Aza-SAHA Derivatives are Selective Histone Deacetylase 10 Chemical Probes That Inhibit Polyamine Deacetylation

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ABSTRACT: We report the first selective chemical probes for histone deacetylase 10 (HDAC10) with unprecedented selectivity over other HDAC isozymes. HDAC10 deacetylates polyamines and has a distinct substrate specificity, making it unique among the 11 zinc-dependent HDAC hydrolases. Taking inspiration from HDAC10 polyamine substrates, we systematically inserted an amino group (“aza-scan”) into the hexyl linker moiety of the approved drug Vorinostat (SAHA). This one atom replacement (C→N) transformed SAHA into a nonselective pan-HDAC inhibitor into a specific HDAC10 inhibitor. Optimization of the aza-SAHA structure yielded DKFZ-748, which has a double-digit nanomolar IC50 against HDAC10 in cells and >500-fold selectivity over the closest relative HDAC6 as well as the Class I enzymes (HDAC1, 2, 3, 8). Potency of our aza-SAHA derivatives is rationalized with HDAC10 co-crystal structures and demonstrated by cellular and biochemical target-engagement, as well as thermal-shift, assays. Treatment of cells with DKFZ-748, followed by quantification of selected polyamines, confirmed for the first time the suspected cellular function of HDAC10 as a polyamine deacetylase. Selective HDAC10 chemical probes provide a valuable pharmacological tool for target validation and will enable further studies on the enigmatic biology of HDAC10 and acetylated polyamines. HDAC10-selective aza-SAHA derivatives are not cytotoxic, which opens the doors to novel therapeutic applications as immunomodulators or in combination cancer therapy.

INTRODUCTION

Histone deacetylases (HDACs), a family of 18 hydrolase enzymes that cleave post-translational acyl moieties from lysines, have received a remarkable amount of attention from biologists and chemists over the past decades, and are the primary targets of five approved drugs (1, 2). The entire family of HDAC isozymes are named histone deacetylases in analogy to the first identified HDAC, histone deacetylase 1 (HDAC1), which was confirmed to be the molecular target of two inhibitors of histone deacetylation, the natural products trapoxin and trichostatin A (3).

Histone deacetylase 10 (HDAC10) is arguably the most intriguing member of the zinc-dependent HDACs due to its unique non-epigenetic enzymatic function and still enigmatic physiological role. Isolated and characterized in 2002 by four groups independently (4–7), HDAC10 is categorized as Class IIB together with its closest relative, HDAC6, an α-tubulin deacetylase (8). The Class IIB enzymes are distinct from the Class I isozymes (HDAC1, 2, 3, 8) as they exhibit poor deacetylase activity on histones and localize primarily in the cytoplasm. Despite decades of intense research on the HDAC family, HDAC10 has received comparatively little attention by the medicinal chemistry community (9–12), resulting in a lack of appropriate pharmacological tools. Indeed, it took 15 years after its isolation before HDAC10 was shown to be a poor lysine deacetylase, which instead recognizes acetylated polyamines as substrates. As such it has been classified as a polyamine deacetylase (PDAC) (13).
While the PDAC activity of HDAC10 is established at a biochemical level, to the best of our knowledge no study has directly linked HDAC10 enzymatic activity to polyamine acetylation levels in a cellular context. Blankenship and Fries may have done so when studying polyamine deacetylase inhibition in the late 1980s, but at the time they could not identify which specific protein(s) were targeted by their inhibitors (17, 15). Since then, our understanding of HDAC10 biology and the physiological function of HDAC10’s putative polyamine substrates within the polyamine metabolome has remained limited (18, 19). HDAC10 knock-out mice are viable and develop normally without signs of disease (20). Still, HDAC10 is reported to be involved in crucial physiological processes, such as autophagy (21, 22), homologous recombination (23), DNA mismatch repair (24), angiogenesis (25), and potentially cell proliferation (26). Furthermore, HDAC10 has been reported to be involved in inflammatory disorders (27) and transplant rejection (20). However, these findings are based on expression analysis, genetic manipulation and non-selective HDAC inhibitors, due to a lack of better tool compounds. Therefore, HDAC10-selective chemical probes are needed to validate HDAC10’s role in physiology and disease and, importantly, to establish a mechanistic link between its activity as a polyamine deacetylase and the higher-level phenotypic observations made after HDAC10 genetic manipulation. Moreover, HDAC10 inhibitors (HDAC10i) have significant potential for therapeutic application in oncology (22)(28)(29) or as immunomodulators (20).

