

Characterization of novel histone deacetylase
inhibitors and specific histone deacetylase 6
inhibition in myeloid leukemia

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Zusammenfassung

Trotz zahlreicher therapeutischer Vorstöße wird akute myeloische Leukämie (AML) mit hohen Rückfallraten und schlechten Überlebensraten assoziiert. Die 5-Jahres-Überlebensrate für Erwachsene / Kinder beträgt 65 % / 30 %. In präklinischen Studien wurden Histondeacetylasen (HDAC) als effektive epigenetische Ziele in AML identifiziert. Allerdings zeigten unselektive HDAC-Inhibitoren (HDACi) nur geringe antileukämische Effekte und schwerwiegende Toxizität. Daher ist ein besseres Verständnis des Mechanismus von HDAC-Inhibitoren und die Identifizierung von synergistischen Therapeutika notwendig. Innerhalb der HDAC-Protein-Familie hat HDAC6 wegen seiner Struktur, welche eine selektive Inhibition erlaubt, ein besonderes Potenzial. HDAC6 reguliert eine Vielzahl krebsrelevanter Stoffwechselprozesse wie die Hitzeschockreaktion, Immunflucht, Zellmobilität und Autophagie. Da die Auswirkungen von HDAC6-Inhibition jedoch äußerst kontextabhängig sind, müssen sie systematisch innerhalb der verschiedenen Leukämie-Subtypen charakterisiert werden.

Diese kumulative Dissertation umfasst vier wissenschaftliche Publikationen. Drei der Studien fokussieren sich auf die Charakterisierung von klassenspezifischen HDACi in einer breiten Auswahl von Leukämiezelllinien. Leitstrukturen mit verbesserten antileukämischen Effekten wurden hinsichtlich ihrer Isoenzymselektivität und Fähigkeit zur Apoptose- oder HDAC-Substrat-Acetylierungsinduktion charakterisiert. Aus diesen Bestrebungen gingen vier äußerst potente Klasse-I/IIb-HDACi (**10h**, **1g**, **4d** und **4m**), ein HDAC6-präferierender Inhibitor (**2b**) und ein hochselektiver HDAC6-Inhibitor (**10p**) hervor. Jeder dieser Inhibitoren zeigte ein unterschiedliches Maß an synergistischer Interaktion mit dem Standard-AML-Chemotherapeutikum Decitabin, allerdings waren **10h**, **2b** und **4d** besonders effektiv.

Die letzte Publikation fokussierte sich auf HDAC6-Inhibition in myeloischer Leukämie. Über CRISPR-Cas9 wurde der Verlust von HDAC6 modelliert, und der präferentielle HDAC6-Inhibitor Ricolinostat wurde für eine pharmakologische Kreuzvalidierung genutzt. Während die Zellen *in vitro* ungehindert wuchsen, war *in vivo* die Leukämiebürde reduziert. Eine Multi-Omik-Analyse via Proteomik, Secretomik, RNA-seq und ATAC-seq zeigte signifikante Veränderungen diverser Stoffwechselwege. Besonders lysosomale Proteine, wie der Tumorsuppressor RNase T2, waren angereichert in den untersuchten Zelllinien. Zudem sensibilisierte Ricolinostat AML-Zellen für CD8+-T-Zell-vermittelte Toxizität. Durch ein Hochdurchsatz-Medikamentenscreening konnte HDAC6 als ein Mediator für Resistenz gegen das Standard-AML-Chemotherapeutikum Cytarabin identifiziert werden. Basierend auf diesen Ergebnissen erscheint HDAC6-Inhibition als potenzielle Behandlungsmöglichkeit für myeloischer Malignome, jedoch bedarf es weiterer Forschung.

Abstract

Acute myeloid leukemia (AML) remains associated with high relapse rates and poor overall survival despite recent therapeutic advances. The 5-year survival rate is approximately 30% in adults and 65% in children. Preclinical studies have identified histone deacetylases (HDACs) as effective epigenetic targets in AML. However, clinical application of pan-HDAC inhibitors has been limited by modest antileukemic efficacy and substantial toxicity. This underscores the need for a more fundamental understanding of HDAC inhibitor (HDACi) mechanisms and the identification of synergistic therapeutic partners. Among the HDAC family, HDAC6 represents a particularly promising target due to its unique structural properties that permit selective inhibition. HDAC6 regulates multiple cancer-relevant processes, including the heat shock response, immune evasion, cell motility and autophagy. Given the context-dependent nature of these effects, systematic evaluation of HDAC6 inhibition across distinct AML subtypes is warranted.

This cumulative dissertation summarizes the findings of four scientific. Three of these studies focus on the characterization of novel class-specific histone deacetylase inhibitors (HDACi) in a broad panel of leukemic cell lines. Lead compounds demonstrating superior antileukemic activity were further analyzed with respect to their HDAC isoenzyme selectivity and their capacity to induce apoptosis or hyperacetylation of HDAC substrates. These investigations identified four highly potent class I/IIb HDACi (**10h**, **1g**, **4d**, and **4m**), one HDAC6-preferential inhibitor (**2b**), and one highly HDAC6-selective inhibitor (**10p**). All inhibitors exhibited varying degrees of synergistic activity with the AML standard chemotherapeutic agent decitabine, with compounds **10h**, **2b** and **4d** showing the most pronounced effects.

The last paper focuses specifically on HDAC6 inhibition in myeloid leukemia. HDAC6 loss models were generated using CRISPR-Cas9, while the HDAC6 preferential inhibitor ricolinostat was used for a pharmacological cross validation. While proliferation *in vitro* was unaffected, *in vivo* experiments showed a significant reduction in leukemia burden. A multi-omics analysis through proteomics, secretomics, RNA-seq, and ATAC-seq identified major shifts in a multitude of pathways. Notably, lysosomal proteins, such as the tumor suppressor RNase T2, were highly enriched across a broader panel of cell lines. Furthermore, ricolinostat induced susceptibility of AML cells to CD8⁺ T cell-mediated cytotoxicity. High-throughput drug screening revealed the specific role of HDAC6 in resistance to the common AML chemotherapeutic cytarabine. Overall, specific targeting HDAC6 may serve as a viable strategy in the treatment of myeloid malignancies, warranting further investigations.

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Abbreviations

Acute lymphoblastic leukemia = ALL

Acute myeloid leukemia = AML

Allogeneic stem cell transplant = allSCT

Catalytic domain 1 = CD1

Catalytic domain 2 = CD2

Chapter 3.1 = C3.1

Chapter 3.2 = C3.2

Chapter 3.3 = C3.3

Chapter 3.4 = C3.4

Chimeric antigen receptor = CAR

Chronic lymphoblastic leukemia = CLL

Chronic myeloid leukemia = CML

Differential drug sensitivity score = dDSS

Hematopoietic stem cells = HSC

Histone acetyltransferases = HAT

Histone deacetylase 6 = HDAC6

Histone deacetylases = HDAC

Inhibitory concentration that stops 50% of cellular viability or growth = IC50

Interferon-alpha = IFN α

Motor binding domain = DMB

NOD SCID Gamma = NSG

Peripheral blood mononuclear cells = PBMCs

Philadelphia chromosome = Ph

Proteolysis targeting chimeras = PROTAC

Reactive oxygen species = ROS

Single-nucleotide polymorphism = SNP

Tyrosine kinase inhibitors = TKi

Ubiquitin-binding zinc finger domain = UZB

1 – Introduction

Leukemia is a malignancy that remains challenging to treat, not only in adults but also in certain forms of pediatric leukemia. It originates from and further disrupts the hematopoietic stem cell system. Since the characteristics of the progenitor often determine crucial aspects of the malignancy, it is essential to examine the hematopoietic system closely.

1.1 - The hematopoietic system

The hematopoietic stem cell system consists of hematopoietic stem cells (HSC) located in the bone marrow [1]. HSCs are the source of all blood cells, which develop downstream through complex differentiation pathways (see Figure 1 [2]). To consistently maintain the foundation of HSC, one of the dividing cells preserves its pluripotent features while the other cell initiates differentiation. The first distinctive cell types represent the myeloid progenitor cells. These progenitors differentiate into various cell types like erythrocytes, megakaryocytes, mast cells, and myeloblasts. Erythrocytes make up to ~80% of all cells in the human body and ~40% of the total blood volume [3]. Their primary function is to supply the constant oxygen demand via hemoglobin-bound transport from the lungs to every body part. Megakaryocytes are relatively large (50-100µm) polyploid cells continuously releasing thrombocytes within their cytoplasm [4]. They subsequently can initiate the coagulation cascade upon tissue damage to prevent further blood loss.

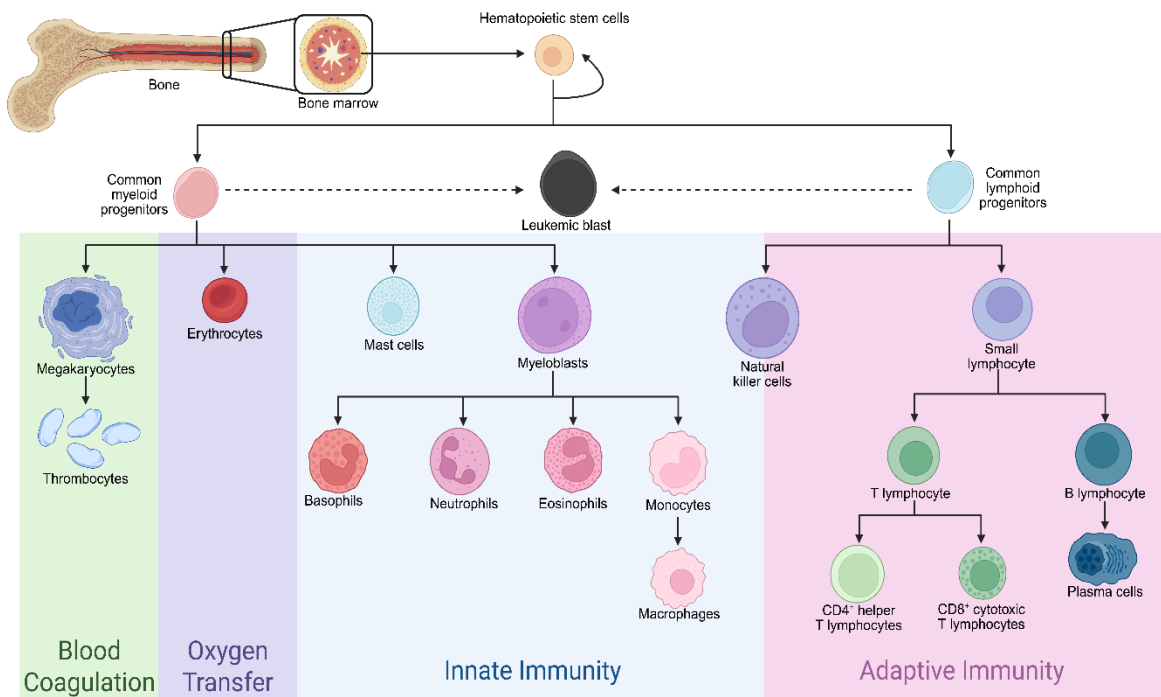


Figure 1: Simplified synopsis of the complex differentiation pathway during hematopoiesis. Hematopoietic stem cells in the bone marrow go through several stages to until they fully differentiate into functional blood components. Created with Biorender.com, based upon [2]

The remaining cell types are critical components in the various intricate processes of the immune system. Upon contact with stimuli like allergens, physical injuries, or microbial pathogens, mast cells release granules with heparin, histamine, and further inflammatory regulators [5]. Myeloblasts further differentiate into basophils, neutrophils, or eosinophils. Basophil cells contribute similarly to mast cells in allergic reactions but also defend against parasites by secreting cytokines that activate eosinophils [6]. Against viruses, Eosinophils secrete granules that contain RNAses to cleave the pathogenic viral mRNA or the genome of RNA viruses. Bacterial or viral infections are initially neutralized by neutrophils and macrophages, which either phagocytose the pathogens or induce inflammatory responses [7]. Macrophages can be divided into pro-inflammatory M1 macrophages, which secrete the radical-producing enzyme iNOS, or anti-inflammatory growth factor-emitting M2 macrophages [8]. Together, these various cell types form the innate arm of the immune response, which acts as a first defense against pathogens to prevent an escalation of the infection.

The adaptive immune system cells emerge from the lymphoblastic progenitor cells and are tasked to neutralize large-scale infections and remember the responsible pathogen in the future. B lymphocytes generate antibodies that act against specific threats and deactivate pathogenic components by antigen binding [9]. Upon contact with their respective antigen, these B cells can differentiate further into rapidly antibody-producing plasma cells. T lymphocytes separate into subtypes like cytotoxic, helper, or regulatory T cells [10]. While helper and regulatory T cells mainly regulate the activity of cytotoxic T cells, this type of cell triggers the cell death of endogenous cells that were either infected by pathogens or underwent malignant transformation. They emit perforins, granzymes, and serine proteases that cleave caspases and induce apoptosis upon antigen recognition. Lastly, natural killer cells were, for a long time, associated with the innate immune system; however, recent results suggest that they seem to bridge the innate and adaptive immune system [11]. While they can operate similarly to cytotoxic T cells, they also carry an arsenal of immune-activating or inhibitory receptors. Collectively, these various cell types carry out essential processes for the survival of the human body.

