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Supporting Information

First Fluorescent Acetylspermidine Deacetylation Assay for HDAC10 Identifies Selective Inhibitors with Cellular Target Engagement**

Daniel Herp, Johannes Ridinger, Dina Robaa, Stephen A. Shinsky, Karin Schmidtkunz, Talha Z. Yesiloglu, Theresa Bayer, Raphael R. Steimbach, Corey J. Herbst-Gervasoni, Annika Merz, Christophe Romier, Peter Sehr, Nikolas Gunkel, Aubry K. Miller, David W. Christianson, Ina Oehme, Wolfgang Sippl, and Manfred Jung*

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Substrate conversion by HPLC

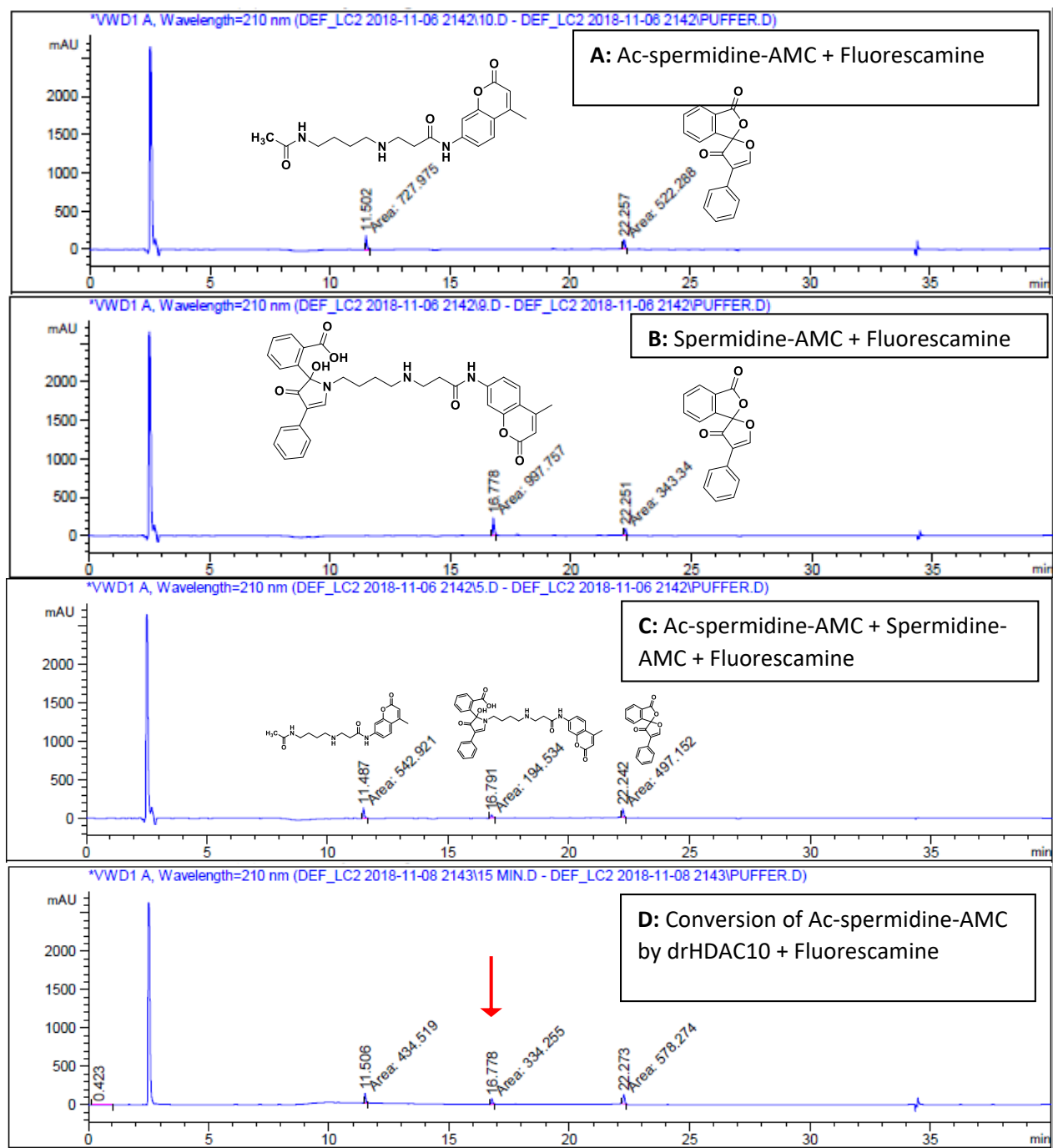


Figure S1. HPLC chromatograms showing substrate conversion. Panels **A-C**: standard solutions, panel **D**: enzymatic conversion: a new peak corresponding to the fluorescamine derivative of the product is occurring around 16.8 min (red arrow) similar to the mixture of substrate and product (**C**). Compare lane **A** as starting point and lane **B** for derivatized product only.