Up to now, no HDAC10i that fulfills the requirements of a selective chemical probe has been described (30). While HDAC10 inhibitors with selectivity over the Class I isozymes are known (2, 9), the primary challenge lies in achieving selectivity over the other Class IIB member, HDAC6. A good example for this is tubastatin A (1), a well-known HDAC6i, which is an even more potent HDAC10i binder (Figure 1, far left box) (9, 31). We therefore used the tubastatin A scaffold as a starting point for the development of selective HDAC10i probes. We showed that an electrostatic interaction between the tertiary amine of the tetrahydro-γ-carboline ring of 1 and the E274 gatekeeper residue in HDAC10 is responsible for its strong binding (9, 21). The gatekeeper residue is unique to HDAC10, and its negative charge provides an attractive interaction with positively charged polyamines (or HDAC10 inhibitors) within HDAC10’s binding site. Ring-opened tubastatin A derivative **DKFZ-480** (2) makes a direct hydrogen bond to E274, which results in enhanced binding affinity. While 2 has improved selectivity over HDAC6 (40-fold vs. 7-fold for tubastatin A), this derives only from increased HDAC10i affinity, with no reduction in HDAC6 potency. We therefore moved on from the tubastatin A scaffold and took inspiration from the remarkable substrate specificity of HDAC10 (32). Polyamines like N-acetylspermidine (3), N-acetylputrescine (4), and N-acetylglutamate (5) are excellent HDAC10i substrates (Figure 1, middle left box). Notably, 3-5 all contain an amine (blue) four to five carbons separated from an N-acetyl group (red). However, not all acetylated polyamines are good substrates as exemplified by N-acetylspermidine (6), an isomer of 3, which differs only by having a three-carbon amine–acetamide separation (Figure 1, middle right box). Furthermore, typical HDAC substrate peptides, which contain an acetylated lysine (7), and simple acetylamides like 8 are poor substrates of HDAC10. Equally informative for our design plans for an HDAC10i chemical probe is the fact that 3-5 are poor substrates of HDAC6. Taken together, these data suggest that HDAC10i has a narrow and unique structural requirement for substrates, which, if appropriately incorporated into an

| HDAC10/6 inhibitors | Good HDAC10 Substrates | Poor HDAC10 Substrates | Design Concept |
|---------------------|------------------------|------------------------|--------------|
| tubastatin A (1)    | N-acetylspermidine (3) | N-acetylspermidine (6) | cap group    |
|                     |                        |                        | SAHA         |
| DKFZ-480 (2)        | N-acetylputrescine (4) | Ki(Ac)-peptides (7)    | Zino-binding |
|                     |                        | N-(N-acetyl)acetamide (8) | group |
|                     |                        |                        | SAHA (9)     |
|                     |                        |                        | aza-SAHA derivatives (11) |

**Figure 1.** HDAC10 inhibitors, substrates, and design concept for a selective HDAC10i inhibitor. Far left box: Dual HDAC10/6 inhibitors (9, 14). Middle left box: Selected acetamides, which are good substrates of HDAC10. Middle right box: Selected acetamides, which are poor substrates of HDAC10. Far right box: Concept to merge the structures of SAHA (9) and 10 (15, 16) to create aza-SAHA derivatives, which should produce a potent and selective HDAC10 chemical probe. $k_{cat}/k_{M}$ values are reported for hHDAC10 and taken from Hai et al. (15).
inhibitor structure, could be leveraged to create an HDAC10-selective chemical probe.

SAHA (9), a pan-HDAC inhibitor, was the first clinically approved HDAC inhibitor (Figure 1, right box). The hexyl portion of SAHA (black) can be thought of as a lysine side chain mimic, which links the hydroxamic acid zinc-binding group (red) to the anilide “cap” group (green). Linear, diamine PDAC inhibitors like 10 have been known for decades (15, 32) and some have recently been crystallized with HDAC10 (33), further supporting that HDAC10 is the cytosolic polyamine deacetylase. We hypothesized that merging SAHA (9) and diamine PDAC inhibitors like 10 into aza-SAHA derivatives (11) would render the SAHA linker into a motif only recognized by HDAC10, effectively converting SAHA from a pan-inhibitor into an HDAC10-selective chemical probe. These compounds, containing both an amino group and a hydroxamic acid, would be highly polar, but the anilide cap group would render them more drug-like than 10, and offer a synthetic handle (R2) for optimization of physicochemical properties. Herein, we report the outcome of this study, which has resulted in the discovery of DFKZ-748, a highly selective HDAC10 chemical probe. Suitable for cellular studies, DFKZ-748 has enabled the first definitive link between cellular HDAC10 inhibition and changes in the acetylation status in the polyamine metabolome.

RESULTS AND DISCUSSION

Aza-SAHA derivatives have diverse HDAC inhibitory profiles. We began by preparing aza-SAHA derivatives, where we “walked” an amino group down the SAHA (9) carbon chain, each time replacing a methylene group and keeping the overall linker length of six atoms the same. This resulted in the β- , γ-, and δ-amino hydroxamic acids (12–14, Figure 2A). One additional compound, ε-amino hydroxamic acid 15, required extension of the linker by one atom so as not to produce a urea, essentially an “insertion” of the amino group into the SAHA linker. Due to a propensity for lactamization, especially for the δ- and γ-aminohydroxamic acids, we prepared all aza-SAHA derivatives as tertiary methylamines.

