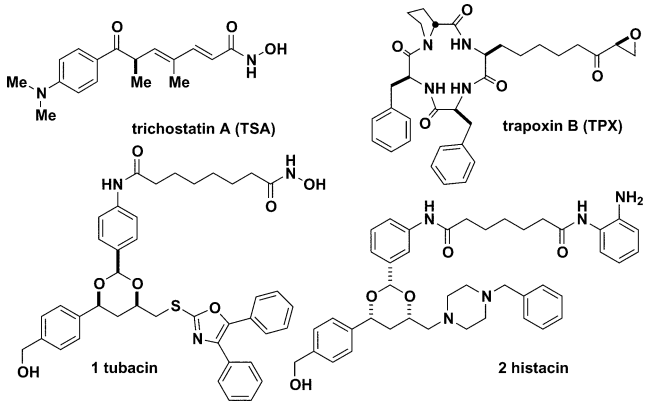

Figure 1. Trichostatin A, trapoxin B, tubacin **1**, and histacin **2**.

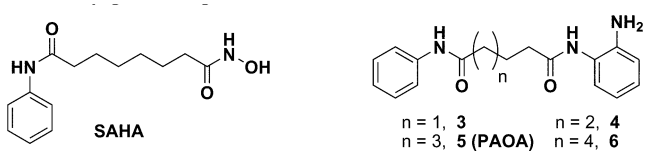

Figure 2. Structures of suberoylanilide hydroxamic acid (SAHA), pimeloylanilide orthoaminoanilide (PAOA), and several analogues.

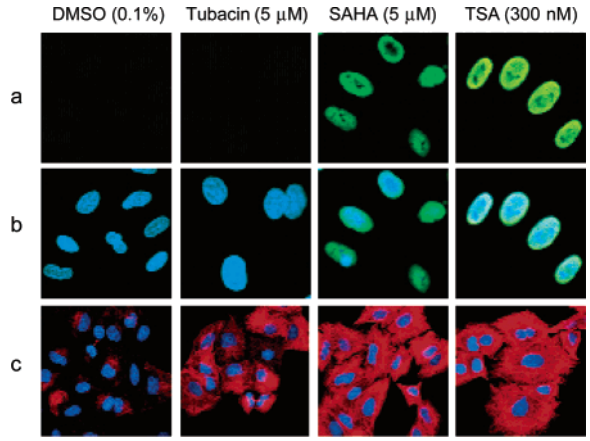

Figure 3. Immunofluorescence microscopy detection in A549 cells of acetylated histones (row a); acetylated histones and Hoechst nuclear staining (row b); acetylated tubulin and Hoechst nuclear staining (row c). Cells were treated for 14 h with the indicated compound (columns).

To explore the role of the *o*-aminoanilide as a potential HDAC paralog selectivity element further, analogues of **5** with various hydrocarbon linker lengths were synthesized and evaluated in A549 cells by immunofluorescence microscopy (Figure 6). As the linker length was increased from three to five methylene units, a corresponding increase in histone acetylation was observed. Furthermore, none of the analogues detectably induced tubulin acetyl-

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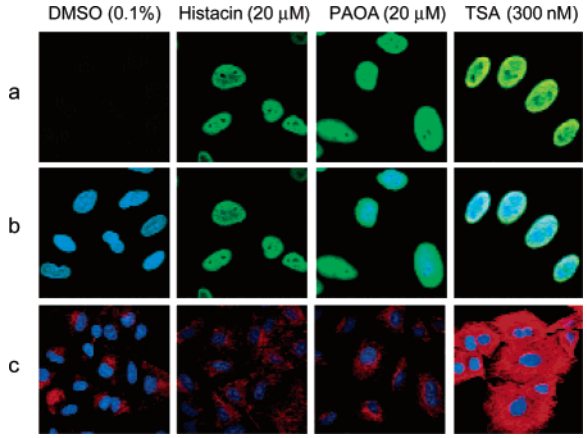

Figure 4. Immunofluorescence microscopy detection in A549 cells of acetylated histones (row a); acetylated histones and Hoechst nuclear staining (row b); acetylated tubulin and Hoechst nuclear staining (row c). Cells were treated for 14 h with the indicated compound (columns).

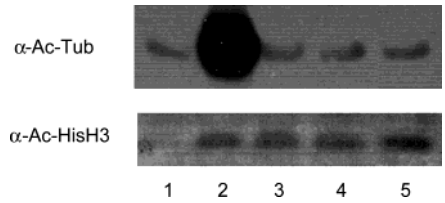

Figure 5. Western blot detection of acetylated tubulin and histone H3 levels in A549 cells after 5 h treatment with the indicated compound. (1) DMSO; (2) TSA (300 nM); (3) PAOA (20 μM); (4) PAOA (50 μM); (5) PAOA (100 μM).

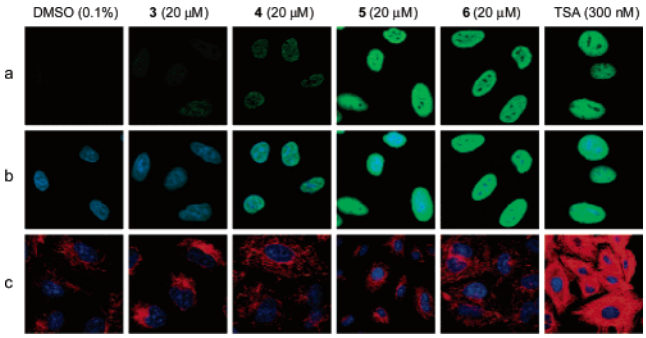

Figure 6. Immunofluorescence microscopy detection in A549 cells of acetylated histones (row a); acetylated histones and Hoechst nuclear staining (row b); acetylated tubulin and Hoechst nuclear staining (row c). Cells were treated for 14 h with the indicated compound (columns).

ation, implying that incorporation of the *o*-aminoanilide element not only biases small molecules toward HDAC inhibition[12] but also renders such inhibitors inactive toward HDAC6 in cells.

Finally, to quantify the selectivity of tubacin, histacin, and their analogues in cells, acetylated tubulin and histone induction were measured for each compound over a range of concentrations by luminescence detection in a cytoblot format (Table 1).[13]

In summary, we have used the structural dissection of two 1,3-dioxanes with in-cell HDAC paralog selectivity to identify key elements for selective HDAC inhibitors. We have demonstrated that *o*-aminoanilides are inactive toward HDAC6 while apparently

Table 1. Activity of Compounds toward Induction of Acetylated Tubulin and Acetylated Histone in A549 Cells[a]

	induction EC_{50}, μM	
compound	Ac-tubulin	Ac-histone
1 (tubacin)	2.9 ± 0.9	217 ± 96
SAHA	1.8 ± 0.5	3.6 ± 0.4
2 (histacin)	NDI	34 ± 5
3	NDI	2534 ± 985
4	NDI	249 ± 48
5 (PAOA)	NDI	36 ± 5
6	NDI	181 ± 47

[a]95% confidence interval reported. NDI = no detectable induction over the tested concentration range.

inhibiting deacetylases that act upon histone substrates. This finding has important clinical implications for the development of HDAC inhibitor-based treatments that do not interfere with microtubule dynamics associated with HDAC6.[4] We have also shown that, in contrast to **1**, the SAHA substructure alone is insufficient for in-cell HDAC6 paralog selectivity. This finding validates our use of 1,3-dioxane chemical diversity to identify paralog-selective HDAC inhibitors.[7]

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Supporting Information Available: Experimental procedures and spectral data for all relevant compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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