Linearity of fluorescence signal

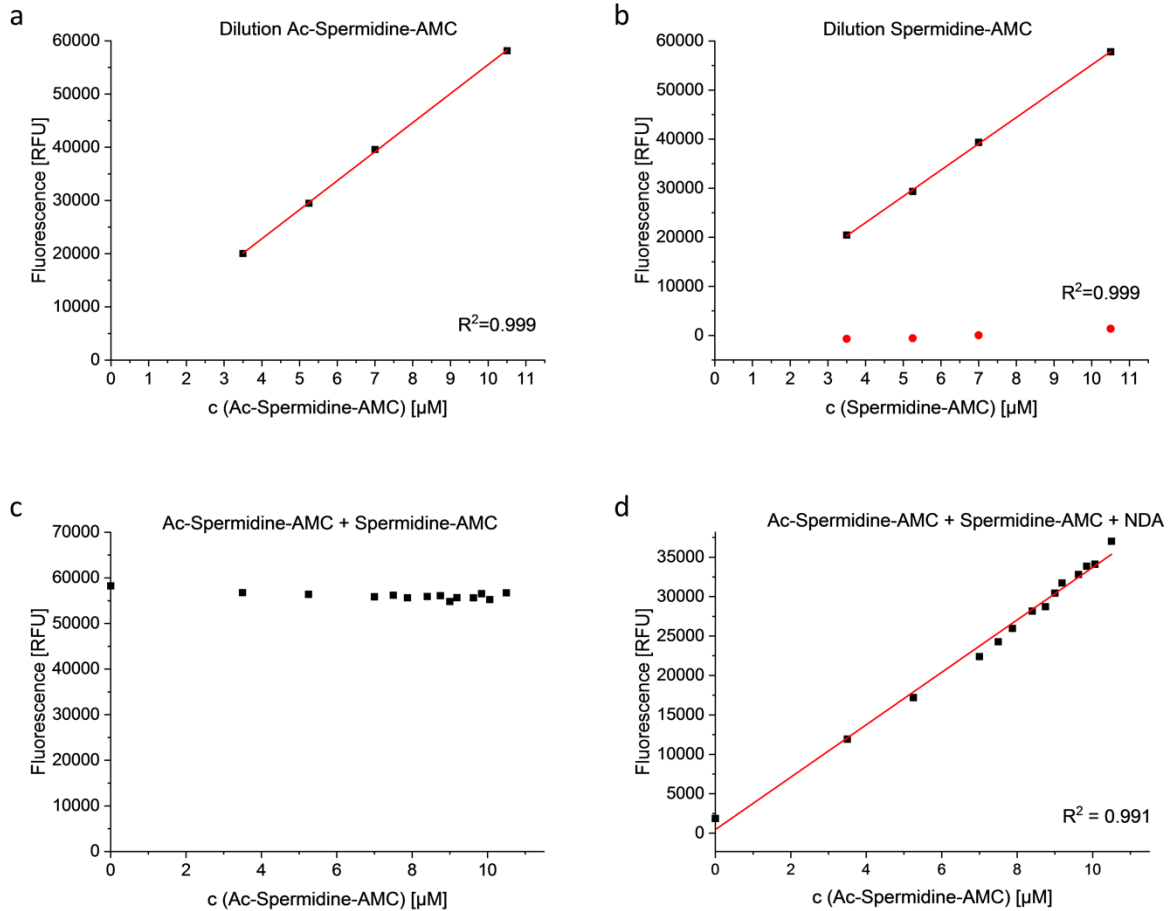


Figure S2: Linearity of fluorescence signal of Ac-spermidine-AMC and Spermidine-AMC. a) Intensities for a dilution of Ac-spermidine-AMC [10.5 μM, 7.0 μM, 5.25 μM, 3.5 μM]; b) Intensities for a dilution of Spermidine-AMC [10.5 μM, 7.0 μM, 5.25 μM, 3.5 μM], black squares before adding stop solution containing NDA, red dots after stop solution and quenching; c) complementation of AC-Spermidine-AMC with Spermidine-AMC to the same total amount of AMC-derivatives results