All four compounds, with SAHA (9) as a benchmark comparison, were screened for HDAC10 binding in a differential scanning fluorimetry thermal shift assay (Figure 2B). SAHA stabilized HDAC10 by 5.0 °C relative to the vehicle control, while β-amino derivative 12 gave a smaller thermal shift of 2.2 °C, indicative of weaker binding. Compared to SAHA, the γ- and δ-amines (DFKZ-711 (13) and 14) induced higher thermal shifts of 8.2 °C and 7.1 °C, respectively. ε-Aminohydroxamic acid 15, which contains a linker length like 10, gave the largest thermal shift of 11.7 °C, suggesting that it may be a potent HDAC10 binder.

In order to more accurately establish HDAC10 binding affinity and to evaluate selectivity profiles with respect to other HDAC enzymes, we tested SAHA and its aza-derivatives against HDACs 1, 2, 3, 6, 8 and 10 (Figure 2C, Table Si). As expected, our data

![Figure 2](image-url)
confirmed SAHA to be a pan-HDAC inhibitor, with moderately higher potency for HDAC1 (pIC\textsubscript{50} = 7.30) and HDAC6 (pIC\textsubscript{50} = 7.72) than for HDAC8 (pIC\textsubscript{50} = 6.16) and HDAC10 (pIC\textsubscript{50} = 6.93). The data for β-aminohydroxamic acid 12 show a nearly complete loss of activity against all isozymes, with a pIC\textsubscript{50} > 5 only for HDAC10. A three-carbon spacer (DKFZ-711), gives a 3.5-fold increase in HDAC10 activity (pIC\textsubscript{50} = 7.48) relative to SAHA. Pleasingly, it also strongly reduces activity against all other tested enzymes, resulting in HDAC10/6 and HDAC10/1 selectivities of 108- and 624-fold, respectively. The δ-aminohydroxamic acid 14 shows a noticeable decrease in affinity compared to SAHA for all measured isoforms, except for HDAC10, resulting in a moderate (<10-fold) selectivity for HDAC10 over HDAC 1, 6, and 8. Lastly, 15 has an over 65-fold higher affinity (pIC\textsubscript{50} = 8.75) for HDAC10 than SAHA, consistent with the thermal shift data. Notably, 15 shows potency similar to SAHA against HDAC 1, 2, 6 and 8, resulting overall in moderate HDAC10/6 and HDAC10/1 selectivities of 13- and 41-fold, respectively. This selectivity profile is similar to that of tubastatin A derivative DKFZ-480 (2).

Structure–Activity–Relationship (SAR) of the Linker Region. Although 15 is the most potent compound of the small aza-SAH series, β-aminohydroxamate DKFZ-711 has the best selectivity profile (34). This confirms the high potency of the previously reported ε-aminohydroxamic acid motif of 10, but reveals that a two carbon shorter amine–hydroxamate separation is superior for selective HDAC10 inhibition. We therefore decided to explore SAR around DKFZ-711. Examination of substituents other than methyl for the tertiary nitrogen (Table 1, orange box) revealed the small methyl substituent in DKFZ-711 to be the best in terms of potency and selectivity. An inverse correlation between potency against HDAC10 and steric bulk was observed (16–21), consistent with the inhibitors residing in the relatively narrow binding tunnel of HDAC10. The basicity of the nitrogen atom in the linker was also probed: 2,2,2-trifluoroethyl derivative 22 (pIC\textsubscript{50} = 5.82) showed almost 10-fold reduced HDAC10 activity as compared to ethyl derivative 16 (pIC\textsubscript{50} = 6.75). At the same time, HDAC6 and Class I activity is changed little by the electron withdrawing substituent, highlighting that a basic linker is important only for HDAC10 binding.

We also constrained the three carbon-linker between the amino group and zinc-binder hydroxamic acid group as trans- and cis-cyclobutanes 23 and 24, respectively, as azetidine 25, and as piperidine 26 (Table 1, green box). Four-membered rings all result in losses of potency and selectivity, with only piperidine 26 showing selectivity similar to lead DKFZ-711, but with reduced HDAC10 activity (pIC\textsubscript{50} = 7.03). Removal of a carbon between the amino and cap groups (27, purple box) has little effect on HDAC10 activity (pIC\textsubscript{50} = 7.54), but increases HDAC6 inhibition (pIC\textsubscript{50} = 6.08), resulting in a loss of selectivity. On the other hand, extension of the DKFZ-711 linker on the cap group side to propyl (28), significantly increases HDAC10 activity (pIC\textsubscript{50} = 8.16). At the same time, activity against all other tested isozymes decreases, resulting in about 2000- and 6800-fold HDAC10/6 and HDAC10/1 selectivities, respectively.

In addition to altering the linker length, we investigated the potential influence of the amide bridging the linker with the capping region (Table 1, gray box). Inverting the anilide in DKFZ-711 to a benzamide gave DKFZ-728 (29), which has improved HDAC10 activity (pIC\textsubscript{50} = 7.97) and decreased off-target activities with about 680- and 2800-fold HDAC6/6 and HDAC10/1 selectivities, respectively. Interestingly, 30, where the benzamide of DKFZ-728 is replaced with a benzenesulfonamide, has a similar HDAC10 activity profile to that of DKFZ-711. Also noteworthy is the 10-fold reduction in HDAC10 potency of methyl amide 31, indicating the importance of the amide NH (vide infra). We therefore employed benzimidazoles in 32 and 33 in order to provide a potentially better hydrogen bond donor. Ethyl-linked 32 exhibits a significant gain in potency (pIC\textsubscript{50} = 8.18) compared to the parent compound DKFZ-711, although the HDAC10/6 selectivity is only slightly improved to 166-fold. Propyl-linked 33 has similar HDAC10 binding (pIC\textsubscript{50} = 8.07) and reduced selectivity compared to 28.