1.2 - Leukemia

The term leukemia describes a pathogenic aberration of hematopoietic cells in the bone marrow [12]. Similar to other malignancies, the tumorigenesis of leukemia is initiated through multiple genetic alterations, but also environmental factors that eventually lead to a pathogenic transformation [13]. Instead of completing the hematopoietic differentiation protocol, these cells stop in an immature state. These blast cells remain mostly

dysfunctional and cannot participate in the normal functions of blood cells. While the disease progresses, the leukemic blasts proliferate faster than the HSC and slowly overtake the bone marrow [14]. Eventually, the blast cells will enter the bloodstream and further infiltrate other organs, making leukemia a systemic disease. Different options, such as microscopy, blood tests, or a lymph node biopsy, can diagnose leukemia. A further indication can be the swelling of particular organs like the kidney, spleen, liver, or brain due to leukemic blast infiltration, which can be seen by ultrasound.

The leukemic cell expansion in the bone marrow suppresses the growth of hematopoietic stem and progenitor cells, resulting in reduced generation of mature blood cells while flooding the blood stream with dysfunctional cells. This impairs the essential functions of the blood and will lead to common symptoms like bleeding, easy bruising, bone pain, fever, fatigue, or a high risk of infections [12]. Rare symptoms are more dependent on the type and progression of the disease and can include symptoms like chills, night sweats, weakness in the limbs, nausea, or weight loss [15]. The infiltration of the central nervous system can lead to a plethora of neurological symptoms like headaches, migraines, seizures, or coma due to the pressure exerted on the brain stem. The primary causes of mortality in leukemia are attributed either to infections, internal bleeding, organ failures of the liver, spleen, or kidney, anemia, complications from the treatment, or a combination of all [12, 16-18]. Therefore, the immediate and correct form of therapy is essential to maximize the chance of survival.

Adult and pediatric leukemia are separated due to inherent differences regarding critical clinical aspects. In the United States alone, roughly 60,000 new cases of leukemia arose in 2024, which accounts for 3.1% of all new cancer cases [19]. While the disease can occur at any stage of adulthood, its prevalence is higher with age. As of 2021, predictions account that approximately 500,000 individuals live with leukemia in the US. The lifelong chance to develop it was predicted at 1.5%. Data from 2014 to 2020 suggest a five-year relative survival rate of 67%. Around 23,000 deaths from leukemia are expected in 2023, which represents 9.4% of deaths by cancer [19]. In pediatric oncology, leukemia accounts for roughly 28% of all childhood cancer cases, with approximately 3,800 new cases under the age of 15. According to the SEER program of the American national institute of health 1 in 1,500 to 2,000 children may be diagnosed with leukemia by the age of 20 [20]. The five-year survival rate is estimated to be 86.3% in 2025 across all subtypes [20]. Based upon these facts, this would mean that of the 3,000 new cases, 450 children would not survive the illness. Male patients seem to have a higher prevalence based on age-adjusted data from 2016 to 2020, with incidence rates of 5.4 for male and 4.2 for female patients per 100,000 [20]. However, all these parameters vary heavily depending on the leukemic subtype.

1.3 - Subtypes of leukemia

Classically, two major attributes are described to classify the four major leukemia subtypes. This either relays to the lineage of the blast cells, which would either make the myeloid or lymphoid, or the progression speed of the disease, which clusters into acute or chronic. These aspects heavily influence the occurrence, survival, and relapse rates (see Figure 2 [19, 21-30]).

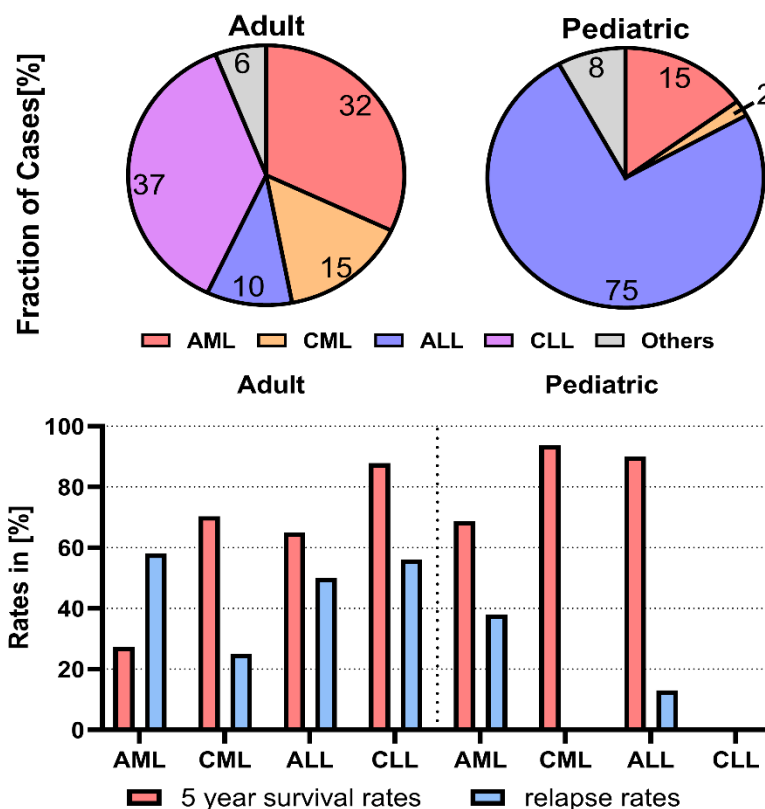


Figure 2: Summary of leukemic incidences, 5-year survival rates and relapse rates divided by subtype in adults and children. */**= insufficient data to give reliable estimations for CLL/AML due to the rarity in the pediatric context. AML = Acute myeloid leukemia, CML= chronic myeloid leukemia, ALL= Acute lymphoblastic leukemia, CLL = Chronic lymphoblastic leukemia. Created with GraphPad Prism and based upon [19, 21-30]

1.3.1 - Acute myeloid leukemia

Acute myeloid leukemia (AML) originates from myeloid progenitor cells and progresses aggressively, which can be fatal within weeks or months if not treated. With 32%, a significant proportion of adult cases can be attributed to AML; however, in comparison, childhood AML only constitutes about 15% of cases [19]. The five-year survival rates remain significantly low, with ~25% for adults and 70% in children, with relatively high relapse rates, going from 50-60% in adults and 30 to 40% in children [21]. Therefore, novel insights into AML are urgently needed to improve the outlook of patients suffering from this disease.

1.3.1.1 - Molecular mechanisms of acute myeloid leukemia

Defining for AML is its heterogeneous genetic background which mainly include single nucleotide polymorphisms (SNP) and gene rearrangements. In case of pediatric AML, the WHO classifies 15 different subtypes by genetic aberrations of *KMT2A*, *RUNX1*, *CBFB*, *NPM1* and other genes [31]. In 2024 Umeda et al. provided a novel genomic framework to categorize 887 pediatric AML patients by contrasting these classifications against molecular categories and identified distinct 23 molecular categories. Most of the found mutations in these patients were either signaling pathway, epigenetics or transcription factor related. A Taiwanese study based on 762 adult AML patients identified 31 recurrently mutated genes that cluster into biological functions like survival signaling via *FLT3* or *NRAS*, epigenetic regulations or loss of tumor suppressor genes [32]. Especially mutations in regards to transcription factors, DNA methylation or chromatin modifications tended to overrepresented. Overall has been demonstrated the that epigenetic dysregulation in AML is an fundamental driver of leukemogenesis [33]. Together these studies highlight the importance of the dysregulation of transcription AML.

Subtype defining frameshift mutations occur in the multifunctional protein *NPM1* (~30% of cases) or transcription factor *CEBPA* (variable occurrence, up to ~20% of cases) [34]. The prognosis of patients varies heavily depending on the cooccurrence with other mutations like *FLT3*, *KIT*, *DNMT3a*. In roughly 30% of AML patients, alterations in *FLT3* are found. However, the clinical implications vary depending on the affected protein domain [35]. Generally, internal tandem duplications of *FLT3* are linked to a poor prognosis, while tyrosine kinase mutations that lead to a constitutively active *FLT3* are more ambiguous regarding prognostic impact. In this case, the co-contributing factor seems to be accessory mutations in other genes. *KIT* has been described in core-binding-factor AML as the most frequently mutated gene, but is also found in other instances of AML [36, 37]. Its impact seems to lead to decreased overall survival and increased relapse rates. While *TP53* is mutated in an abundance of cancer types, it appears to be relatively stable in initial AML [37]. Specifically in cases of increased genomic instability, e.g., induced via therapy, *TP53* is prominently mutated, leading to further complex cytogenic abnormalities, chemoresistance, and poor outcomes. Defining for AML is often the co-occurrence of mutated genes, since some studies imply tightly regulated interactions between the mutated proteins [38].

Researchers have identified numerous chromosomal alterations that affect the biological background of the disease and have clinical implications for the patient's survival. Recurrent subtypes carry fusion genes of either *KMT2A*, *RUNX1*, *RARA*, or *MECOM*. *KMT2A*

rearranged cases of AML (5-15%, depending on age) are another highly heterogeneous subtype where the prognosis depends on the fusion partner [39, 40]. The oncogenic protein modifies the methylation patterns of the histones and, therefore, alters the epigenetic landscape. This leads to a hypomethylation of gene regions of stem cell and leukemogenesis-associated proteins like S100A8/9, which induces an overexpression of these proteins [41]. *RARA* is altered in roughly 30% of AML cases and functionally prevents any differentiation downstream of the myeloid lineage. Recently, 14 different fusion translocations with *RARA* and various partners have been described, which promote resistance against differentiation induction through all-trans retinoic acid [42]. While fortunately relatively rare, the prognosis given to patients with *MECOM* alterations remains dismal [43]. Despite its rarity, this subtype is recognized as its own subclass of AML since it remains challenging to achieve complete remission in the corresponding patients. Typically, *MECOM* maintains the replication of hematopoietic stem cells. However, it can also carry out the same function as an oncogene [44]. All these types of mutations and translocations build a complex to navigate interdependent landscape which still impedes the treatment of AML.

1.3.1.2 - Treatment of acute myeloid leukemia

For 40 years, AML has been treated via the commonly referred to “7+3 regimen” chemotherapy [14]. Critical components of the therapy are the adenosine nucleoside analog cytarabine and anthracyclines like idarubicin or daunorubicin. Cytarabine acts by being falsely integrated into the DNA during synthesis, which subsequently inhibits its progression and leads to apoptosis. Anthracyclines, on the other hand, intercalate into the DNA, block topoisomerase II, or induce free radicals, which makes their effect cell cycle-independent. However, both drugs come with severe side effects like central nervous system toxicity, pancytopenia, and cardiotoxicity [45, 46]. The therapy begins with an induction phase that includes seven days of treatment with cytarabine combined with daunorubicin or idarubicin on the first three days [14]. This is followed by a consolidation phase with high doses of cytarabine twice daily for five days. Several variations of these regimens have been tested and consolidated that high dose of cytarabine is favorable. After reaching initial complete remission - if possible - the patient often receives an allogeneic stem cell transplant (alloSCT), which improves the antileukemic efficacy of the therapy and reduces relapse rates through graft-vs-leukemia reactions [47]. Still, this form of therapy is hampered since some subclasses seem unaffected, which can be traced back to mutations in *RARA*. In total, the success of these therapies is limited, and AML still has significantly unfavorable results compared to other leukemic subtypes.

Due to the increased insight into the genetic background and biology of AML, several novel protocols tailored to the disease are being investigated in clinical trials. In the "7+3 regimen" protocol, numerous studies found favorable results with adenosine nucleoside analogs with cladribine, fludarabine, or clofarabine [48-50]. For older patients with *TP53* mutations who are unfit for high-dose therapy, epigenetic modulators like the hypomethylating agents decitabine or azacytidine are being explored [51]. A valuable addition to these patients could be the BCL2 inhibitor venetoclax, which seems to significantly prolong the survival of these high-risk patients [52]. For patients with *FLT3* variants in some clinics, it has become common practice to add FLT3 inhibitors like midostaurin and gilteritinib, while other FLT3 inhibitors are investigated in clinical trials [35, 53]. A novel targeted approach utilizes tamibarotene, a RARA agonist, in combination with classical chemotherapeutics and accomplished high complete remission rates [54]. An alternative approach in promyelocytic AML to classical chemotherapy is the induction of differentiation via ATRA [55]. A recent study even improved the efficacy of this approach by designing epigenetic modifiers which overcame ATRA therapy resistance [56].

From the emerging field of immunotherapy, the antibody-drug conjugate gemtuzumab ozogamicin seems to be another valuable tool for treating AML [57]. The antibody gemtuzumab targets CD33, which is overexpressed in most AML cases, while the bound calicheamicin- γ_1 derivative induces DNA damage in the targeted cells. While these novel protocols seem to be significant improvements, it is likely that further optimized strategies could emerge from additional studies. A field lacking improvement due to the heterogeneity of AML is the generation of chimeric antigen receptor (CAR) T-cells [58]. These findings highlight that research is still needed to optimize the relatively old form of therapy for treating AML.