in a stable signal; d) Simulation of enzymatic conversion by quenching of the fluorescence signal of Spermidine-AMC by adding NDA dilution to the complemented mixture of substrate and deacetylated product.

IC₅₀ curves

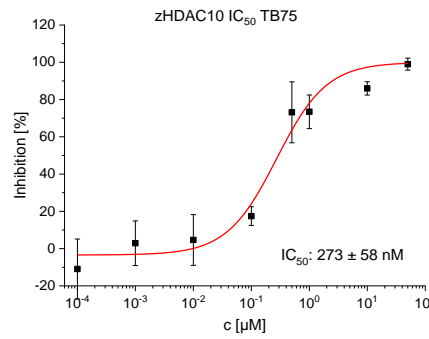
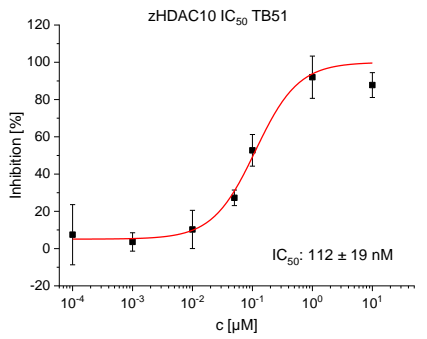
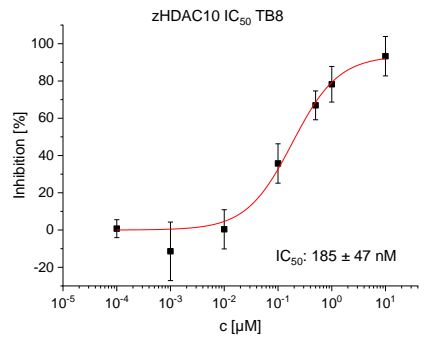
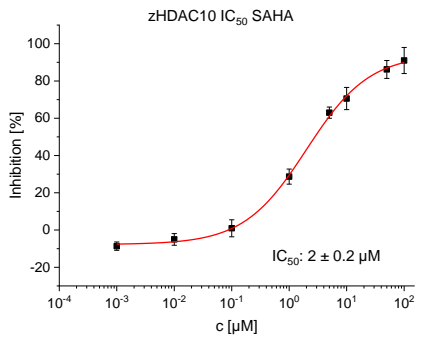
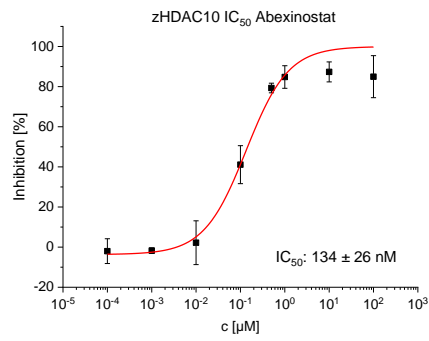
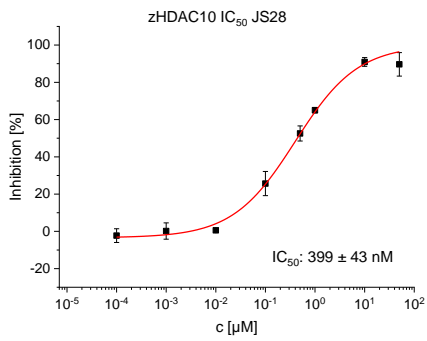
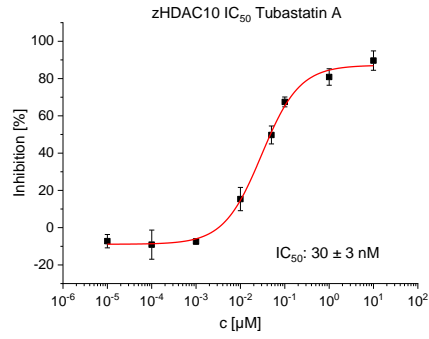
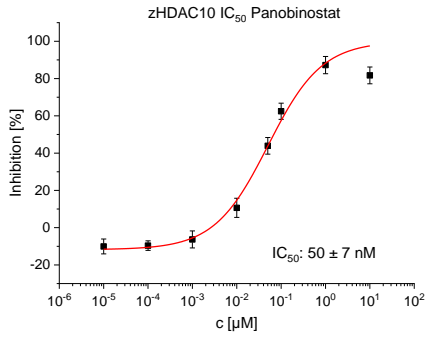