SAR of the Capping Region. We next optimized the cap group of DKFZ-728. Compounds 28 and 33 have the highest HDAC10 potency and largest selectivity factors from Table 1; however, DKFZ-728 also has an excellent biochemical selectivity profile as well as superior cellular selectivity (vide infra). We reasoned that keeping the linker as short as possible was favorable and that cap group diversification of DKFZ-728 by amide bond formation would be simpler than for benzimidazole 33.

Structures of cap SAR compounds are shown in Figure 3, together with a graphical summary of data (red dots) as a plot of potency vs. HDAC10/6 selectivity (see Table S4 for complete assay data). Selected compounds from Figure 2 and Table 1 are also included for reference (other colors). Many derivatives had decreased selectivity, especially 2-methyl indole compounds 34 and 35. Potency, on the other hand, could be well modulated with differently sized cap groups. Fused bi- and tricyclic (hetero)aromatic ring systems provide a gain in HDAC10 potency, but often at the price of slightly reduced selectivity over HDAC6, as seen with the indazoles 36 and 37 and anthracene 38. The highest gain in potency with retention of selectivity could be achieved by naphthamide cap groups in 39–42, while biphenyl 43 resulted in a loss of selectivity and potency despite the increased hydrophobic surface area. Among the naphthamides, DKFZ-748 (41) had the best HDAC10 selectivity, superior over its regioisomer 42. We also probed the three aromatic substitution vectors of benzamide DKFZ-728 with aniline (44 and 45) and phenol functionalities (46 and 47), but no beneficial vector for additional H-bond donor/acceptors was identified. Constraining the cap group phenyl ring either in plane with the amide (47) or out of plane (48) did not show increased HDAC10 binding or selectivity over HDAC6.
Table 1. pIC$_{50}$ values of γ-amino hydroxamic acids$^a$.

| Cmpd.   | HDAC1$^b$ | HDAC2$^b$ | HDAC3$^b$ | HDAC6$^b$ | HDAC8$^b$ | HDAC10$^c$ | SF 10/6 | SF 10/1 |
|---------|-----------|-----------|-----------|-----------|-----------|-----------|---------|---------|
| DKFZ-711 | 4.68$^d$  | 4.13$^d$  | 4.11$^d$  | 5.44$^d$  | 5.27$^d$  | 7.48$^e$  | 110     | 620     |
| 16      | 4.26      | <4        | <4        | 5.25      | 5.25      | 6.75      | 32      | 310     |
| 17      | 4.40      | <4        | <4        | 5.48      | 5.65      | 6.62      | 14      | 160     |
| 18      | 4.08      | <4        | <4        | 5.26      | 5.11      | 6.38      | 13      | 200     |
| 19      | <4        | <4        | <4        | 4.89      | 5.34      | 6.16      | 19      | >500    |
| 20      | 4.06      | <4        | <4        | 5.34      | 5.13      | 6.12      | 5.9     | 110     |
| 21      | 4.62      | <4        | <4        | 6.04      | 5.64      | 5.60      | 0.36    | 10      |
| 22      | 4.71      | 4.49      | <4        | 5.23      | 5.22      | 5.82      | 3.9     | 13      |
| 23      | 4.69      | <4        | <4        | 5.54      | 4.89      | 6.32      | 6.1     | 43      |
| 24      | 4.85      | 4.59      | <4        | 5.69      | 5.10      | 6.93      | 18      | 120     |
| 25      | 4.29      | 4.09      | <4        | 5.15      | 4.71      | 6.84      | 49      | 360     |
| 26      | <4        | <4        | <4        | 5.02      | 5.14      | 7.03      | 100     | >2000   |
| 27      | 4.72      | 4.55      | <4        | 6.05      | 5.55      | 7.54      | 29      | 660     |
| 28      | 4.32      | <4        | <4        | 4.85      | 4.59      | 8.16      | 2000    | 6800    |
| DKFZ-728 | 4.50$^a$  | 4.07$^a$  | <4        | 5.02$^a$  | 4.96$^a$  | 7.97$^a$  | 890     | 2900    |
| 30      | 4.58      | <4        | <4        | 5.24      | 5.24      | 7.50      | 180     | 820     |
| 31      | 4.23      | <4        | <4        | 5.01      | 4.77      | 6.97      | 90      | 550     |
| 32      | 4.96      | 4.64      | <4        | 5.96      | 5.25      | 8.18      | 170     | 1700    |
| 33      | 4.43      | 4.30      | <4        | 4.88      | 4.98      | 8.07      | 1500    | 4300    |

$^a$ Determined by four-parameter dose-response non-linear regression from one experiment with eight dose levels in triplicates with HDAC Glo assay$^b$ or FRET assay$^c$. $^d$ Mean of two individual experiments, each conducted in triplicate. $^e$ Mean of at least three individual experiments. $^f$ Curve fit analysis not convergent at 100 nM. SF (selectivity factor) = 10$^{(pIC_{50}(HDAC10) - pIC_{50}(HDAC6 or 11))}$ rounded to two significant figures. Activity profile for the best HDAC10 inhibitors are highlighted in bold. For an extended version of this table with 95% confidence intervals, see Table S2.