1.3.2 - Chronic myeloid leukemia

Similar to AML, chronic myeloid leukemia (CML) stems from the myeloid lineage, but its progression is much less rapid [59]. At the beginning of the disease, which is termed the chronic phase, the cells are dormant, and even if untreated, patients can live years without complications. In the accelerated phase, the proliferation rate of the cells increases, and the first symptoms arise. At a certain point, the cells reach the blast crisis state, which requires medical intervention. CML is more common in adults (15%), but it is quite a rare disease in children (2%) [19, 22]. The five-year survival rate in children and adults is relatively high, with 70%, or respectively, >90%, while relapse rates remain low [22]. This stems from the effective therapeutic options tailored explicitly towards the CML cells.

1.3.2.1 - Molecular mechanisms of chronic myeloid leukemia

The genetic trademark aberration that leads to CML is the t(9;22)(q34;q11.2) translocation, which is generally called the Philadelphia chromosome (Ph) [60]. It is found in 95% of all CML patients and emerges from the reciprocal switching of the tails of chromosomes 9 and 22. The root of this process is the *BCR* gene, which is known to break and cause different translocations with *ABL1* recurrently. Several domains from *BCR* gene are added to the end of the *ABL1* gene in the de novo *BCR::ABL1* fusion gene [61]. This increases the signaling capacity of the tyrosine kinase domain and makes it constitutively active. The altered signaling disturbs the balance between proliferation, differentiation, and apoptosis pathways and induces immortality and arrest in an immature stage. So far, three different isoforms for *BCR::ABL1*, called p190, p210, and p230, have been described [62]. Here, the number in the name correlates to the length of *BCR*, which is added to *ABL1*. Interestingly, each of these variants is connected to a different type of leukemia. While p210 is most commonly identified in CML and p230 is associated explicitly with neutrophilic CML, p190 is rarely found in this disease [62, 63]. This implies that the amount of added *ABL1* gene causes different signaling functionalities between these three variants.

While *BCR::ABL1* is understood as an essential driver in CML, systematic use of next-generation sequencing has identified other noteworthy recurrently altered proteins. Studies furthermore established that the stage of CML (chronic /accelerated phase or blast crisis) seems to influence which gene variations can arise. In the chronic phase, the most common mutations affected epigenetic modifiers [64]. *ASXL1*, for example, is mutated in various myeloid disorders and seems to be a driver mutation in roughly 15% of CML patients in the chronic phase [65, 66]. These variants emerge from truncations of the C-terminal domain that induce a gain-of-function regarding epigenetic regulations [66]. Unfortunately, this effect seems to worsen the prognosis of patients by promoting therapy resistance. Studies have found that in more than 90% of patients who reach blast crisis, additional relevant pathological mutations are found [67]. Interestingly, 90% of identified genes are associated with epigenetic modifications and gene regulation. *ASXL1* and the common leukemic driver gene *RUNX1* are mutated in roughly 30% of blast crisis cases. *RUNX1* acts as a transcription factor, and the multitude of different loss-of-function mutations suggests a tumor suppressor function. Therefore, while *BCR::ABL1* kinase is a key oncogenic driver in CML, the impact of additional mutations should not be underestimated.

1.3.2.2 - Treatment of chronic myeloid leukemia

Modern treatment regimens of CML heavily rely on small-molecule tyrosine kinase inhibitors (TKi) [59]. Their potency in suppressing the disease is probably one of the biggest success

stories in targeted drug design. The foundation for this class was found by Buchdunger et al. in 1996 and could inhibit the BCR::ABL1 kinase selectively [68]. Later, the compound was named imatinib, and only after 5 years it was approved by the Food and Drug Administration and the European Medicine Agency [69]. Chemical modifications of imatinib's base structure gave rise to the second and third generations of TKI that broadened the spectrum of targeted kinases. Prominent examples include dasatinib, which affects kinases like KIT and SRC, or ponatinib, which addresses several additional tyrosine kinases like EGFR, FGFR, PDGFR, and VEGFR [70, 71].

The therapy protocol depends mainly on the phase of the disease [72]. In the chronic phase, first-in-line options include imatinib, nilotinib twice a day, or dasatinib. Based upon the reaction, either the dose or the inhibitor is adjusted, and only in specific cases of *BCR::ABL1* mutations is ponatinib used. Further adaptations include the administration of interferon-alpha ($IFN\alpha$) or alogSCT. In blast crisis, the effectiveness of single-agent TKI therapy with imatinib remains limited, while dasatinib and especially ponatinib show favorable results [73]. However, these cases still have dismal overall survival rates that rarely exceed a few months. Multiple studies tried to incorporate, with success, standard chemotherapeutics, such as cytarabine, mitoxantrone, decitabine, or azacytidine into the blast crisis TKI regimen [74, 75]. Targeted inhibitor combinations using TKI with BCL2 inhibitors like venetoclax or JAK inhibitors like ruxolitinib seem promising and are currently being investigated [76, 77]. Epigenetic targeted inhibitors could be especially effective in ASXL1⁺ AML [66]. On the other hand, gemtuzumab ozogamicin has gained further traction in different trials since the CML stem cells also tend to overexpress CD33 [57, 78]. In conclusion, TKIs have revolutionized the standard care of CML patients; however, for patients in blast crisis, the situation remains dire, and better treatment combinations are needed.

1.3.3 - Acute lymphoblastic leukemia

In contrast to the previously described entities, acute lymphoblastic leukemia (ALL) originates from lymphoid progenitor cells [79]. Two major subtypes emerge from the B- or T-cell lineage to become B-ALL or T-ALL. This has a significant effect on the biological background and necessitates treatment protocols that are complementary to the lineage. Similar to AML, both subtypes progress rapidly and can be fatal in a short period. ALL makes up 10% of new cases in adults; however, in children, it is the major contributor to overall cases, with ~75% [19]. While the overall 5-year survival remains dismal in adults with ~60%, children have a much better prognosis with ~90% [23, 24]. The high rate of relapse of 50% seems to contribute to the lower survival rates in adults, while children relapse at a significantly lower rate of 10 to 15%. B-ALL, separated from T-ALL, makes up

~75% / ~85% of ALL cases in adults and children, while the remaining ~25% / ~15% of ALL cases can be attributed to T-ALL. Fortunately, B-ALL cases have a high 5-year survival of 85% to >90% in adults and children due to advances in therapy protocols. However, adult cases of T-ALL have a relatively poor prognosis with ~50% five-year survival, while children tend to have better outlooks of ~85% survival [25-28].

1.3.3.1 - Molecular mechanisms of B-cell acute lymphoblastic leukemia

B-ALL can emerge from several genetic aberrations like aneuploidy, gene translocations/rearrangements or driver mutations. Aneuploidy describes cases where altered numbers of chromosomes, either hyperdiploidy or hypodiploidy, when more or fewer than 46 chromosomes are detected [80]. These subtypes can be further classified into different subclusters depending on the number of chromosomes. While hyperdiploid leukemias are clustered into low hyperdiploid (n=47-57) or high hyperdiploid (n=51-63), hypodiploid leukemias are characterized either as high hypodiploid (n=40-44) or low hypodiploid (n=30-39). The subtype also often correlates with specific genetic alterations and immunophenotypes. High hyperdiploid leukemias, for example, are often accompanied by mutations of the histone modifier *CREBBP* or *EZH2*, which leads to epigenetic dysregulation [81, 82]. The concrete origins and regulatory mechanisms involved in hyper- and hypodiploidy are elusive and need further investigation [83]. While high hyperdiploidy is quite common in pediatric (~30%) and adult (~10%) ALL, fortunately, the diagnosis is quite favorable, with 90% overall survival [24]. Hypodiploidy is present in ~5% of B-ALL cases, but in comparison with hyperdiploid cases, it has a worse prognosis with 30 to 50% overall survival [84].

Regarding genetic rearrangements, they are fairly common B-ALL and often are used as identifiers to cluster subtypes. A well-known example is the t(12;21)(p13;q22) translocation, which leads to the combination of the *ETV6* and *RUNX1* genes [85]. The emerging *ETV6::RUNX1* fusion gene is the predominant chromosomal lesion in childhood ALL with ~25% of all cases. Both *ETV6* and *RUNX1* in their original form are crucial transcription factors that regulate the growth and differentiation of HSC. Their original function is lost in these cells, but proteins like *EPOR*, *IGF2BP1*, and *RAG1* are dysregulated, affecting differentiation, survival, and immune evasion. The translocation can occur early in utero, which leads to long-living dormant preleukemic clones [86]. Only by acquiring additional mutations can these cells develop into pathogenic leukemic cells. In general, this subtype is connected to a good prognosis with >90% overall survival, but at the same time, 20% of patients will relapse [85]. With 5%, another frequently rearranged gene is *KMT2A* [87]. Like AML, it has multiple potential fusion partners, but *AFF1* is generally the most likely to

translocate to t(4;11)(q21;q23) and generate the *KMT2A::AFF1* fusion gene. This leads to an overexpression of anti-apoptotic genes like BCL2 or the proto-oncogene MYC [88]. The prognosis for adult *KMT2A-AFF1*⁺ patients remains extremely poor, with 18% 5-year survival, and seems to depend highly on the treatment [87]. It has also been observed that ALL patients with *KMT2A-AFF1* can relapse with a phenotype switch from ALL to AML [88]. Remarkably - similar to CML - B-ALL patients can also develop the *BCR::ABL1* fusion gene by generation of the Ph chromosome. While rarely found in pediatric cases (1-3%), it is quite often found in older patients (25%) [24]. It functions by constitutively sending pro-survival signals via RAS, PI3K/AKT, and the JAK/STAT pathway [61]. Unfortunately, despite the advances in treating *BCR::ABL1*⁺ patients, event-free survival remains comparably low at 84% [24]. A similar subtype shows a comparable expression profile but lacks Ph chromosome and, therefore, the *BCR::ABL1* fusion gene. The Ph-like subtype has a relatively poor prognosis with ~60% five-year event-free survival since it lacks the therapeutic options that *BCR::ABL1* offers.

A study with 120 candidate genes in 187 high-risk B-ALL patients identified frequently mutated genes [89]. These lesions mainly cluster into gain/loss of function mutations of oncogenes/tumor suppressors or altered kinase activity. With ~30%, *NRAS* and *KRAS* mutations are predominantly found in these high-risk cases. Their genes belong to the RAS GTPase family, and several studies linked the pathogenic version to differentiation, cell growth, survival, and motility modulation [90]. Downstream signaling pathways are the PI3K-AKT and RAF-MEK-ERK pathways, which are constitutively activated. Furthermore, these genes have been linked to increased chances of relapse and, in turn, a worse prognosis [91]. Commonly altered tumor suppressor genes include *PAX5* (~25%), *TP53* (~7.5%), and *IKZF1* (~6%). *PAX5*, in the case of B cell development, is a crucial regulator of differentiation and cellular maintenance [92]. It is often even described as a driver gene, and many different mutation types, like point mutations, copy number variations, or translocations, have been observed [93]. Usually, *PAX5* acts as a transcription regulator and represses genes not connected to the B cell lineage while inducing the appropriate genes. The use of *PAX5* as a prognostic tool is ambivalent since, in adults, it seems favorable, but in children, this subtype is connected to an unfavorable prognosis [94]. The last cluster of frequently found mutated genes contains kinases like *JAK2*, *FLT3*, and *PTPN11*, which influence cell survival and proliferation.

An interesting factor in B-cell-derived ALL is the involvement of B-cell receptor signaling. This signaling cascade would normally induce the expression of antibodies and proliferation upon contact with a specific antigen. Recent studies imply that it can contribute to the disease. One subtype of precursor B-ALL heavily depends on the pre-B cell receptor [95]. It emerges from the process that is called V(D)J recombination and should be expressed

only transiently [96]. In the conventional context, it would induce proliferation and further downstream differentiation. However, due to the disturbed metabolism of leukemic cells, the receptor's differentiation function is blocked, and it contributes to proliferation by modulating PI3K/AKT signaling or MYC [95]. Often, a dependency on the pre-B cell receptor is found in subtypes with specific aberrations like *TCF3::PBX1* [97]. In contrast to other diseases like lymphoma, the adult version of the B-cell receptor does not seem to play a significant role in B-ALL.