Figure S3. IC₅₀ curves for drHDAC10 of different HDAC inhibitors; one experiment performed in quadruplicate, error bars represent standard deviation of the mean, error of IC₅₀ value is shown as SEM of the non-linear regression.

Molecular docking

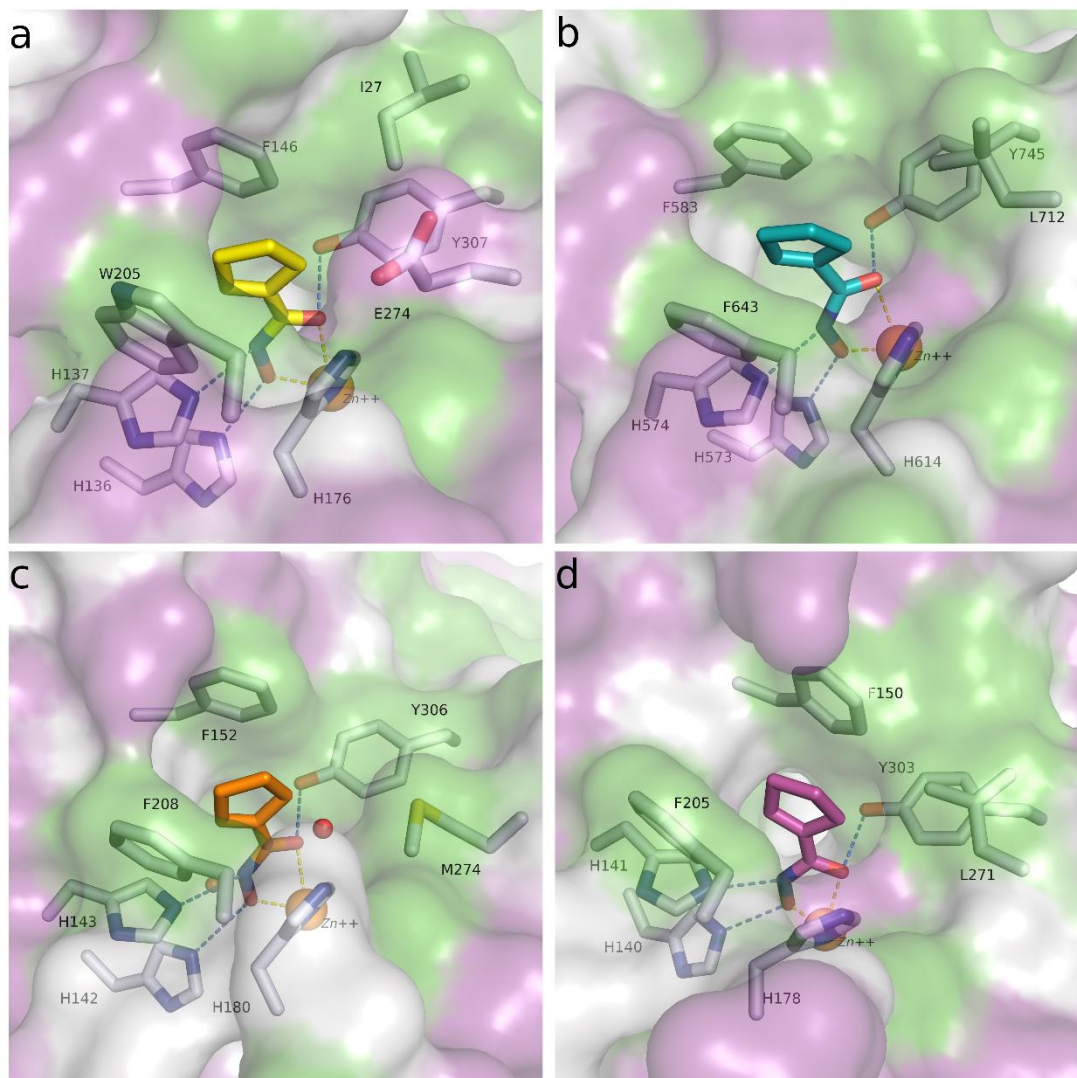


Figure S4. a) Predicted binding mode of **BRD9757** (yellow sticks) in drHDAC10 (PDB ID 6UHU), b) X-ray structure (PDB ID 6CSS) of drHDAC6 in complex with **BRD9757** (teal sticks), c) Predicted binding mode of **BRD9757** (orange sticks) in HDAC8 (PDB ID 2V5X), d) Predicted binding mode of **BRD9757** (magenta sticks) in