Identification of mercaptoacetamide-based HDAC6 inhibitors via a lean inhibitor strategy: screening, synthesis, and biological evaluation

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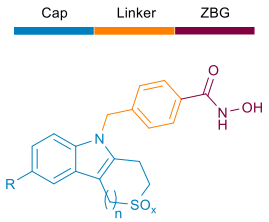
Non-selective inhibition of different histone deacetylase enzymes by hydroxamic acid-based drugs causes severe side effects when used as a (long-term) cancer treatment. In this work, we searched for a potent zinc-binding group able to replace the contested hydroxamic acid by employing a lean inhibitor strategy. This instructed the synthesis of a set of HDAC6-selective inhibitors containing the more desirable mercaptoacetamide moiety. Biological evaluation of these new compounds showed an IC_{50} in the nanomolar range, dose-dependent HDAC6 inhibition in MM1.S cells and improved genotoxicity results, rendering these new inhibitors valuable hits for applications even beyond oncology.

Histone deacetylases (HDACs) are polyvalent regulators of protein function and thus control vital cellular processes such as cell proliferation, transcription, cell migration, and cell death.[1-3] These enzymes catalyze the removal of acetyl groups on lysine residues, a crucial post-translational protein modification.[4-7] HDACs can be classified into two major groups. Group 1 comprises eleven zinc-dependent isozymes, categorized as class I (HDAC1-3 and 8), class II (HDAC4-7 and 9-10), and class IV (HDAC11), while group 2 includes class III HDACs or sirtuins, which are NAD^+-dependent.[8] However, this classification and the HDAC nomenclature are challenged in the face of recent findings about their substrates, ranging far beyond histones.[1, 9] While involved in normal cellular functioning, HDACs are also associated with human diseases such as cancer,[10, 11] neurodegeneration and immune disorders.[12-16] These observations instigated the development of HDAC inhibitors as a therapeutic strategy. To date, four HDAC inhibitors (Panobinostat, Belinostat, Vorinostat, Romidepsin) have been FDA-approved for use in hematological cancers,[17-20] and a fifth one (Chidamide/Epidaza) is approved by the NMPA (formerly CFDA),[21] for use in hematological and breast cancer. Of note, these agents are all pan-inhibitors, targeting several isozymes of the HDAC family simultaneously. This often culminates in severe side effects, such as hematological toxicity (i.a. thrombocytopenia, neutropenia) and cardiovascular problems (i.a. arrhythmia). Selective targeting of one HDAC family member for a specific condition could overcome these side effects, since different HDACs are implicated in different diseases, making simultaneous inhibition of multiple HDAC enzymes unnecessary and even detrimental.[22, 23]

Histone deacetylase 6 (HDAC6) is unique among HDACs since it is localized in the cytoplasm, where it has many non-histone interaction partners[24, 25] in contrast to nuclear HDACs. This differential action mechanism is also reflected in HDAC6 knockout mice, which are viable and develop normally, contrary to the lethality resulting from the genetic deletion of class I HDACs.[26] Because literature supports that HDAC6 is dysregulated and/or implicated in several cancers and neurodegenerative disorders.[12, 27-31], pharmacological inhibition of this specific enzyme holds great therapeutic potential and could also limit side effects considerably.[32]

Many potent HDAC6-selective inhibitors (HDAC6i) have been developed in the last decade, including the Tubathians (Figure 1) by our group, which showed high selectivity over other HDACs and pronounced nanomolar activity.[33-41] As most HDACi, HDAC6i share a common pharmacophore structure, consisting of a cap group for interaction with the protein surface, a linker, and a zinc-binding group (ZBG), which chelates the zinc ion in the catalytic center. Up to now, structural variations have mainly been focussed on the cap group and linker unit, while the majority of published inhibitors bears a hydroxamic acid (HAA) zinc-binding moiety.[33-35, 37, 42, 43] Although hydroxamic acids are indeed powerful metal chelators, they are also suspected of mutagenicity and non-specific metal binding, causing side effects and hampering medicinal applications, especially beyond oncology.[44] Considering the pivotal role of HDAC6 in e.g. neurological diseases, the development of a safe and potent non-HAA HDAC6-selective inhibitor is expected to have a high therapeutic value.[14, 45]