1.3.3.2 - Molecular mechanisms of T-cell acute lymphoblastic leukemia

Comparing the mutational landscape of B-ALL and T-ALL, one can see that both entities have their own profile. Dysfunctions of the T-cell receptor have been researched but have yielded no significant results thus far [98]. A study from 2017 looked at 264 T-ALL-derived samples and identified 106 putative driver genes, which highlights the genetic heterogeneity of this disease [99]. The most frequently mutated gene was *NOTCH1*, with ~75% of samples. *NOTCH1* is a transmembrane receptor molecule that normally transverse into the nucleus to induce the expression of NOTCH-regulated genes after cell-cell interactions [100]. Upon acquiring mutations, the transformed receptor gains ligand independence, which leads to its constant activation and signaling. Target genes of NOTCH signaling, including *HEYL*, *HES1*, and *CCND1*, are connected to increased cell cycle progression and proliferation [101]. Loss-of-function mutations of tumor suppressors *CDK2A* and *CDK2B* are observed in roughly 70% of the studied T-ALL samples. These proteins normally inhibit cyclin-dependent kinases like *CDK4* or *CDK6* in the case of *CDKN2A* [102]. The deregulation of these kinases, in turn, increases proliferation. In 14% of samples, tumor suppressor *PTEN* mutations were identified, which led to increased proliferation via deregulated *AKT*. Unfortunately, these cases are also connected to relatively poor outcomes [103]. Other lesions include genes *FBXW7*, *PFH6*, *USP7*, and *DNM2*. Most of these genes are connected to increased proliferation, either by losing a tumor suppressor function or increased signaling activity. However, other studies have also highlighted the role of proteins that regulate the epigenetic landscape [104]. Proteins like *DNMT3A*, *EZH2*, *TET2*, or *EP300* are altered in >35% of pediatric and >60% of adult T-ALL, and especially *DNMT3A* or *EZH2* lesions are attributed to a poor prognosis and chemoresistance [105, 106]. Chromosomal translocations that lead to the expression of chimeric fusion proteins are rare compared to AML or ALL and have only recently gained interest [107].

1.3.3.3 - Treatment of acute lymphoblastic leukemia

Similar to AML, ALL treatment follows the induction, consolidation, and maintenance methodology and can take up to two years [108]. The first consolidation extends 4 weeks with a combination of vincristine, glucocorticoid steroids, anthracyclines, and asparaginase. The alkaloid vincristine acts as an antimetabolic agent by inhibiting the polymerization of actin and, therefore, directly disrupting the cytoskeleton [109]. While it is relatively unspecific and causes severe side effects due to its target, low doses of vincristine have still proven to be a reliable component in multiple treatment protocols. Glucocorticoids like dexamethasone or prednisolone have a plethora of clinical uses due to their immunomodulatory, anti-inflammatory, and antileukemic properties, despite their poorly understood mechanism [110]. A key factor for determining the appropriate next of therapy is whether the malignant cells are susceptible or resistant to glucocorticoids. At the same time, glucocorticoids cause numerous severe side effects, such as severe infections due to immune suppression, hypertension, or hyperglycemia [111].

Anthracyclines like idarubicin or doxorubicin induce effects and risks that are similar to AML, but have ultimately proven to be also effective in this disease [112]. The final component of the standard care is the enzyme asparaginase, which follows an intriguing mechanism of action. Instead of directly damaging the cancer cells, the enzyme catalyzes L-asparagine degradation to L-aspartic acid and ammonia in the serum [113]. Since the cancer cells lack the enzyme to synthesize L-asparagine, this effectively starves the cancer cells while healthy cells are unaffected. Therefore, protein synthesis is impaired, and apoptosis is induced. Despite the lack of direct cytotoxicity, side effects like hepatotoxicity, thrombosis, or hyperglycemia are still attributed to Asparaginase [114]. All four drugs need to be administered at carefully evaluated doses and adapted depending on the response of the patient [108]. After achieving complete remission, the induction phase is followed by the consolidation regimen, which consists of a longer-than-six-month rotational schedule of high doses of cytarabine, methotrexate, asparaginase, and other drugs. The concluding step is the maintenance phase, during which methotrexate and mercaptopurine are given every three months for up to two years.

While the previous paragraphs broadly describe the general standard care protocol, the subtype of ALL and genetic background can lead to significant modifications. An important example would be *BCR::ABL1*⁺ B-ALL, where TKIs have been established as an integral addition to the standard chemotherapy regimen [115]. First- and second-generation TKIs, like imatinib or dasatinib, have proven their effectiveness and have led to a dose reduction of the harmful chemotherapy backbone. Conversely, the Ph-like subtype lacks the *BCR::ABL1* kinase as a target and is attributed with a poor prognosis. This reduces the effectiveness of the TKI. However, ponatinib, as a multitarget inhibitor, still affects other

kinases [116]. Other studies are investigating the potential use of JAK inhibitor ruxolitinib due to the implication of JAK mutations [117]. As for hypodiploid leukemia-afflicted patients, recent investigations highlighted the potential of PI3K inhibitors [118].

T-ALL follows a comparable treatment protocol used for B-ALL and shares its sensitivity to Asparaginase [119]. Extensive investigations focused on the nucleoside analog nelarabine and showed that it is especially potent in early relapse T-ALL combined with the alkylating agent cyclophosphamide [120]. Additionally, due to their high presence, the NOTCH family has been deemed an ideal target in T-ALL [100]. NOTCH1 inhibition via γ -secretase inhibitors has been established and showed a transient antileukemic effect in a clinical trial, but was offset by gastrointestinal toxicity [121]. A considerable factor could be that some of the regularly found lesions of genes like PTEN confer resistance [122].

A rapidly developing field in ALL is the use of immunotherapy. Monoclonal antibodies that target CD19, CD20, CD22, or CD52 could be used to improve the outcome of the conventional chemotherapy [123]. Unconjugated antibodies that bind to CD22, like epratuzumab, have been clinically investigated, but failed to deliver a significant effect [124]. A far more superior approach is the generation of synthetic bispecific antibodies which bind e.g. the CD3 of T cells and an overrepresented antigen of the leukemic cells simultaneously [125]. The colocalization of T cell and leads to an effective and specific antileukemic immune response. Especially noteworthy in the context of B-ALL is blinatumomab which complemented or even conventional B-ALL chemotherapy protocols in clinical trials [126-128]. Drug-conjugated antibodies, like inotuzumab ozogamicin, significantly improved survival in other trials [129]. Generating CAR T-cells that specifically work in B- or T-ALL has been quite successful. Trials with CD19-specific CAR T-cells showed excellent complete remission rates despite not being supplemented by any additional treatment [130].

1.3.4 - Chronic lymphoblastic leukemia

Chronic lymphoblastic leukemia (CLL) is a slowly progressing disease that derives from the lymphoblastic lineage. While exceedingly rare in children (2%), it is quite often found in adults (40%) [19]. Due to its slow progression, the five-year survival is very high, with >92% in adults [29]. However, the relapse rates still reach up to 40%, which necessitates improved therapeutic strategies [30]. Culturing CLL cells is quite challenging since they depend on a particular tumor microenvironment and otherwise undergo spontaneous apoptosis [131]. While generally, some progress has been made to establish a more robust CLL cell line model, it is still not applicable in a broader, general use [132]. Therefore, CLL does not play a significant role in this dissertation and is not explained in greater detail.

1.4 - Epigenetic regulation via histone modifications

Life depends on the complex interplay of the many cellular components, like proteins. While the genomes determine which proteins can be expressed, these rather minute differences do not explain why we differ so much from individual to individual. Therefore, how much these proteins are expressed also matters. While different promoters can modulate the expression rate, additional mechanisms are still necessary. Major contributors to the regulation of gene expression are epigenetics. This term comprises two distinct processes. First is the methylation of cytosine, which happens mainly in the CpG sites of the promoter or enhancer regions [133]. The added methyl group represses the binding of the transcribing enzymes and prevents transcription. Moreover, methylated cytosines attract methyl-CpG-binding domain proteins, which interact with nucleosome remodeling complexes, which can further silence these regions [134].

These nucleosome remodeling proteins are the second core component of epigenetics. Roughly 150 bp of DNA are wrapped around eight histone molecules that form repetitive units of nucleosomes [135]. Two of each H2A, H2B, H3, and H4 histone subcomponents form the complete histone protein, each with a tail that sticks out of the protein. Different residues can be either added by epigenetic writers or removed by epigenetic erasers to the tail [136]. The residue further determines how the conformational structure of the chromatin changes due to how tightly the chromatin wraps around the histone. Intermolecular forces at the lysine tails determine, furthermore, whether the histones induce condensation or decondensation of the nucleosome. Commonly known histone posttranslational modifications include methylation, phosphorylation, or acetylation. However, recent studies also identified other processes like lactylation, succinylation, or malonylation [136]. Therefore, the field of epigenetics is quite complex and still evolving.

1.4.1 - Histone deacetylases

Histone deacetylases (HDAC) belong to the class of epigenetic erasers and are the counterpart of histone acetyltransferases (HAT). They catalyze the reversible deacetylation or, respectively, acetylation of the N-terminal histone lysine tails (see Figure 3 [137, 138]). Deacetylation increases the ionic interactions between positively charged histones and negatively charged DNA [137]. Therefore, the intermolecular attraction of both molecules increases, leading to a stronger bond between them. The DNA wraps more tightly around the histones, which initiates nucleosome formation and, therefore, prevents transcription machinery access to the specific location. HATs, on the other hand, reverse this process and acetylate the lysine tails, which induces a more negative polarity, weakens the molecular adhesion, and increases accessibility and transcription. Classical HDACs are zinc-dependent and carry a Zn^{2+} ion in their catalytic domain. Their structure adopts a α/β fold, which is similar to other metalloenzymes [139]. Further details, such as the protein structure, deacetylation target sites, and protein interactions, are specific to the individual HDAC. Some HDACs have a broader target spectrum and deacetylate cytoplasmic proteins or transcription factors.

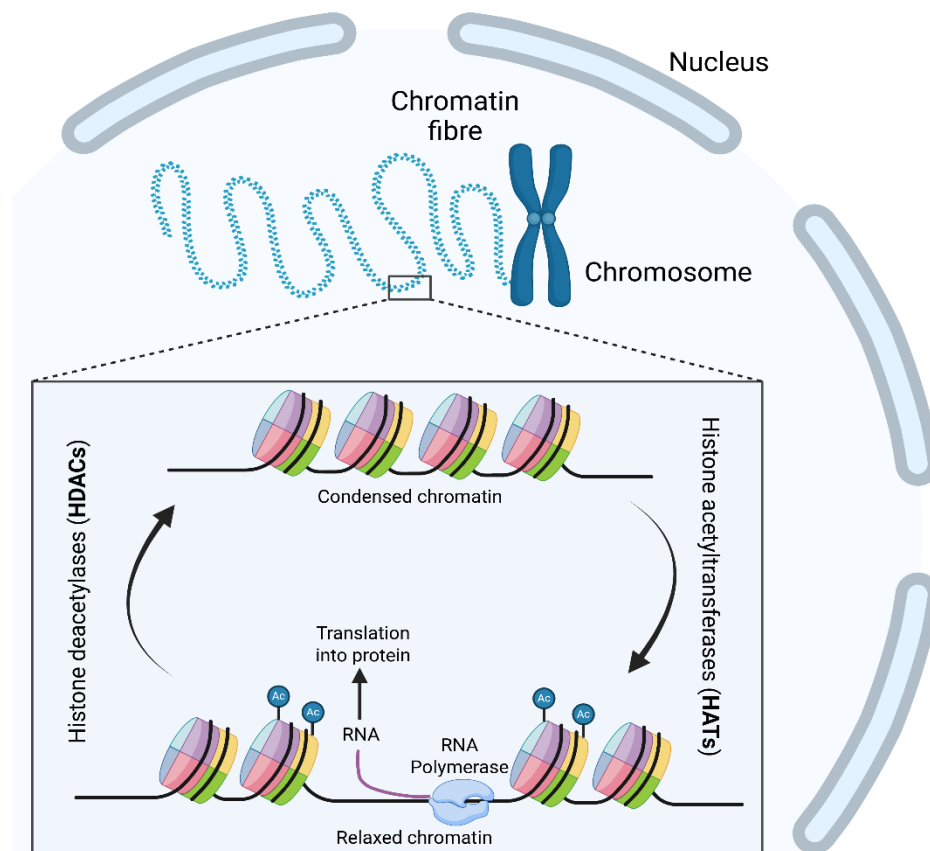


Figure 3: Epigenetic regulation of gene transcription via histone tail acetylation by histone acetyltransferase or deacetylation by histone deacetylase. Created with Biorender.com, based upon [137-138]

HDACs were first identified in yeast, and all 18 human HDACs were categorized, depending on their yeast homology, into five classes. Classes I, IIa, IIb, and IV are the classical zinc-dependent enzymes. However, class III are atypical since they depend on NADPH and are called sirtuins [140]. Since the inhibition and mechanisms of sirtuins are vastly different from the classical HDACs, they are not explained further. Class I of the HDAC family consists of HDAC1, HDAC2, HDAC3, and HDAC8 (see Figure 4 [141-144]). All four proteins are comparably small and localized in the nucleus. The first three representatives of this subcluster share high levels of homology and are predominantly found in cooperative protein homo- or heterodimer complexes like CoREST, SMART or Sin3A [141, 145]. While their main focus is the deacetylation of histones, they can further target proteins like p53, E2F, or STAT3. A systemic knockout (KO) of HDAC1 and HDAC3 is lethal in mouse embryos, while a loss of HDAC2 can seemingly be compensated by other HDAC [146-148].