HDAC1 (PDB ID 5ICN). The surface of the proteins is colored according to lipophilicity; green for hydrophobic and magenta for hydrophilic. Side chains of binding site residues are shown as white sticks and the catalytic zinc ion as orange spheres. Hydrogen bonds and salt bridge interactions are depicted as blue-dashed lines and coordination of the zinc ion by the ligand as yellow-dashed lines.

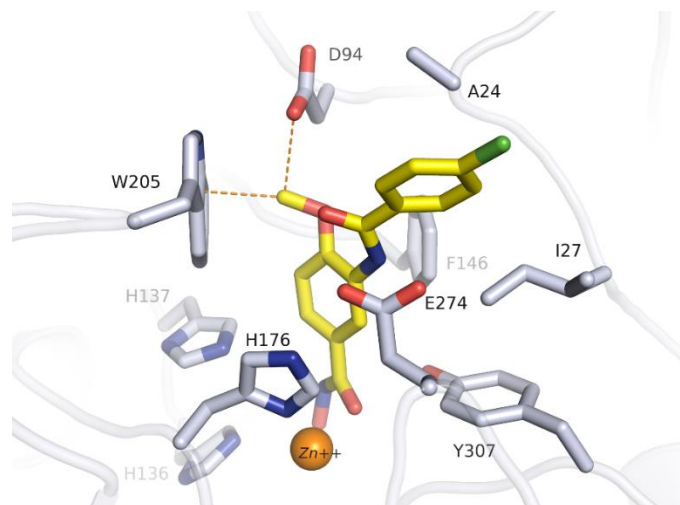


Figure S5. The obtained docking pose of the *m*-substituted benzhydroxamate TH68 (**38b**) shows clashes with residues in the binding site of drHDAC10 (PDB ID 6UHU).