In this work, we aimed to diversify the promising class of Tubathians towards non-oncological therapeutic applications by incorporating a suitable non-hydroxamic acid zinc-binding group. Wagner *et al*. have demonstrated that HDAC inhibitors don't necessarily require the classical cap group-linker-ZBG constellation to attain potent inhibition.[46] Therefore, a lean inhibitor, composed of only a linker unit and the zinc-binding group under investigation, can serve as an efficiently obtained proxy to evaluate the biological potential. Here, a lean inhibitor strategy allowed for efficient experimental validation of the proposed alternative chelators, before engaging in the synthesis of the corresponding full-length inhibitors. Careful selection of the linker moiety, however, is key, as it was shown to influence zinc-binding properties, positioning in the catalytic cavity, and isoform selectivity.[47] We selected the phenyl group as the linker in our HDAC6 inhibitors, since it confers selectivity by exploiting the wider tunnel size compared to class I HDACs and because π-π stacking interactions with the aromatic moieties in the substrate channel contribute to the ligand efficiency.[48] A set of metal binding groups was selected from literature based on their successful incorporation in Vorinostat, furnishing potent HDAC inhibitors, or use in the inhibition of other metalloenzymes.[49-55] Phenyl moieties bearing these ZBGs were purchased or obtained via a short synthesis (Table 1). *N*-hydroxybenzamide was included as a reference compound (Entry 1).



Tubathians

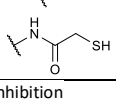
Tubathian A: R = H, n = 1, x = 2

Figure 1 Structure of Tubathians, a class of HDAC6-selective inhibitors exhibiting low nanomolar activity (HDAC6 IC_{50} = 1.9 - 22 nM).[34, 39]

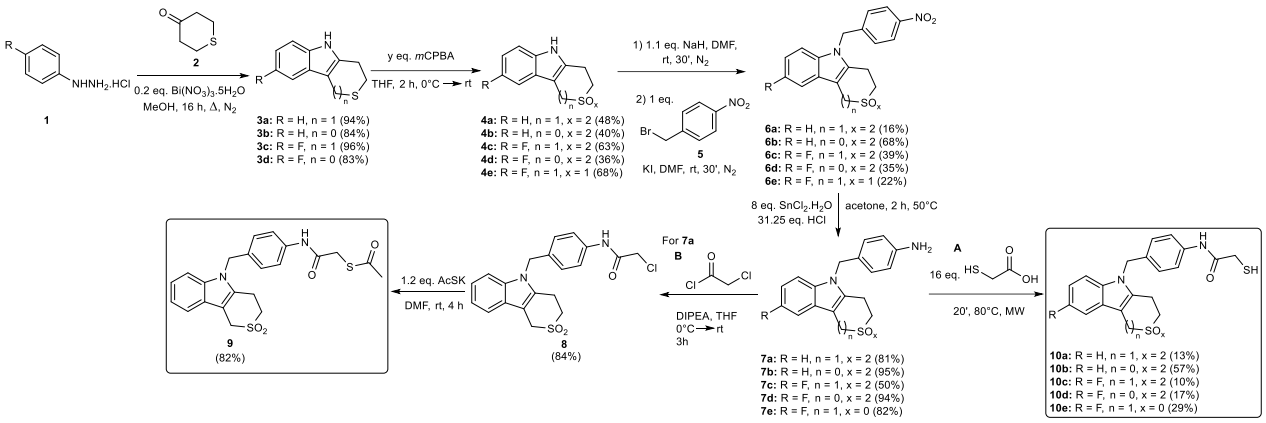
Validation of the metal chelating properties by means of an enzymatic inhibition assay showed that only mercaptoacetamide (MAA) emerged as a suitable substitute for hydroxamic acid (71% inhibition at 10 µM, Table 1, Entry 7). Also, in contrast to hydroxamic acid, there has been no report of MAA-born genotoxicity,[54] and no phenyl MAA-based HDAC6 inhibitors have been synthesized so far. The cap-less counterpart of the Tubathians showed less inhibition than Tubathian A (88% and 99%[39] inhibition at 10 µM, respectively), demonstrating the added value of the cap group. Quite surprisingly, no activity was observed for the trifluoromethyl ketone-based compound (Table1, Entry 2), although docking studies had predicted a favorable binding mode,[36] and HDACi containing an alkyl trifluoromethyl ketone had shown micromolar activity before.[49, 56] Similar conclusions can be drawn for boronic acid. These observations also underline the complementary benefit of the lean inhibitor strategy. Thiophenol (Table 1, Entry 3) was included since zinc ions are thiophilic and thiol derivatives were reported before as inhibitors of HDACs, including the FDA-approved Romidepsin, and other zinc-dependent enzymes.[50, 57-59] Because the free thiol is required for zinc chelation, the methylated analog (Table 1, Entry 4) was included as a negative control.[57] Results did not show any inhibition at 10 µM, possibly because the orientation or position in the catalytic site did not allow efficient chelation of the zinc ion. Together, this again supports experimental validation of promising ZBGs via lean inhibitors.