Overall, the cap group modifications did not increase HDAC10/6 selectivity beyond that of DKFZ-728, 28, and 33. Still, the SAR study suggests that HDAC10 is remarkably tolerant towards differently sized and substituted (hetero)aromatic cap groups and demonstrates that physicochemical properties and potency can be modulated with different cap groups, while maintaining excellent HDAC10 selectivity, as long as the γ-amino linker is employed.
Figure 3. Biochemical potency versus selectivity plot of HDAC10 inhibitors. Cap group modifications of DKFZ-728 modulate HDAC10 potency, but do not achieve an increase in selectivity beyond that of DKFZ-728, 28, and 33. Dot size represents ligand efficiency (LE = 1.37 x pIC_{50}(HDAC10) / number of heavy atoms) (35). Colors represent from which "box" from Table 1 and Figures 2 and 3 the compounds originate: Blue dots: "aza-scan" compounds (Figure 2); Red dots: cap modifications based on DKFZ-728 (Figure 3); Grey dots: cap attachment amide modifications (Table 1); Purple dots: amine to cap amide linker length variations of DKFZ-728 scaffold (Table 1); Green dots: cyclic γ-amine linkers (Table 1). For a tabulated and extended version of this plot with 95% confidence intervals, see Table S2.
Crystal Structures of Linear HDAC10i Complexed with Humanized \textit{Danio rerio} HDAC10. In order to gain insight into the structural features of HDAC10 binding, we solved crystal structures of SAHA, 14, DKFZ-711, and DKFZ-728 bound to zebrafish (\textit{D. rerio}) HDAC10, which has been “humanized” by the introduction of two mutations near the binding site (36).

The 2.10 Å-resolution crystal structure of the HDAC10-SAHA complex (Figure 4A) reveals bidentate hydroxamate-ZnII coordination with C=O---ZnII and N=O---ZnII separations of 2.4 Å and 2.1 Å, respectively. The hydroxamate carbonyl oxygen accepts a hydrogen bond from Y307, the hydroxamate NH group donates a hydrogen bond to H137, and the ZnII-bound hydroxamate N-O group accepts a hydrogen bond from H136.

Lacking functionality, the hexyl side chain of SAHA makes no polar interactions in the active site. The NH of the anilide capping group donates a hydrogen bond to a water molecule that in turn donates a hydrogen bond to the active site gatekeeper, E274. The ZnII ligand H176 donates a hydrogen bond to E274 as well, as also observed in the HDAC10--tubastatin A complex (11), thereby forming a hydrogen bond network between H176 and the anilide NH group. Water-mediated hydrogen bond networks with H176 are also observed in other HDAC10--inhibitor complexes (11, 37).

The 2.15 Å-resolution crystal structure of the HDAC10--14 complex (Figure 4B) reveals bidentate hydroxamate-Zn II coordination with C=O---ZnII and N=O---ZnII separations of 2.1 Å and 2.3 Å, respectively. Inhibitor binding is further stabilized by hydrogen bonds between the hydroxamate carbonyl oxygen and Y307, the hydroxamate NH group and H137, and the ZnII-bound hydroxamate N–O– group and H136.

The tertiary amine installed at the δ-position of the inhibitor is protonated and hence positively charged at physiological pH. This amino group appears to be stabilized by a
weak cation–π interaction with W205 and a long-range electrostatic interaction with gatekeeper residue E274 (the N–O separation of 4.2 Å is too long for hydrogen bonding). As in the SAHA and tubastatin A structures, E274 forms a hydrogen bond with H176.

In contrast with the binding of SAHA, in which the anilide NH group makes a water-mediated hydrogen bond with E274, the anilide NH group of 14 makes a direct hydrogen bond interaction with E274. Additionally, the sidechain of E24 interacts with the benzamide C=O group, so it is possible that E24 is protonated to accommodate this interaction.

The crystal structure of the HDAC10–DKFZ-711 complex (Figure 4C) was determined at 2.25 Å resolution, revealing that only the hydroxamate N–O group coordinates to the catalytic Zn²⁺ ion with an N–O---Zn²⁺ separation of 2.0 Å. The hydroxamate C=O---Zn²⁺ separation is 2.8 Å, which is too long to be considered an inner sphere coordination interaction. The inhibitor hydroxamate group is further stabilized by hydrogen bond interactions with Y307, H136, and H137. Because the hydroxamate NH and N–O groups are equidistant to H136 (N---N and N---O separations are 2.6 Å), the directionality of bifurcated hydrogen bonding is ambiguous.