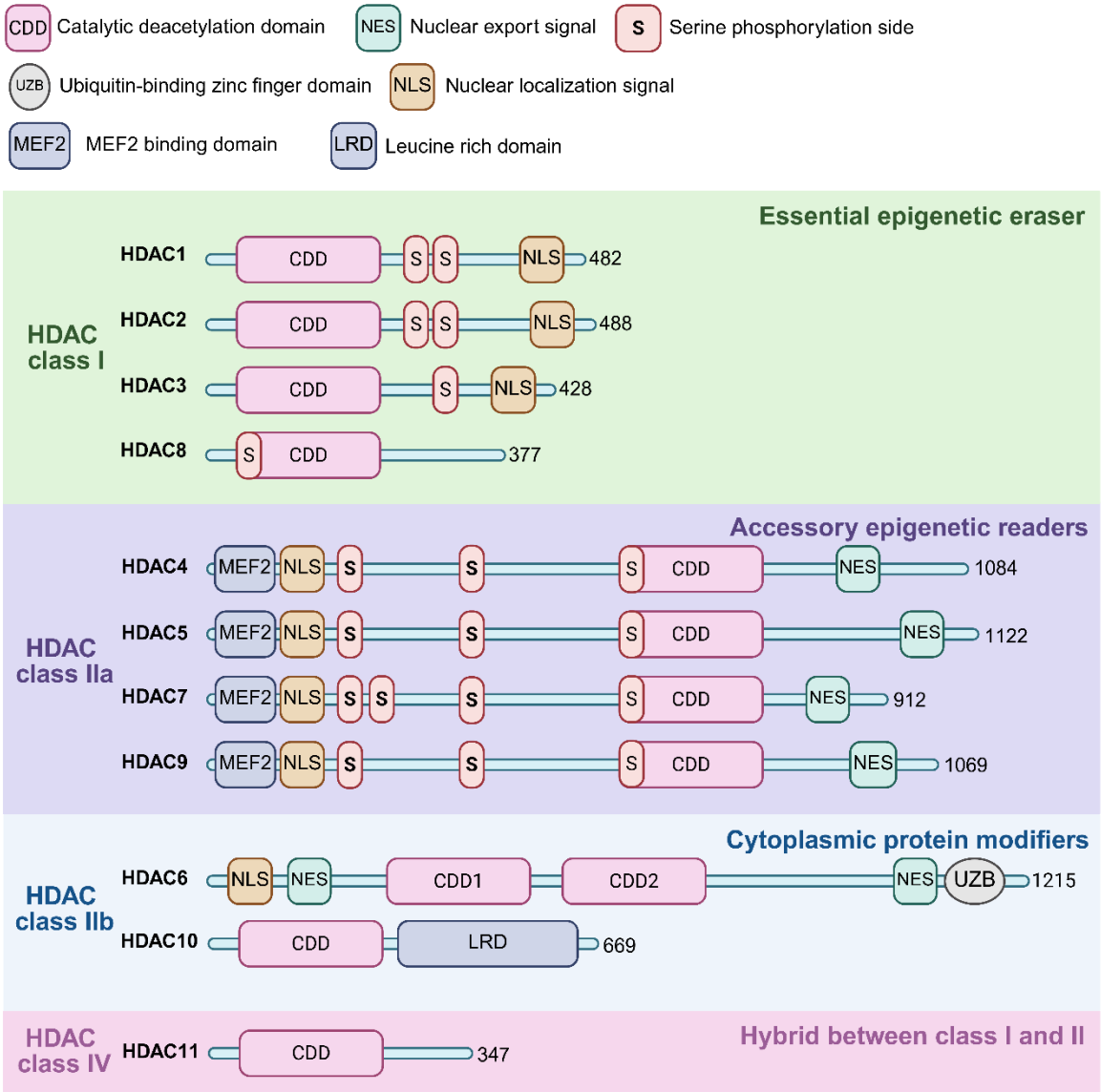


Figure 4: Structural comparison of the different HDAC clustered into the different HDAC family. Numbers indicate the amino acid length of the respective protein. Created with Biorender.com, based upon [141-144]

HDAC8, on the other hand, has distinct features compared to the other class I members. Since it does not contain a C-terminal protein-protein interaction domain, it does not associate with other proteins to form complexes [149]. Its catalytic activity against histone molecules is lower than that of the other class members, but it also targets other nuclear proteins like SMC3, p52, or ERRA. Furthermore, it was proven that HDAC8 can catalyze the deacetylation of long-chain fatty acyl lysine [150].

HDAC4, HDAC5, HDAC7, and HDAC9 belong to class IIa (see figure 4). In contrast to class I, they possess a nuclear export signal, which is regulated by the phosphorylation of serine and allows them to enter the cytoplasm [151]. Nuclear localization domains reverse this process and allow the proteins to enter the nucleus. Regarding the protein size, class IIa is significantly bigger. Their catalytic activity against acetylated lysine histone tails is much lower due to a mutation that switched an essential tyrosine with histidine [152]. Thus, it has been questioned whether their actual target has yet to be determined or if these HDACs are just pseudoenzymes. However, it has been described that their function could be seen as epigenetic readers that are used in larger protein complexes to identify acetylated lysine domains [153]. HDAC class IIb is unique from the other classes and comprises HDAC6 and HDAC10. Structurally, both differ significantly from each other. Since HDAC6 and its inhibition is a major focus of this dissertation, its unique structure and functions are explained in more detail later. Like the class IIa members, HDAC10 can be found both in the nucleus and cytoplasm. Where it significantly differs is its transcriptional repressor function. Rather than deacetylating histone tails, HDAC10 seems to catalyze the hydrolysis of acetylpolyamine [154]. Finally, class IV only consists of HDAC11, the smallest family member. It primarily localizes in the nucleus, and similar to HDAC8, it catalyzes the hydrolysis of acyllysine of long-chain acyl groups [150]. These points highlight that the HDAC family members, despite their homology, act through various mechanisms and have different targets.

1.4.2 - Structure and function of histone deacetylase 6

Histone deacetylase 6 (HDAC6) is a unique member of the HDAC family due to its structure and function. Not only is it the largest family member, but it also possesses two catalytic domains (see Figure 5 [155, 156]). Both can catalyze lysine deacetylation. However, novel insights have shown that both have different targets, and their mechanisms are not fully understood [157]. Catalytic domain 1 (CD1) seems more substrate-specific by targeting only C-terminal acetylated lysine residues, while catalytic domain 2 (CD2) displays a broader specificity, which also includes internal acetyl-lysine residues [158]. In a study from Ariyaratne et al. in 2025 a proteomics-based trapping approach identified 94 unique CD 1 client proteins (e.g. HUWE1, UBR5, DDX3X), 8 which were shared by both domains, and

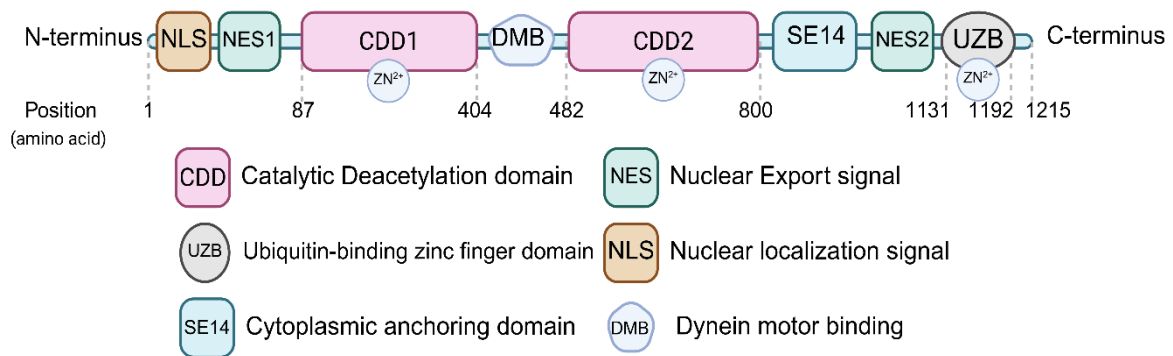


Figure 5: Summary of the structural domains of HDAC6 and their position in the protein. Created with Biorender.com, based upon [155,156]

112 CD2 client proteins (e.g. α -tubulin, cortactin, HSP90) [159]. Therefore, both domains seem to have a mostly individual client spectrum which indicates distinct biological functions. While both domains seem to predominantly act on cytosolic proteins it has been shown that both CD1 and CD2 have the ability to catalyze histone deacetylation, at least *in vitro* [158]. In addition to these catalytic domains, HDAC6 contains a ubiquitin-binding zinc finger domain (UZB) that enables interaction with ubiquitinated proteins. Together these three functional domains allow HDAC6 to act as a multifunctional protein which is deeply entrenched in many biological pathways.

HDAC6 further contains a nuclear localization signal, two nuclear export signals, and a cytoplasmic anchoring domain [160]. In principle, HDAC6 can shuttle between the nucleus and the cytoplasm through these features. Most of the protein tends to localize in the cytoplasm due to the anchoring domain and specific acetylation motifs delivered by HATs like EP300 [161, 162]. Via the dynein motor binding domain (DMB), HDAC6 can interact with dynein motor proteins. This permits the protein to use the cytoskeleton to move through the cytoplasm to transport bound cargo. In addition to these functional protein domains, HDAC6 possesses various posttranslational modification sites. These enable functional regulation or localization through phosphorylation via GSK-3 β or EP300 acetylation [162, 163].

The described domains and modification sites enable HDAC6 to modulate various cellular functions and pathways including:

- Gene expression regulation via histone deacetylation
- Cellular motility via α -tubulin and cortactin deacetylation
- Heat shock response via heat shock complex dissociation and HSP90 deacetylation
- Aggresomal accumulation and degradation via autophagy modulation
- Proliferation through PTEN and EGFR deacetylation
- DNA damage response and apoptosis via TP53 or LH1 deacetylation
- Immune modulation via PD-L1 and NF- κ B deacetylation

1.4.2.1 - Gene expression regulation

While HDAC6 is primarily localized in the cytoplasm during proliferation, specific conditions can induce its translocation into the nucleus. Acetylation sites within HDAC6 that EP300 targets enable nuclear translocation via importin- α and simultaneously diminish its activity on α -tubulin but not histones [162]. Here, especially, CD2 seems to be active since the N-terminal lysine residues of the histones are affected [158]. It should be noted that HDAC6's direct effects on histones are still rare since its primary targets are localized in the cytoplasm. Furthermore, it is difficult to distinguish its effect from other family members like HDAC1, HDAC2, or HDAC3. To some degree, HDAC6 seems to be able to form complexes that could also contribute to the epigenetic reading and writing properties. A prominent example is the CoREST complex, which can incorporate HDAC6 next to HDAC1, HDAC2, and LSD1 under specific cases [164]. A significant cross-talk was found between HDAC6 and LSD1, a lysine demethylase [165]. Recent evidence further revealed that the KAT5-EP400 complex uses HDAC6 to enhance its binding to its target genes [166]. However, HDAC6 interacts with transcription factors like RUNX2, NF- κ B, and LCoR. To repress the p21 promotor, RUNX2 recruits HDAC6 from the cytoplasm via its carboxy terminus [167]. NF- κ B was observed to utilize HDAC6 to repress the H⁺-K⁺ ATPase α 2 subunit gene in mice [168]. A broader effect of HDAC6 can be observed in conjunction with LCoR, since it enhances the repression of estrogen-inducible genes in breast cancer [169]. In conclusion, the regulation of gene expression by HDAC6 remains poorly understood and appears to be highly context-dependent.

1.4.2.2 - Cell motility

Two very important components of the cytoskeleton are α -tubulin and cortactin, and simultaneously client proteins of HDAC6 (see figure 6). Microtubules are built from the polymerization of many α -/ β -tubulin molecules that connect in specific patterns [170]. At lysine 40 of α -tubulin, α -tubulin acetyltransferases can add acetyl residues while HDAC6 removes them [171, 172]. These modifications seemingly play a significant role in the stability of the microtubules. However, the exact mechanism remains unclear. Still, there seems to be a definitive connection between HDAC6 and the microtubule dynamics [173, 174]. A general assumption is that α -tubulin acetylation stabilizes the microtubule structure, whereas deacetylation destabilizes it. Through this effect, the cells can rapidly reshape their cytoskeleton and fundamentally increase their motility.