Table 1 Percentage of HDAC6 inhibition by the lean inhibitors composed of a phenyl linker and varying zinc-binding groups (ZBG) at 10 µM.

Lean inhibitor: 

Entry	ZBG	% inhibition (10 µM)[a]
1		88
2		N.I.
3		N.I.
4		N.I.
5		N.I.
6		N.I.
7		71

[a] N.I. = No Inhibition



Scheme 1 Synthesis route towards mercaptoacetamides **10a-e** and thioester prodrug **9**. For the synthesis of sulfones **4a-d**, y = 3, to obtain sulfoxide **4e**, y = 1. MW = microwave.

The above results warranted the actual synthesis of MAA Tubathian analogs. The synthesis of the cap group was previously optimized in our research group,[33, 34, 36] involving a Fisher-indole reaction to furnish the tricyclic sulfides **3a-d** (Scheme 1). Subsequent oxidation of the sulfur atom with 3-chloroperbenzoic acid (*m*CPBA) gave sulfones **4a-d** (y = 3) and sulfoxide **4e** (y = 1). The aromatic linker unit was introduced through *N*-benzylation with 4-nitrobenzyl bromide, which was reduced with tin(II) chloride ($SnCl_2$), yielding amines **7a-e.** The envisioned mercaptoacetamide products **10** were obtained by reaction of amines **7a-e** with thioglycolic acid under microwave conditions (Scheme 1, route A). Of note, sulfoxide **6e** was reduced concurrently with the nitro group, which was not observed for the sulfones **6a-d**. Direct alkylation of sulfide **3c** to surpass the seemingly redundant oxidation-reduction steps to obtain **7e** resulted in a complex reaction mixture, and efforts to preserve the sulfoxide moiety under reducing conditions were to no avail. In total, five first-in-class phenyl MAA-based HDAC6i **10** were thus obtained, with variations in cap group substitution (R = H, F), ring size (n = 0, 1) and oxidation state of the sulfur atom (x = 0, 2). IC_{50} values were determined to assess the potency of these new compounds to inhibit HDAC6 deacetylase activity (Table 2). Low micromolar to nanomolar activities were observed, demonstrating that a significant activity improvement resulted from the addition of a cap group: 71.2% inhibition with 10 µM of *N*-phenylmercaptoacetamide (Table 1, Entry 7) versus 95% inhibition with 10 µM of compound **10a**.

By comparing structures and corresponding activities, we concluded that *S*-oxidation conferred a substantial improvement in the inhibition profile (IC_{50} **10e** vs IC_{50} **10a**), as was observed for the Tubathians.[34, 60] The six-membered thiaheterocycle showed superior activity over its five-membered counterpart (IC_{50} **10c** vs IC_{50} **10d**). However, this effect was not as pronounced for the compounds without fluorine (IC_{50} **10a** vs IC_{50} **10b**). Functionalisation of the cap group with fluorine did not improve the *in vitro* inhibition potential (IC_{50} **10a** vs IC_{50} **10c** and IC_{50} **10b** vs IC_{50} **10d**).

Table 2 *In vitro* enzyme inhibition results of mercaptoacetamides **10a-e** towards HDAC6.

Compound	**10a**	**10b**	**10c**	**10d**	**10e**
IC_{50} (µM)	0.13	0.24	0.23	1.10	1.30

The best-performing inhibitor **10a** was then selected for further biological evaluation. First, selectivity against other HDAC isozymes was evaluated (Table 3), with **10a** displaying moderate selectivity against HDAC3 and high selectivity with respect to HDACs 1, 2, 4, 5, 8, and 9. This selectivity profile proves that structure **10a** performs as an HDAC6-selective inhibitor.

Table 3 *In vitro* enzyme inhibition of compound **10a** against HDAC1-9 and fold selectivity compared to HDAC6 (IC_{50} isozyme/IC_{50} HDAC6).