In contrast to 14, DKFZ-711 contains a tertiary ammonium group not at the δ-, but at the γ-position of the inhibitor. Even so, the tertiary ammonium group is also stabilized by a weak cation–π interaction with W205 and does not make a direct hydrogen bonding interaction with E274. However, it donates a hydrogen bond to water molecule #194 which in turn donates a hydrogen bond to E274. No long-range electrostatic interaction between the ammonium cation and E274 exists, but the gatekeeper residue forms a hydrogen bond with the Zn²⁺ ligand H176. The anilide capping group makes no direct hydrogen bonds with protein residues.

The crystal structure of the HDAC10–DKFZ-728 complex (Figure 4D) was determined at 2.20 Å resolution and reveals bidentate hydroxamate-Zn²⁺ coordination with C=O---Zn²⁺ and N–O---Zn²⁺ separations of 2.4 Å and 1.8 Å, respectively. The hydroxamate moiety makes the same array of hydrogen bond interactions with active site residues as observed in the other complexes: The C=O group accepts a hydrogen bond from Y307, the NH group donates a hydrogen bond to H137, and the Zn²⁺-bound N–O group accepts a hydrogen bond from H136.

Like DKFZ-711, DKFZ-728 contains a tertiary ammonium group at the γ-position of the inhibitor. Interactions are similar in that it appears to make a weak cation–π interaction with W205, but contrary to DKFZ-711, DKFZ-728 makes a long-range electrostatic interaction with gatekeeper E274. Here, too, E274 forms a hydrogen bond with H176. DKFZ-728 differs from DKFZ-711 in that the amide bond has been “inverted” to an anilide to a benzamide. This benzamide NH group donates a bifurcated hydrogen bond directly to E274, which is not observed for the anilide NH in DKFZ-711. Moreover, the benzamide capping group adopts a different conformation in comparison with that of DKFZ-711 as well as 14 (Figure 5).

Finally, the benzamide carbonyl of DKFZ-728 replaces a water molecule within a surface water network that is found in both 14 and DKFZ-711.

We incorporated a tertiary ammonium cation into the SAHA scaffold to mimic N'-acytelylsermidine, an excellent substrate of HDAC10. On the basis of previous crystal structures of HDAC10 bound by polyanime 10 (33) and tubastatin A (33), we expected that the ammonium cations of potent binders would directly interact with E274, the gatekeeper residue. To our surprise, the distinguishing feature of the three newly solved aza-SAHA structures is that each ammonium establishes a cation–π interaction with W205. Despite the ammonium cations being located at γ- or δ-positions relative to the hydroxamic acid, they are in approximately the same location relative to W205 in each complex, suggesting that the cation–π interaction acts as an anchor around which the molecules orient themselves (Figure 5).

In the crystal structure of 10 bound to HDAC10 (PDB 6UHV), the secondary ammonium of 10 is at the ε position and makes a bifurcated hydrogen bond with E274. Presumably, compound 15, with an ε-amine linkage like 10, also makes a direct H-bond interaction between the ammonium and E274. As seen with tubastatin A derivative 2, such a polarized H-bond to the gatekeeper residue provides excellent potency toward HDAC10; however, binding to the gatekeeper residue is apparently not sufficient for diminishing potency against other HDAC isozymes, as 15 shows little difference to SAHA with respect to HDACs 1, 2, 3, 6, and 8. This potentially provides a rationale for why we were never able to abolish HDAC6 activity in the tubastatin A scaffold. Instead, the cation–π interaction with W205 appears to be the critical feature for determining selectivity. Other HDAC isozymes lack a tryptophan at this position and presumably -711, -728, and their derivatives have unmatched electrostatics with the relatively non-polar surfaces of other HDAC isozymes (35).

These crystal structure data help to rationalize the SAR data surrounding the linker amine and cap group amide. In the case of β-amine derivative 12, the linker length is
such that it is geometrically impossible for the hydroxamic acid to bind the zinc at the bottom of the binding tunnel simultaneous to the ammonium cation making a cation-π interaction with W205, resulting in a very weak binder. It is reasonable to assume that conformationally restricted γ-amino hydroxamic acid inhibitors with amino cyclobutane (23 and 24) or azetidine linkers (25) do not allow for optimal location of the ammonium cation to engage in the cation-π interaction with W205. The important contribution of hydrogen bonding from the cap group amide NH to the gatekeeper E274 is reflected in the distinct gain of HDAC6 affinity by inversion of the linker amide from the anilide in DKFZ-711 to the benzamide orientation in DKFZ-728. Contribution of the NH hydrogen bond donor was confirmed by the nearly 10-fold loss of HDAC6 activity by methylation of the DKFZ-728 amide in 31.