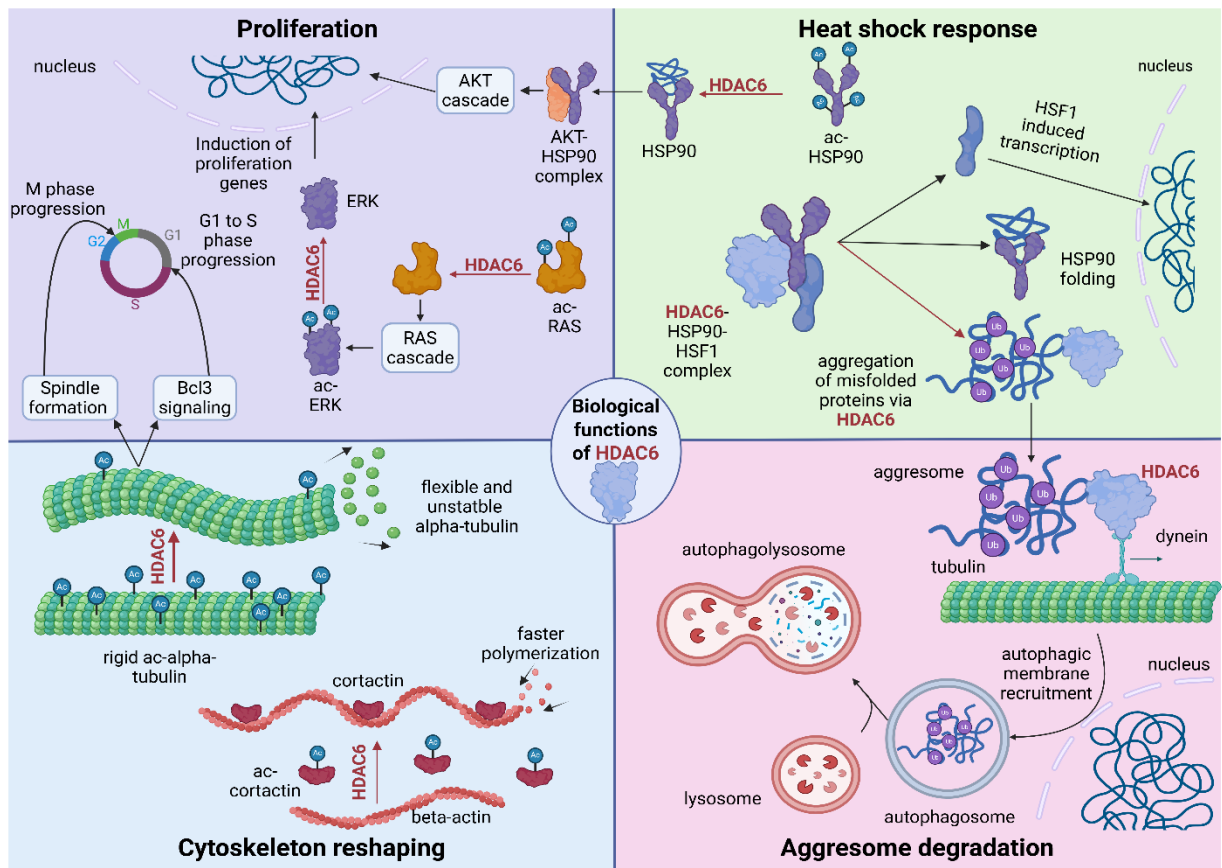


Figure 6: Overview of prominent cellular functions of HDAC6 in regards to cellular motility, proliferation, heat shock response, and aggresome degradation. Created with Biorender.com based upon the sources from the HDAC6 subchapters

Similar mechanisms are observable for cortactin. Cortactin contains F-actin binding repeats, which enable it to interact with actin filaments. EP300 can acetylate the lysine residues in this domain, to discourage the translocation to actin-rich areas and binding of actin strands [175]. When successfully bound, the actin filament gets stabilized, and this promotes its elongation through polymerization [176]. HDAC6 can deacetylate the F-actin binding repeats and, therefore, facilitates cortactin-actin interactions. Increased actin polymerization speed is a key factor in cellular motility. A regulator of HDAC6 activity on both α -tubulin and cortactin is GRK2. This G protein-coupled receptor kinase phosphorylates the S670 position of HDAC6 [177]. This posttranscriptional modification induces the translocation and activity of HDAC6 to the lamellipodia of migrating cells. Further HDAC6 phosphorylation sites that alter its protein structure and catalytic capabilities in this regard have been recently identified [178]. Since the cytoskeleton is an important cornerstone of organization and metabolism, it implies that HDAC6 has a significant biological effect.

1.4.2.3 - Heat shock response

Certain stimuli, like intense heat, oxidative stress, radiation, or organic and inorganic compounds, can induce the rapid expression of the so-called heat shock response genes [179]. These chaperones and cofactors are tasked with folding and maintaining the functional structures of their client proteins. A core component of this pathway are HSP90 and HSF1. HSP90 is a major chaperone with an inducible (HSP90 α) and a constitutive form (HSP90 β). HSF1, on the other hand, is a transcription factor that induces the expression of the heat shock response genes. In cells that are not stressed, HDAC6 associates into a complex with HSP90, HSF1, and VCP/p97 [180] (see figure 6). When external stimuli prevent the correct folding of proteins, and they accumulate in the cytoplasm, HDAC6 initiates the dissociation of the complex. The released HSF1 translocate into the nucleus and induces the expression of essential heat shock genes like *HSP90AA1* (HSP90 α) or *HSPA1A* (HSP70).

HSP90 furthermore contains certain acetylation sites that HDAC6 can deacetylate. This affects how HSP90 can bind to its co-chaperones, ATP, and client proteins, and studies have shown that it directly impairs its chaperoning function [181, 182]. Without HDAC6, HSP90 client proteins would face ubiquitination and, subsequently, degradation. However, HDAC6 is a client of HSP90-mediated folding and, therefore, dependent on the chaperone [183]. Phosphorylation of serine 22 of HDAC6 via GSK-3 β is associated with cellular stress and could, therefore, directly influence the chaperoning function of HSP90 [163]. Furthermore, the UZB is a relevant non-direct interaction partner of HSP90 due to its clearance of misfolded proteins that are often even HSP90 client proteins. These results highlight that HDAC6 and HSP90 are deeply interconnected and essential to ensure vital cellular functions.

1.4.2.4 - Aggresome degradation via autophagy

Damaged, misfolded or protein junk is routinely degraded via the proteasome, however, if overwhelmed or dysfunctional, the accumulating microaggregates can become toxic and affect the cell viability [184]. In response, cells can activate the aggresomal pathway. This allows them to specifically organize the distributed particles at one spot by accumulating them into aggresomes. Subsequently, the aggresomes are cleared with the help of autophagy. HDAC6 has multiple crucial tasks in forming and digesting these aggresomes (see Figure 6). Upon dislocation from HSP90 and HSF1, HDAC6 can bind to ubiquitin of ubiquitinated proteins through UZB. After recruiting dynein via the DMB, HDAC6 transports its bound cargo along the microtubules to the microtubule organization center [185]. HDAC6

then deacetylates cortactin, which initiates the aggresome formation by stacking the protein aggregates at one spot [186].

After completion of the aggresome formation via chaperones TRIM50 and p62, the autophagosome engulfs the aggresome and fuses with the lysosome. Here, HDAC6 again recruits and deacetylates cortactin, which binds the two vesicles to the actin filament and allows their fusion to build the autophagolysosome [187]. A further contributor to the autophagosome formation is the c-Jun NH2-terminal kinases, which can be activated via HDAC6 [188]. The contained hydrolases start digesting the aggresome and clear the accumulated junk. HDAC6 involvement in this process extends even further since it also regulates autophagy. It can deacetylate LC3B-II, a regulator of autophagy, which is necessary to induce autophagy successfully [189]. Due to its interaction with HSP90, HDAC6 furthermore indirectly targets and seemingly stabilizes essential lysosomal proteins like LAMP2 [190]. In summary, highlight these findings that HDAC6 is involved in healthy protein homeostasis beyond its involvement with the heat shock response.

1.4.2.5 - Proliferation

While not primarily known as a key regulator of proliferation, HDAC6 can target a plethora of signaling pathways that are connected to proliferation. PI3K/AKT signaling can be modulated by HDAC by multiple means. HDAC6 can deacetylate PTEN at K163, which deactivates its suppressive influence on AKT signaling [191]. In some circumstances, HDAC6 can even affect the total levels of PTEN [192]. Indirectly, HDAC6 is connected to AKT itself since HSP90-mediated chaperoning depends on the deacetylated form of HSP90 [181]. Recent studies have shown that two of HDAC6's potential substrates are ERK1 and ERK2. Both initiate the ERK/MAPK pathway, and it has been proven that deacetylation via HDAC6 increases their activity [193]. At the same time, HDAC6 has phosphorylation sites that ERK can target to stimulate cellular migration [194].

Since the acetylation level of tubulin affects the stability of growth factor receptors like EGFR, and EGFR signaling to ERK1/ERK2 is pivotal for its activity, another facet of regulation via HDAC6 is tangible [195]. The EGFR-RAS-RAF-MEK-ERK axis interconnects this since HDAC6 can deacetylate RAS to induce RAF phosphorylation [196]. Another mechanism of action of HDAC6 on proliferation is via Wnt-signaling through the deacetylation of β -catenin [197]. When β -catenin translocates from the cytoplasm into the nucleus, it induces the expression of proto-oncogenes like c-Myc. HDAC6 also regulates, at some points, the progression through the cell cycle due to the tubulin acetylation level. Tubulin deacetylation prevents the inhibition of Bcl-3 genes via CYLD proteins and subsequently leads to G1- to S-phase progression via NF- κ b [198, 199]. For the spindle

formation during the M-phase, the tubulin acetylation level is crucial for assembling the correct structure [200]. In total, HDAC6 can induce pro-proliferative impulses through various pathways.

1.4.2.6 - DNA damage response and apoptosis

Upon increased cellular stress levels, like DNA damage, p53 is induced as a response and accumulates in the nucleus. If the damage is repairable, p53 modulates other cell cycle progression genes to halt the cell cycle at the G1/S or G2/M phase [201]. When successfully repaired, the cells usually progress through the different phases. However, in case of irreversible damage, p53 acts as a tumor suppressor and induces the expression of pro-apoptotic genes. Class I HDAC and HDAC6 have recently been proven they deacetylate p53 at several sites [202, 203]. These acetylation markers affect the stability and transcriptional activity of p53. A major effector in this regard is lys-381/382, which, if deacetylated, inactivates the protein [203]. Further interaction between p53 and HDAC6 happens in conjunction with RUNX2 complexes. The RUNX2/p53 complex can counteract the expression of p53-regulated genes and, therefore, acts as a direct counteragent. This complex can further recruit HDAC6 to amplify its repressive functions to counteract DNA damage-induced apoptosis [204].

Other studies have shown that HDAC6 can act on DNA repair by the key mismatch repair protein MLH1. It can deacetylate lys-33 or lys-241, decreasing MLH1's affinity for the MutS α -MutL α complex [205]. Together, these components would ensure the strand integrity by correcting mutations; however, through HDAC6 deacetylation, the DNA damage tolerance is increased. Further mismatch repair proteins that are targeted through HDAC6 are MSH2 and MSH6. Recent studies found that their deacetylation via HDAC6 decreases their stability and induces proteasomal degradation via ubiquitination [206]. This impairs the mismatch repair mechanism and increases error tolerance. These findings highlight that HDAC6 can modulate DNA repair and have an anti-apoptotic function under specific circumstances.

1.4.2.7 - Immune modulation

Recently emerging studies have shown that HDAC6 potentially regulates important immunomodulatory pathways and could play a role in immunotherapy. A prominent example is PD-1/PD-L1 signaling, which regulates the reactivity of immune cells to prevent autoimmunity. A key regulator of this pathway is STAT3. Recent studies established that HDAC6 controls the expression of PD-L1 in melanoma patients via STAT3 [207]. Upon

HDAC6 loss, STAT3 transcription induction of PD-L1 was reduced. However, this was not directly linked to STAT3 acetylation. This would imply an indirect influence of HDAC6 on STAT3 and, subsequently, PD-L1. The publication further established changes in the upregulation expression of MHC class I and other tumor-associated antigens like gp100, MART1, TYRP1, and TYRP2.

Further studies have proven the effect of HDAC6 on the PDL-1 expression and regulation of T cell activity in melanoma and multiple myeloma [208, 209]. Regarding T cell activity, HDAC6 furthermore seems to amplify the expression of IL4, IL5, IL6, IL10, and IL13 [210]. These Th2 cytokines suppress Th1 cell activation and the expression of IFN γ and IL17. All of the mentioned components are critical for the response of cytotoxic CD8⁺ T cells regarding cancer and other diseases. Other significant immune-relevant pathways include the pro-inflammatory NF- κ B or IL1 signaling. Not only does HDAC6 interact with NF- κ B but it also controls IL1 induction and downregulates I κ B, which abrogates NF- κ B signaling [211]. Both are driving positive modulators of inflammation. While HDAC6 is a relatively novel player in immune modulation, some compelling evidence has emerged that would make it an attractive target in this context.

1.4.3 - Histone deacetylase in the hallmarks of cancer

The hallmarks of cancer describe a subset of biological functions that are essential/altered upon oncogenesis. Traditionally, this includes proliferation, metastasis formation, angiogenesis, differentiation, immune evasion, and inflammation [212]. While more and more subsets of these hallmarks are identified, this dissertation only focuses on the traditionally known pathways within the context of HDAC (see Figure 7 [213]). Alteration of HDAC activity has been described in several different contexts of cancer, including both increased and decreased [137]. Here, the cellular and mutational background seems to be a deciding factor. A not-to-be-underestimated factor is also the ratio of HDAC and interaction within complexes. The defining problem of cancer is its ability to go rapidly through the cell cycle and proliferate. In this regard, HDAC1, HDAC2, and HDAC3 have been established as core regulators, while the other HDACs seem more circumstantial [213, 214].

Due to its targets, such as α -tubulin and cortactin, HDAC6 has been recognized as an essential player regarding cellular motility. Since establishing metastasis (in solid tumors) requires that the cells leave their original location and migrate to spread the disease, HDAC6 could be a major effector [215]. Other HDACs that have amplifying functions in metastasis formation include HDAC1 and HDAC2 [213]. Angiogenesis describes the

formation of additional blood vessels. Solid cancer tumors often induce angiogenesis to increase their growing nutrient and oxygen demand. Through various mechanisms, HDAC has been attributed to be pro-angiogenesis. In oxidative stress, the heat shock response is a key player that can further induce angiogenesis [216]. Not only HDAC6 but also HDAC4, HDAC5, and HDAC7 seem to have some influence on the HSF1 acetylation and subsequently angiogenesis. Cancer cells tend to be immature and not fully differentiated, enabling them to adapt their metabolism and proliferate faster. The role of HDAC in differentiation is highly specific to every tissue [137]. However, an often-occurring effect of HDAC inhibition is the induction of differentiation.