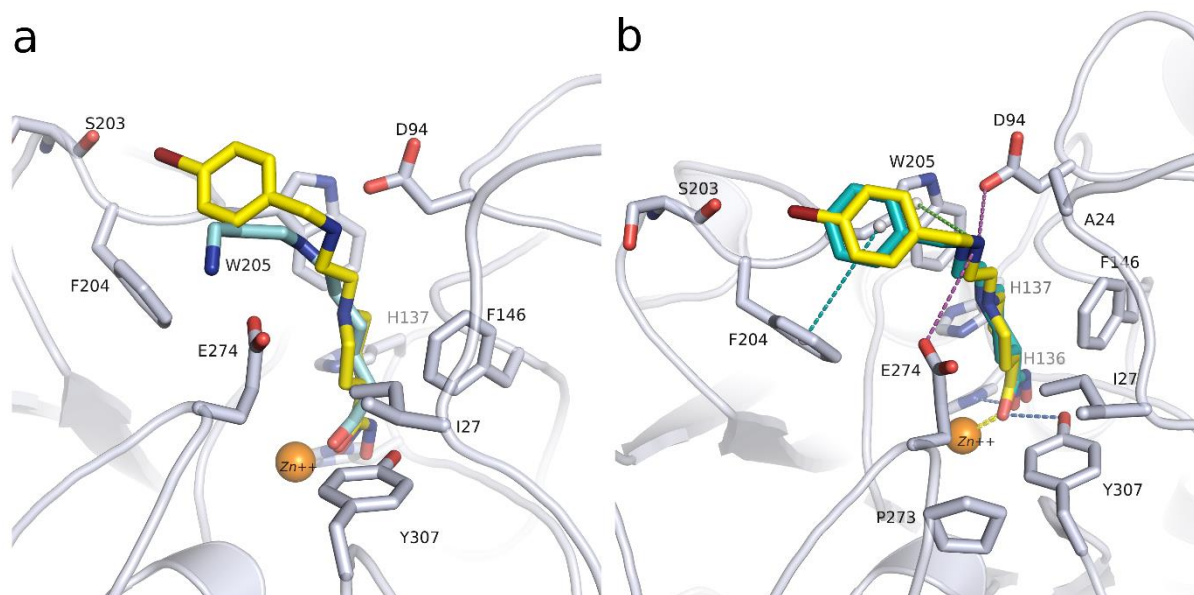


Figure S6. a) Predicted binding mode of **48b** (yellow sticks) in drHDAC10 (PDB ID 6UHU) overlapped with the experimentally determined binding mode of N⁸-acetylspermidine analogue inhibitor (cyan sticks) taken from the respective crystal structure (PDB ID 6UHV), b) predicted binding mode of **48b** (yellow sticks) and **48a** (cyan sticks) in drHDAC10 (PDB ID 6UHU). Binding site residues are shown as white sticks and the catalytic zinc ion as orange spheres. Hydrogen bonds interactions are depicted as blue-dashed lines, salt bridge interactions as magenta-dashed lines, cation- π interactions as teal-dashed lines, and coordination of the zinc ion by the ligand as yellow-dashed lines.

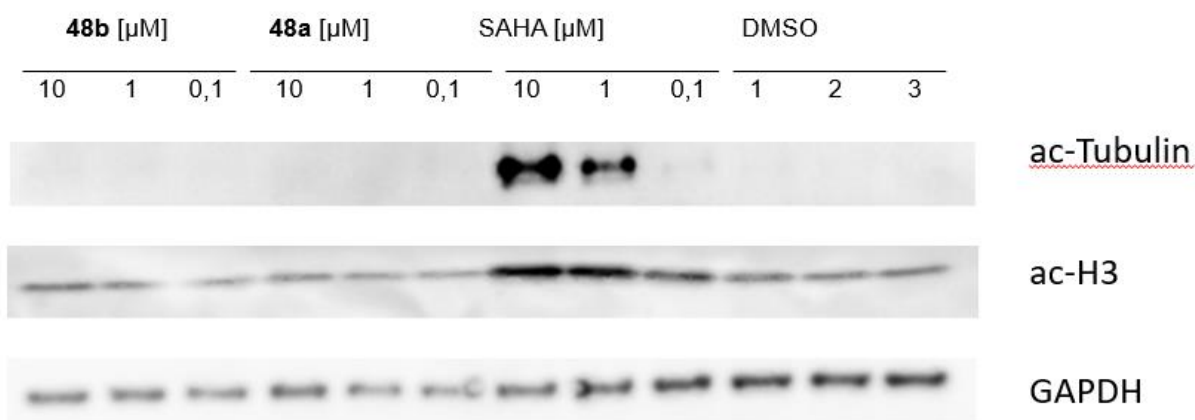


Figure S7. Acetylation level of HDAC substrate proteins as measured by Western blotting in HL60 cells. Ac-Tubulin is the prototypical HDAC6 substrate, ac-H3 the class I (HDAC1) substrate. Selective inhibitors **48a-b** do not result in hyperacetylation whereas positive control SAHA (vorinostat, **6**) results in robust hyperacetylation of both substrates.

Table S1: Dilution ratios for HPLC calibration curve

Spermidine-AMC	Ac-spermidine-AMC	Fluorescamine
0 [μM] / 0 μl	100 [μM] / 50 μl	300 [μM] / 50 μl
5 [μM] / 2.5 μl	95 [μM] / 47.5 μl	300 [μM] / 50 μl
10 [μM] / 5 μl	90 [μM] / 45 μl	300 [μM] / 50 μl
12.5 [μM] / 6.25 μl	87.5 [μM] / 43.75 μl	300 [μM] / 50 μl
20 [μM] / 10 μl	80 [μM] / 40 μl	300 [μM] / 50 μl
25 [μM] / 12.5 μl	75 [μM] / 37.5 μl	300 [μM] / 50 μl
50 [μM] / 25 μl	50 [μM] / 25 μl	300 [μM] / 50 μl
75 [μM] / 37.5 μl	25 [μM] / 12.5 μl	300 [μM] / 50 μl
100 [μM] / 50 μl	0 [μM] / 0 μl	300 [μM] / 50 μl