HDAC ISOZYME	**IC_{50} (µM)**	**FOLD SELECTIVITY**
HDAC1	28.1	216
HDAC2	> 30	> 231
HDAC3	1.93	15
HDAC4	> 30	> 231
HDAC5	20.6	158
HDAC6	0.130	1
HDAC7	N.C.[a]	N.C.
HDAC8	29.6	228
HDAC9	> 30	> 231

[a] Not Calculated (no inhibition observed at concentrations tested 0.3 nM – 30 µM).

Docking of the MAA analog **10a** of Tubathian A in catalytic domain CD2 of human HDAC6 (PDB 5EDU)[61] showed a monodentate binding mode (Figure 1 and 2, ESI), like the HAA counterpart, with a comparable binding energy (7.6 and 8.1 kcal/mol, respectively).

The sulfur and oxygen atom of the MAA engaged in hydrogen bonds, and while an additional hydrogen bond of the MAA NH group was expected for alkyl linkers,[62] this was not observed in the docking of compound **10a**.

To examine whether a mercaptoacetamide may serve as a safer alternative to hydroxamic acid in terms of mutagenicity, we evaluated **10a** using an Ames fluctuation test. No positive effects could be detected at the concentrations tested (5, 10, 50, and 100 μM, see ESI), indicating that compound **10a** is not mutagenic and that a mercaptoacetamide functionality could indeed represent a superior ZBG in that regard.[34]

Considering that thiols are prone to dimerization, which could affect cellular activity, the thioacetate prodrug of compound **10a** was synthesized as well (Scheme 1, route B) to be included in cellular assays. To that end, amine **7a** was transformed to chloroacetamide **8** through reaction with 2-chloroacetyl chloride. Addition of potassium thioacetate then yielded the thioester prodrug **9**.[63] The HDAC6 inhibitory activity of mercaptoacetamide **10a** and its respective prodrug **9** was analyzed on a cellular level in MM1.S cells by determining their ability to induce an increase in the protein levels of acetylated α-tubulin (an HDAC6 substrate) *via* Western blot (Figure 3, ESI). Acetylated histone H3 and histone H3 were included as a control for inhibition of nuclear HDACs to monitor potential off-target effects. Tubastatin A, a known HDAC6-selective inhibitor, was used as a positive control. To prevent oxidative dimerization of **10a**, a combination of the MAA and a reducing agent (tris(2-carboxyethyl)phosphine, TCEP) was included as well.[62, 64] The results in Figure 3 (ESI) showed a clear difference between the MAA **10a**, the acetyl prodrug **9**, and the combination of compound **10a** with TCEP. More specifically, the MAA **10a** triggered a marginal increase in acetylated α-tubulin protein levels, while the prodrug and the combination treatment induced a pronounced dose-dependent effect. Increased acetylated histone H3 levels were only observed for the prodrug **9** at the highest concentration (50 μM), confirming HDAC6-selective activity of the compounds. A possible explanation could be that the acetyl group makes the prodrug more effective in penetrating the MM1.S cells, resulting in a higher effective concentration in the cell, which caused off-target effects at 50 μM.[53] In contrast to the literature observation that a dimerized MAA could serve as a prodrug,[53] our results point to a loss of activity upon dimerization of compound **10a** in solution and upon storage in DMSO. Taken together, mercaptoacetamide **10a**, when combined with TCEP to avoid dimerization, and the corresponding prodrug **9** demonstrate promising cellular activity since they can selectively inhibit HDAC6 in MM1.S cells.

In conclusion, our lean inhibitor strategy to find a suitable alternative for the hydroxamic acid zinc-binding group in HDAC6 inhibitors to overcome related drawbacks was efficient and useful, as it resulted in the identification of the mercaptoacetamide substitute. This approach could also represent a valuable strategy for the identification of novel inhibitors for other metalloenzymes. Furthermore, we showed that the traditional HDAC inhibitor pharmacophore has its merits, as evidenced by the more pronounced activity of the indole-containing hit structures compared to the lean inhibitor. In that way, a unique phenyl mercaptoacetamide-based HDAC6 inhibitor was synthesized, exhibiting an IC_{50} in the nanomolar range with a good to excellent HDAC6 selectivity profile. In line herewith, Western Blot analysis corroborated selective activity in a dose-dependent manner in MM1.S cells. Furthermore, initial screening of the mutagenic potency indicated that the mercaptoacetamide represents a potentially safer alternative to the hydroxamic acid. In summary, we developed and evaluated a novel, potent non-hydroxamic acid HDAC6-selective inhibitor that represents a potential hit for the therapeutic application of HDAC inhibitors beyond oncology, such as in a neurological disease context.

There are no conflicts to declare.

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