In contrast to 14 and DKFZ-728, the amide NH group of DKFZ-711 does not form a hydrogen bond with HDAC6. Even so, DKFZ-711 has higher affinity and selectivity for HDAC6 than 14. Possibly, water #194 contributes to increased affinity. Structures of inactivated HDAC6 complexed with substrates reveal that 1–2 water molecules engage the secondary ammonium group of N8-acetylspermidine and the primary ammonium group of N-acetylputrescine in hydrogen bond interactions that dictate substrate specificity (37). The implicated protons on these ammonium cations point directly to water molecules that are also stabilized by hydrogen bonds with E274, much like the proton on the tertiary ammonium cation of DKFZ-711 that donates a hydrogen bond to water #194.

**Confirmation of Intracellular HDAC6 Engagement and Enzymatic Inhibition.** With an array of potent and selective compounds in hand, we tested selected substances for target engagement and selectivity over HDAC6 in a cell-based bioluminescence resonance energy transfer (BRET) assay (38). BRET data (plotted in Figure 6 and listed in Figure S3) confirms the excellent HDAC6
selectivity of the γ-amino hydroxamic acid motif, verifies our SAR data, and proves cellular target engagement. Some compounds like 28 showed reduced potency and selectivity in the cellular assay format, while inhibitors with large, hydrophobic cap groups like DKFZ-748 and 38 perform well in both the biochemical and cellular HDAC10 assay. Most amino hydroxamic acids show only 10–20% of their biochemical activity in the cellular BRET assay. This can be attributed to the high polarity, and resulting moderate cell permeability of the compounds (Table S4). Less polar HDAC10 binders like SAHA or tubastatin A retain over 50% of their biochemical potency in the cellular assay system (Table S3). We selected DKFZ-728, DKFZ-748 and 37 for further biological testing due to their high selectivity, high cellular target engagement, and good ligand efficiency.

Selective HDAC10 inhibitors have no effect on Histone and Tubulin Acetylation, and Lack Cytotoxicity. For further confirmation of HDAC10 selectivity, we investigated the effects of our inhibitors on the acetylation status of potential off-targets by Western blot in the human neuroblastoma BE(2)-C cell line, using SAHA and tubastatin A as reference compounds (Figure 7A). Little to no increase in acetylation of the HDAC1 substrate histone H3 is detected even at 100 μM DKFZ-728, about 40-fold above the concentration required for complete cellular HDAC10 target occupancy (~2.5 μM). We also observe low effects at 10 μM with the more potent inhibitors DKFZ-748 and 37. Similar results were obtained for the HDAC6 target α-tubulin: while tubastatin A and SAHA treatment both resulted in hyperacetylation of tubulin at low micromolar concentrations, aza-SAHA inhibitor doses sufficient for full cellular HDAC10 inhibition did not affect tubulin acetylation. Only the highest tested concentration of DKFZ-748 (100 μM) induced significant acetylation out of the three aza-SAHA derivatives.

Previously, we measured acidification of the lysosomal compartment, using a pH-dependent fluorescent probe, as a marker for cellular HDAC10 interference (9, 39), due to the involvement of HDAC10 in autophagy. Surprisingly, the potent and highly selective HDAC10i DKFZ-728 did not lead to lysosomal acidification (Figure 7B), a phenotype observed after HDAC10i knockdown and with comparably potent, but less selective HDAC10i binders like many tubastatin A derivatives (9) or the pan inhibitor SAHA. Another unexpected observation with our selective HDAC10 inhibitors is a lack of growth inhibition or cytotoxic effects in HEK293T, HeLa, and BE(2)-C cells (Figure 7C). Although DKFZ-728 is stable in cell culture with BE(2)-C cells for at least 72 h (Figure S1), we observed no reduction in cell viability linked to HDAC10 inhibition.
This is particularly surprising, since our selective HDAC10 inhibitors differ from the cytotoxic SAHA only by an appropriately placed amino functionality, and still show comparable intracellular target engagement in a cellular assay as well as acceptable ADME profiles (Table S4). To expand beyond the three cell lines we had tested, one-dose NCI-60 screening was performed with DKFZ-748 at 10 µM, which resulted in an average viability of 99 ± 8% over all 60 cell lines with the lowest 10th percentile covering 72–91% viability (for complete data see SI). We also observed no cooperative toxicity in BE(2)-C cells by co-treatment of 5 µM 37 with the Helmholtz Drug Repurposing Library, a collection of over 5000 pharmaceuticals and bioactive compounds, covering a wide range of biological targets. After manual curation of hits, we tested 12 drugs with synergistic effects in the screen, but none of these hits could be reproduced and validated (see SI).