Table S2: Dilution ratios for determination of linearity

Ac-spermidine-AMC [μM]	Spermidine-AMC [μM]	Fluorescence [RFU]
0	10.5	186.,25
3.5	7	11923.25
5.25	5.25	17197.25
7	3.5	22397.75
7.5	3	24276
7.875	2.625	25970.75
8.4	2.1	28166
8.75	1.75	28723
9	1.5	30456.75
9.1875	1.3125	31729.75
9.625	0.875	32802.75
9.84375	0.65625	33851.75
10.0625	0.4375	34106.5
10.5	0	37021.75

Table S3. Data for determination of Z'-factor of three experiments on three different days

	Day 1		Day 2		Day 3	
	Positive control	Negative control	Positive control	Negative control	Positive control	Negative control
Mean (RFU)	38225	51427	33509	45089	37410	48455
Standard deviation (RFU)	1074	920	849	981	967	874
Z'-factor	0.55		0.53		0.50	

Table S4. Activity of the different HDAC isotypes on the pan-deacetylase substrate ZMTFAL

Enzyme [μ L/well]	Conversion of ZMTFAL [%]			
	hHDAC1	hHDAC6	hHDAC8	drHDAC10
1	15.9	3.3	-	88.5
0.5	3.1	1.9	-	65.1
0.1	0.0	0.4	100	13.4
0.01	0.0	0.0	38.7	0.3
0.005	-	-	15.0	-
0.001	-	-	3.1	-

Table S5. Conversion of the HDAC10 substrate by the different enzymes. Enzyme amounts normalized to equi-effective deacetylation of pan-substrate according to Table S4.

Subtype	Enzyme [μ L/well]	Conversion of Ac-spermidine-AMC [%]
hHDAC1	1	3.5
hHDAC6	1	5.8
hHDAC8	0.005	3.7
drHDAC10	0.1	93.3

Table S6. X-ray Crystallographic Data Collection and Refinement Statistics^a

HDAC10 Complex	HDAC10-DH79
Space group	<i>P</i> 3 ₁ 21
a,b,c (Å)	80.9, 80.9, 247.5
α, β, γ (°)	90, 90, 120
R_{merge}^b	0.139 (1.291)
R_{pim}^c	0.056 (0.506)
CC_{1/2}^d	0.994 (0.664)
Redundancy	7.0 (7.3)
Completeness (%)	99.8 (99.9)
I/σ	8.6 (2.0)
Refinement	
Resolution (Å)	53.38–2.18 (2.26–2.18)
No. reflections	49895 (4923)
R_{work}/R_{free}^e	0.180/0.210 (0.253/0.288)
Number of Atoms ^f	
Protein	4863
Ligand	51
Solvent	264
Average B factor (Å ²)	
Protein	43
Ligand	46
Solvent	44
Root-Mean-Square Deviation	
Bond lengths (Å)	0.007
Bond angles (°)	0.9
Ramachandran Plot (%) ^g	
Favored	96.23
Allowed	3.46
Outliers	0.31
PDB Entry	7U59

^aValues in parentheses refer to the highest-resolution shell of data.

^b $R_{\text{merge}} = \frac{\sum_h \sum_i |I_{i,h} - \langle I \rangle_h|}{\sum_h \sum_i I_{i,h}}$, where $\langle I \rangle_h$ is the average intensity calculated for reflection h from i replicate measurements.

^c $R_{\text{p.i.m.}} = \frac{(\sum_h (1/(N-1))^{1/2} \sum_i |I_{i,h} - \langle I \rangle_h|)}{\sum_h \sum_i I_{i,h}}$, where N is the number of reflections and $\langle I \rangle_h$ is the average intensity calculated for reflection h from replicate measurements.

^dPearson correlation coefficient between random half-datasets.

^e $R_{\text{work}} = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}$ for reflections contained in the working set. $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively. R_{free} is calculated using the same expression for reflections contained in the test set held aside during refinement.

^fPer asymmetric unit. ^gCalculated with MolProbity.