Since cancer cells behave detrimental to the individual's health and often miss integral compatibility components, they tend to induce an immune response initially. However, over time, low immunogenic clones will escape the immune system and, to a certain degree, reprogram immune cells to aid the tumor. Many HDAC family members play a significant role in the innate and adaptive immune system at different points [217]. Connected to the immune system is the process of inflammation. While this is typically intended to combat cancer, it has been shown that tumor cells can also utilize it for their benefit. Here, HDAC3 and HDAC6 are key contributors [137]. One standout throughout all these different functions and pathways is HDAC6, since it seems to be universally involved. While this could lead to the interpretation that HDAC6 is a high-value target, one should not forget that its

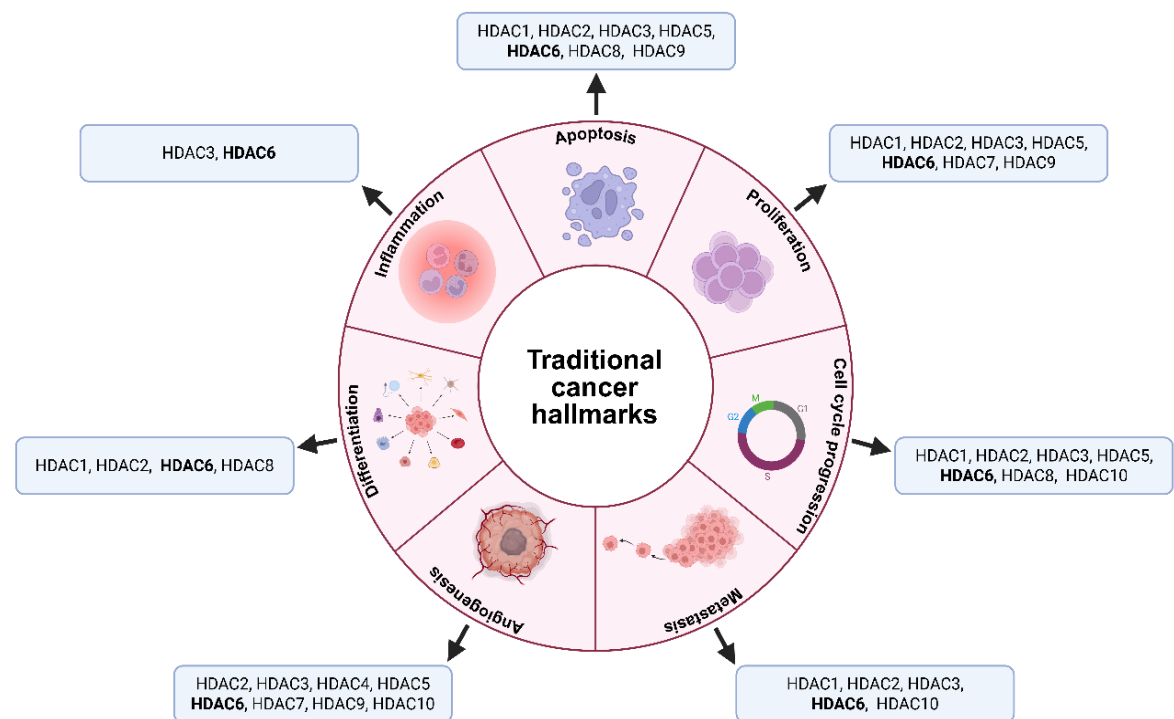


Figure 7: Involvement of the HDAC family members in the hallmarks of cancer. Created with Biorender.com, based upon [213]

involvement is highly circumstantial. In general, these points highlight why the HDAC family has gained a lot of focus in clinical research.

1.4.4 - Histone deacetylases in the hematopoietic system and leukemia

HDACs play a significant role in the control of cell metabolism and differentiation. In the context of the hematopoietic system, many HDAC family members play key roles in inducing differentiation into specific branches and even their survival. HSCs themselves seem highly dependent on the function of certain HDACs. Core actors of HSC maintenance are the class I HDACs. Especially HDAC1 and HDAC2 sustain the HSC homeostasis and the bone marrow integrity [218]. While they have, to a high degree, overlapping functions, it has been revealed that they contribute differentially to HSC development. HDAC3, on the other hand, seems to be more ambiguous in its function. In a mouse study, it was demonstrated that it is essential for HSC self-renewal in mice. However, cord blood studies found that HDAC3 can also restrict cellular expansion [219, 220]. HDAC8 can complement the role of HDAC1 and HDAC2 and helps to maintain the hematopoietic population. It becomes exceptionally important in cellular stress, modulating p53 activity to ensure HSC survival [221]. Other HDAC family members have not been directly implicated in playing a direct role in HSCs' homeostasis.

Further involvement of HDAC includes the control of differentiation of the many branches of the hematopoietic system. HDAC1 and HDAC2 seem to be key drivers that regulate the differentiation of every lineage to some degree. Especially in the myeloid lineage, it is influential since it seems to repress the differentiation of common myeloid progenitors into granulocyte-monocyte progenitors to favor the megakaryocyte-erythrocyte progenitor lineage [222, 223]. Together with the CoREST complex, it can further influence the differentiation of erythroid, megakaryocytic, and granulocytic lineages. HDAC3, on the other hand, has more involvement in the lymphoid lineage. The loss of HDAC3 leads to a depletion of both B and T cells via cell cycle arrest of the earliest lymphoid progenitors [220]. For the correct differentiation from monocytes to conventional dendritic cells, HDAC4 plays an important role [224]. HDAC5 and HDAC6 contribute to the differentiation and survival of the erythroblast lineage [225, 226]. An essential contributor to the terminal differentiation of macrophages is HDAC7, which happens in conjunction with HDAC1 [227]. HDAC11 has been shown to play an indispensable role in the biology and differentiation of neutrophils [228]. In conclusion, the HDAC family is deeply involved in the development and survival of several components of the immune system.

1.4.4.1 - HDAC in AML

Due to their origin, leukemic cells seem to share the dependency on HDAC of their healthy counterpart. Throughout all different leukemia subtypes, overexpression of several HDACs has been observed [229]. The origin of leukemia is often attributed to genetic lesions and epigenetic dysregulation. Often, these mutations directly contribute to these epigenetic shifts. In AML, ~35% of cases carry mutations that directly affect the epigenetic regulation [32]. While mutations of HDACs themselves are comparably rare, often, the translocated fusion proteins like PML::RAR α , RUNX1::ETO1, or MLL fusions recruit HDAC family members. In the case of PML::RAR α , HDACs will be utilized to deacetylate p53 [230]. Furthermore, the oncoprotein blocks the differentiation through RAR gene repression mediated by HDACs [55]. RUNX1::ETO uses HDAC to silence AML1 target genes, which are typically necessary to limit proliferation and prevent oncogenic transformation [231]. HDAC1, HDAC2, and HDAC3, in particular, contribute to blocking differentiation by RUNX1::ETO [232]. These three HDACs are also necessary for MLL-fusion-induced chromatin remodeling. MLL's repressive domain can bind to HDAC1 and HDAC2 and amplify chromatin hypoacetylation to repress transcription [233, 234]. Further important interactions between different HDAC family members and AML-relevant proteins include p53, SMC3, MEF2, and the SMRT complex [235]. HDAC6 specifically seems to drive AML through hedgehog signaling-induced ciliogenesis *in vivo* [236]. A connection between HDAC6 and improved DNA repair mechanism upon doxorubicin treatment has been also reported [237]. Therefore, many links have already established that HDACs are necessary to establish and maintain the AML phenotype.

1.4.4.2 - HDAC in CML

CML leukemogenesis is highly dependent on the BCR::ABL1 fusion gene. Fusion proteins like BCR::ABL1 often depend on HSP90-mediated chaperoning. Due to HDAC6's ability to directly affect HSP90's catalytic activity via acetylation, it indirectly controls the stability of BCR::ABL1 [238]. Studies have shown that HDAC6 is upregulated in CML, and its activity on PTEN directly modulates the activity of AKT signaling [239]. Another connection was established between HDAC6, STAT5, and the stability of tumor driver c-MYC in CML [240]. Significant for the cellular survival of imatinib-resistant CML were both HDAC1 and HDAC2. Upon their loss, cells induced apoptosis since their aberrant p53 acetylation was lost [241]. HDACs potentially increase the expression of anti-apoptotic proteins like XIAP in BCR::ABL1⁺ cells [242]. However, compared to AML, less concrete evidence is available to establish that HDAC inhibitors are a viable treatment tool in CML.

1.4.4.3 - HDAC in ALL

HDACs function in ALL is similarly essential. Likewise, to the myeloid lineages, the survival of ALL cells depends on functional HDAC1 and HDAC2 [243]. Expression profiling of B-ALL and T-ALL compared to healthy controls revealed general upregulation of HDAC2, HDAC3, HDAC6, HDAC7, and HDAC8 [244]. B-ALL specifically displayed elevated levels of HDAC5, while in T-ALL, HDAC1 and HDAC4 expression were amplified. Overexpression of HDAC3 and HDAC7 was associated with an overall worse prognosis. In MLL-rearranged ALL, HDAC4, and HDAC5 seem to be driving factors [245]. However, compared to other subtypes, the exact mechanism and interdependencies of HDAC regarding ALL are poorly characterized. Similar to CML, evidence regarding ALL is still limited, and additional research is necessary.

1.4.5 - Histone deacetylase inhibitors

Over the last decades, a rather large variety of small-molecule HDAC inhibitors (HDACi) have been identified, designed, and synthesized [246].

1.4.5.1 - Structure and selectivity

A crucial aspect regarding HDACi is their structure, which defines their HDAC inhibition profile through binding the Zn^{2+} ion in the catalytic domain [247]. The determining factor regarding selectivity and potency is how the inhibitor interacts with the structure of the

Table 1: Binding affinity to the HDAC family members of a representative selection of HDAC inhibitors

HDAC class	Class I				Class IIa				Class IIb		Class IV
HDAC Inhibitor	HDAC1	HDAC2	HDAC3	HDAC8	HDAC4	HDAC5	HDAC7	HDAC9	HDAC6	HDAC10	HDAC11
Panobinostat	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Vorinostat	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Quisinostat	++++	++++	++++	++++	++++	++++				++++	++++
Ricolinostat	++	+++	++	++					++++		
Tubacin									++++		
HPOB	+	+	+	+					++	+	
TMP269					++	++	+++	+++			

The number of + indicates the binding affinity/potency of the inhibitors to the specific HDAC, the color indicates the class, data provided by selleckchem from [246]

catalytic center, catalytic tunnel, and pocket rim. Due to the enzyme homology, it is rather difficult to selectively target one specific HDAC without inhibiting the other class members [248] (see Table 1 [246]). However, intelligent drug design leads to inhibitors that are at least class-specific. It should be noted that selectivity, in this case, is not a strict line but a more flowing transition. This means that increasing the concentration of the HDACi can negatively influence the selectivity by overcoming the binding bias through oversaturation. Three general classifications based on the selectivity profile are in use [248]. Pan-HDACi can bind unselectively all HDAC family members with little to no bias for specific enzymes. Preferential inhibitors bind to one or more classes but not all, while selective inhibitors primarily target one or more HDAC within one class. Structurally, HDACi have 3 reoccurring domains: a cap domain, a zinc-binding domain, and a linker domain. The cap group often contains (hetero-)aromatic structures with hydrophobic moieties and regulates specificity through interaction with the enzyme rim [249]. The zinc-binding region chelates the Zn^{2+} ion in the catalytic domain, highly affecting the inhibitor's potency [250]. The linker domain connects the cap and zinc binding group and inertly affects selectivity and potency since its length modulates interaction with the catalytic tunnel.

Based on the zinc-binding domain, five fundamental organic carbon backbones have been found [247]. The most prominent group that yielded the most FDA-approved candidates functions via a hydroxamic acid [248]. In a bidentate manner, the functional group's hydroxyl and carbonyl oxygen atoms establish a charge-based interaction with the Zn^{2+} ion. This process is called Zinc coordination. FDA-approved examples include the potent pan-inhibitors panobinostat (withdrawn in 2022), belinostat, and vorinostat. A more specific HDAC class I/IIb, but less toxic HDACi, is HPOB. Another noteworthy group is the benzamides. The amide carbonyl oxygen coordinates weakly with the Zn^{2+} , while the carbonyl group forms a comparably weak monodentate [138]. While this makes them less potent, benzamide-based HDACi are often more selective than the hydroxamic acid-based inhibitors [251, 252]. The only FDA-approved example would be chidamide, and other more prominent examples include CI994 or entinostat, which are all class I selective. Macrocyclic peptide-based inhibitors are the most complex form of HDACi [253]. In the case of romidepsin - the only FDA-approved HDACi of this class - a thiol group coordinates with the Zn^{2+} . The last two binding methods include short fatty acids like valproic acids or, so far, quite experimental ketone inhibitors. Neither group has provided any FDA-approved drugs and is, therefore, not further discussed.