**Selective Inhibition of HDAC10 Leads to Spermidine N^4-Hyperacetylation in Cells.** The fact that our selective HDAC10 binders induce no proliferative phenotype in cells, in spite of demonstrated cellular target engagement, begged the question whether they inhibit HDAC10 enzymatic activity. To our knowledge, there is no report on the enzymatic inhibition of native cellular HDAC10 since its classification as the cytosolic polyamine deacetylase. We therefore employed a targeted metabolomics LC-MS/MS approach to measure cellular acetylated spermidine species: N-acetyl-, N^6-acetyl- and N^1,8-diacytlylspermidine were quantified in BE(2)-C cells treated with DKFZ-748, inactive control ester 49 (see Table S2), or vehicle after 24 h (Figure 8A and Figure S2). We observed significant accumulation of N^6-acetyl- and N^1,8-diacytlylspermidine in a dose-dependent manner with DKFZ-748, but no accumulation of N-acetylspermidine. Effective dose-levels align with the pIC_{50} for cellular target engagement (BRET pIC_{50} of DKFZ-748 = 7.66), and the inactive amino ester 49 has no effect on acetylspermidine levels. HDAC10 knockdown resulted in a similar metabolic profile (Figure 8B) with significant accumulation of only N^6-acetyl- and N^1,8-diacytlylspermidine. In contrast to pharmacological inhibition, HDAC10 knockdown decreased N-acetylspermidine relative to the control, which might be a result of regulatory processes of polyamine metabolism involving the HDAC10 protein. Notably, these data confirm the previously reported (13) substrate specificity of recombinant HDAC10 in cellulo, since only the two polyamines with a four carbon spacer between the acetamide and the amine accumulate upon inhibitor treatment.
CONCLUSIONS

We report the rational design of HDAC10 inhibitors with unprecedented selectivity over HDAC6 and Class I iso-
ymes. Compounds with an amino functionality inserted into the linear linker region of the pan-inhibitor SAHA
have distinct HDAC isozyme selectivity profiles depending on the location of the amino group. While linker variations
resulted in a dispersed selectivity profile, cap group modi-
fications based on the HDAC10-selective linker resulted in
a mostly clustered SAR. We identified the flexible, slender γ-amino hydroxamic acid scaffold as the driver for HDAC10
selectivity, which is enhanced by an appropriately located
hydrogen bond donating NH bridging the linker to a flat,
bicyclic hydrophobic capping region. Structural insights from HDAC10-inhibitor complexes, together with the
SAR study paint a clear picture of the HDAC10 active site and provide a good understanding of the struc-
tural features for potent and selective HDAC10 in-
hibition. Definitive explanation of why the γ-amino hydroxamic acid scaffold so dramatically reduces Class I and
HDAC6 affinity is a matter for further investigations; how-
ever, our SAR study suggests that an amino functionality
close to the catalytic center is generally detrimental for
HDAC binding, but HDAC10 is special in this regard, since
it can tolerate an ammonium ion closer to the catalytic
center than any other isozyme and still benefit from a weak
cation–π-interaction with W205.

We demonstrate potent HDAC10 binding biochemically
with a target engagement FRET assay (best inhibitors with
pIC50 > 8) and by thermal shift of recombinant HDAC10.
Intracellular target engagement is shown with a BRET as-
say (best inhibitors with pIC50 > 7.5) and cellular inhibition
of HDAC10 specific PDAC activity is confirmed by targeted
metabolomics of acetylated spermidines. Over 500-fold
HDAC10/6 selectivity for the best inhibitors is observed in
cell-free and cellular target engagement assays. Selectivity
over Class I enzymes is measured to be several 1000-fold in
a biochemical assay format and is confirmed by Western
blotting against histone acetylation. Selectivity over HDAC6
is confirmed by Western blotting against α-tubulin
acetylation. Therefore, we conclude that γ-amino hydroxamic
compounds, like DKFZ-748, fulfill the requirements of a
selective chemical probe (40) for HDAC10 and are
valuable tool compounds to investigate HDAC10 biol-
ology and the role of acetylated polyamines. Furthermore,
we believe this scaffold can be utilized as a platform to de-
velop covalent and multifunctional chemical probes spe-
cific for HDAC10.

Selective HDAC10i exhibit surprisingly low cytotoxicity
in cancer cell lines and HEK293T compared to the HDAC6/10
inhibitor tubastatin A. This may be beneficial for future
therapeutic applications of selective HDAC10i as immuno-
modulators, as previously suggested (20) by the long-term
allograft survival of HDAC10−/− mice receiving fully MHC-
mismatched cardiac transplants. That inhibition of
HDAC10 enzymatic function is not cytotoxic, and does not
sensitize neuroblastoma (BE(2)-C) cells toward drug treat-
ment, suggests that a so far undescribed scaffolding or
adaptor function of HDAC10 may exist. The development
of selective HDAC10 degraders (41) and rescue experiments
using mutant, enzymatically inactive HDAC10 could help
to clarify these currently unanswered questions.

ASSOCIATED CONTENT

Supplementary tables and figures, detailed experimental pro-
cedures, compound characterization, and 1H and 13C NMR
spectra of newly synthesized substances. (PDF)
NCI60 Screen Data (XLSX)
Sensitization screen with repurposing library data (XLSX)

ACCESS CODES

The atomic coordinates and crystallographic structure fac-
tors of enzyme-inhibitor complexes have been deposited in
the Protein Data Bank (www.rcsb.org) with accession codes
as follows: HDAC10-SAHAl complex, 7SGG; HDAC10-
compound 14 complex, 7SGI; HDAC10-DKFZ-711
complex, 7SGJ; HDAC10-DKFZ-728 complex, 7SGK.

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Notes
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