Designing selective inhibitors hinges on considering the structural diversity of the different HDACi. While class I HDACs have highly conserved motifs, their catalytic tunnel differs slightly from each other. HDAC2, for example, has a deeper-reaching catalytic tunnel, while the ones of HDAC3 and HDAC8 are smaller with no additional room in the lower periphery [254]. A core difference between class I/IIb HDAC and class IIa HDAC is the conserved substitution of tyrosine against histidine in the catalytic center. This potentially affects the inhibitor binding due to altered interaction patterns when coordinating. However, HDAC class IIa are anyhow less catalytically active, and therefore, potential modes could arise from blocking the interaction with protein complex partners [255, 256]. A special case is again HDAC6, which has several peculiar structural characteristics. Its binding pocket is significantly wider (17.5 Å) when compared to HDAC1 (12.5 Å) and not as deep (see Figure 8, [257]). This enables inhibitors with a small linker and a bigger cap group to still interact with HDAC6, but does not accommodate other HDACs. Another facet of HDAC6's role is its multiple potential targeting sites. The structural difference between CD1 and CD2 necessitates two individual drug design approaches [157]. Therefore, even within HDAC6, CD1 and CD2 can be selectively targeted. Additionally, the selective inhibition of the UZB of HDAC6 is feasible and could be an interesting therapeutic option [258].

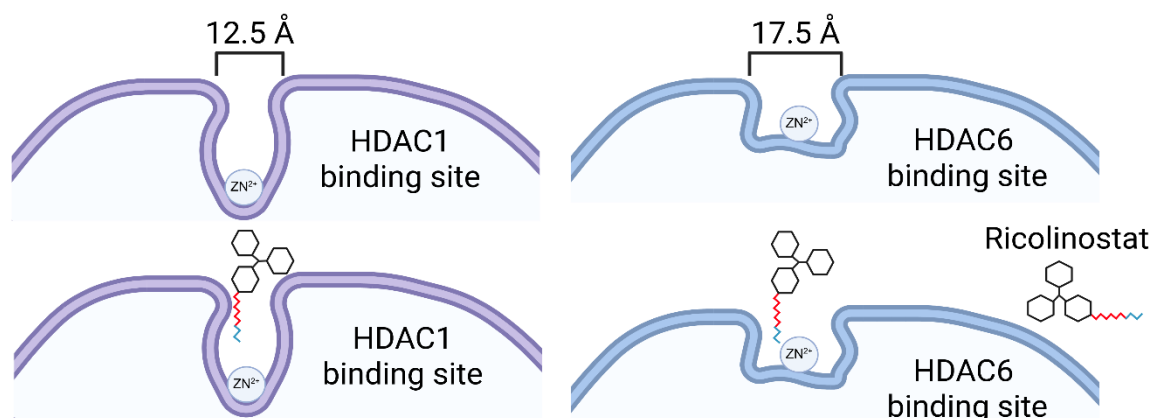


Figure 8: Comparison of the structural differences of the binding pocket of HDAC1 and HDAC6 and their binding via Ricolinostat Created with Biorender.com, based on information from [257]

1.4.5.2 - HDAC inhibition in the context of leukemia

Due to the wide functional spectra of the HDAC family and the significant differences in selectivity, it is highly difficult to predict the effect of HDACi on leukemia. Compared to solid malignancy, hematopoietic tumor cells are highly susceptible to HDAC inhibition in general [217]. AML has garnered special focus in the scientific community regarding leukemia subtypes and HDACi. Myeloid malignancies and especially AML are comparably more susceptible to HDAC inhibition than ALL [259]. A sound explanation would be that AML often depends on mutations that specifically dysregulate conventional epigenetic patterns.

Since hypomethylating agents are already widely used in the clinic, targeting HDAC is a logical next step as an alternative epigenetic target. However, in the lymphoid diseases, HDACi have shown some promising potential, but the results were less consistent [260].

Pan-HDACi have been proven to be especially potent when used against leukemia or lymphoma cells. Usually, their inhibitory concentration that stops 50% of cellular viability (IC₅₀) is located at the lower nM level of the scale. Selective HDACi have, on the other hand, a more circumstantial effect. Inhibitors that target class I HDAC induce differentiation/DNA damage/cell cycle arrest, and apoptosis [229, 260]. Their IC₅₀ profile highly varies and can be located from the lower nM to μ M scale. Class IIa preferential inhibitors can induce apoptosis, but tend to do so at higher concentrations [261]. Increasing the specificity towards class IIa seems to reduce the direct cytotoxic effect of the compound [262].

Class IIb and especially HDAC6 seem to be more challenging to categorize. Some studies found a cytotoxic effect in HDAC6 preferential inhibitors [237, 263, 264]. However, a critical study that deals with off-target cytotoxicity revealed that the loss of HDAC6 did not alter the reaction toward the HDAC6 preferential inhibitor ricolinostat [265]. Therefore, a direct contribution of HDAC6 to ricolinostat's cytotoxicity seem unlikely. Evidence of a direct cytotoxic effect of HDAC6 inhibition has come into question, and a valid criticism is that at higher concentrations, many HDACi lose their specificity and target class I HDAC. Still, HDAC6 inhibition could be a valuable tool in combination therapies. Synergistic effect has been shown between an experimental HDAC6i and vincristine or doxorubicin in ALL cells [237]. The combination of HDAC6 and LSD1 inhibitors induced a higher susceptibility to doxorubicin in AML cells [266]. Another prominent example in CML would be the positive interaction between imatinib and HDAC6i-induced effects in CML [239, 240]. Since all of these drugs are commonly used in chemotherapy, this highlights that despite HDAC6's ambiguous cytotoxic effect, there is still interest in it as a drug target.

Noteworthy efforts have also been put into improving the understanding of the interplay between HDACi and the immune system. For example, it has been shown that the therapeutic differentiation induction in AML via panobinostat depends on the epigenetic activation of plasmacytoid dendritic cells [267]. However, these interactions are highly complex since every immune cell type is differently affected, and the different inhibition profiles of the inhibitors discourage generalizations. HDAC6 specifically has gained a lot of attention as an immunomodulatory agent. Through its ability to regulate the PD-L1 stability, HDAC6 can diminish the effect of T-cell and macrophage activation in melanoma [207, 268, 269]. In the context of T-cells, it seems that especially naïve T-cells benefit from HDAC6 inhibition due to increased functionality [270]. Studies with the HDAC6 selective inhibitor

nexturastat A revealed potential synergy with anti-CD47 antibodies in melanoma [268]. Similar effects have been observed in CLL models [271]. In conclusion, HDACi, especially HDAC6i, are promising and have the potential to complement immunotherapeutic approaches.

1.4.5.3 - Clinical applications of histone deacetylase inhibitors

Currently, vorinostat, romidepsin, belinostat, and chidamide are approved for clinical use against (relapsed) cutaneous and/or peripheral T-cell Lymphoma. While panobinostat was approved for treating multiple myeloma, the FDA reversed this decision in 2022. Still, it is used in Europe as an alternative for patients who do not respond well to standard chemotherapy. Against leukemia, according to clinicaltrials.gov, to this time point, more than 140 trials are/have been conducted with HDAC inhibitors. The majority of these trials have been done specifically in AML patients. Pan-HDACi, in particular, have been the primary focus of these trials due to their potent cytotoxicity. However, the success of vorinostat, panobinostat, and belinostat in clinical trials has been limited [272-274]. While they were relatively safe to use, the response to the drugs did not meet the expectations set by preclinical experiments.

Therefore, efforts were moved from monotherapy to protocols of synergistic drug combinations. Prominent combinations in AML so far included pan HDACi together with daunorubicin/idarubicin, cytarabine, azacytidine, decitabine, or bortezomib [275-279]. Still, these pan HDACi show, in some cases, significant adverse effects in these clinical trials [280]. Both romidepsin and belinostat treatment led to severe infections and tumor lysis syndrome. Panobinostat could induce cardiac ischemia and hemorrhage, while vorinostat was responsible for pulmonary embolisms, deep vein thrombosis, and hyperglycemia. Most of these severe side effects could be traced back to the immune system or, at the same time, related to immune cells. A reasonable explanation would be that these pan-HDACi also interfere with the metabolism of healthy immune cells. Therefore, more selective HDACi could be helpful in addressing only the HDAC that specifically contributes to tumor growth. So far, ricolinostat is the furthest advanced HDAC6 preferential inhibitor in clinical trials. While ricolinostat, similar to the pan-HDACi, did not perform well as a solo agent, it did show some potential with bortezomib and dexamethasone in multiple myeloma [281]. Ongoing trials are investigating the effect of ricolinostat and BCR inhibitors in CLL. These findings highlight the clinical potential of HDAC inhibitors; however, further research is needed to fully realize and validate their therapeutic efficacy.

2 - Aims

AML remains the most difficult leukemia subtype to treat, with overall prognosis inferior to other forms. Although HDACi showed promise in preclinical studies, their clinical efficacy as monotherapies has been limited, with minimal therapeutic benefit and substantial toxicity. Advancing the field requires both a refined understanding of HDAC biology in leukemia and the development of more selective inhibitors, ideally applied in rational drug combinations. HDAC6 represents a distinct target, as its inhibition produces context-dependent effects that extend beyond direct cytotoxicity to include immunomodulation and chemosensitization. Accordingly, this thesis aimed to elucidate the functional role of HDAC6 in leukemia and to explore its therapeutic potential:

First Aim: Characterization of Novel Class-Specific HDAC Inhibitors and Identification of Synergistic Drug Combinations

Experimental HDAC inhibitors, provided by the Department of Pharmaceutical Chemistry at the University of Düsseldorf (Prof. Kurz) and the University of Bonn (Prof. Hansen), will be systematically characterized with respect to their isoform selectivity and anti-leukemic activity. Lead compounds identified from these libraries will undergo detailed evaluation, including validation of HDAC target engagement, elucidation of cell death mechanisms, and comparative assessment against established, commercially available HDAC inhibitors. Furthermore, the potential of nucleoside analogs such as decitabine and clofarabine as synergistic partners in combination treatment strategies for AML samples will be investigated.

Second Aim: Investigating the potential of HDAC6 selective inhibition in the diverse subtypes of Leukemia

Given the highly context-dependent effects of selective HDAC6 inhibition, this study will aim to identify the leukemia subtype that could most benefit from HDAC6 inhibition as a synergistic addition to existing therapeutic regimens. Utilizing a database-driven approach, myeloid leukemias like AML and CML will be prioritized as a promising candidate for further investigation. To characterize the molecular and cellular consequences of HDAC6 inhibition, genetically engineered HDAC6 KO models in leukemia cells will be generated. These models will be systematically analyzed using integrated multi-omics approaches, including proteomics, secretomics, transcriptomics, and ATAC-seq, to elucidate the underlying mechanisms and to evaluate the therapeutic potential of HDAC6 loss in leukemia cells.

3 - Cumulative publications

The core of this dissertation includes the following paper with shared first authorship (indicated by #):

3.1 Development of Fluorinated Peptoid-Based Histone Deacetylase (HDAC) Inhibitors for Therapy-Resistant Acute Leukemia

Nina Reißing[#], **Julian Schliehe-Diecks[#]**, Paris R. Watson, Melf Sönnichsen, Abigail D. Cragin, Andrea Schöler, Jing Yang, Linda Schäker-Hübner, Arndt Borkhardt, David W. Christianson, Sanil Bhatia^{*}, Finn K. Hansen^{*}

Journal of Medicinal Chemistry - November 9, 2022

Impact Factor (2025): 7.3

DOI: <https://doi.org/10.1021/acs.jmedchem.2c01418>

3.2 Preferential HDAC6 inhibitors derived from HPOB exhibit synergistic antileukemia activity in combination with decitabine

Maik Tretbar[#], **Julian Schliehe-Diecks[#]**, Lukas von Bredow, Kathrin Tan, Martin Roatsch, Jia-Wey Tu, Marie Kemkes, Melf Sönnichsen, Andrea Schöler, Arndt Borkhardt, Sanil Bhatia^{*}, Finn K. Hansen^{*}

European Journal of Medicinal Chemistry – June 5, 2024

2-Year Impact Factor (2024): 6.0

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3.3 Deciphering the Therapeutic Potential of Novel Pentyloxyamide-Based Class I, IIb HDAC Inhibitors against Therapy-Resistant Leukemia

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3.4 Epigenetic remodeling via HDAC6 inhibition amplifies anti-tumoral immune responses in myeloid leukemia cells

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3.1 - Development of fluorinated peptoid-based histone deacetylase (HDAC) inhibitors for therapy-resistant acute leukemia

This study aimed to establish novel peptoid-based HDACi with different residues at multiple positions to increase selectivity and potency. Central to the synthesis was a microwave-based Ugi-four-component reaction, which combined an amine, an isonitrile, carboxylic acid, and paraformaldehyde. Ultimately, the novel inhibitors carried a fluorine residue in the linker domain, while the capping group consisted of a peptoid with multiple residues. From this approach, 16 inhibitors emerged, which were characterized in an enzyme assay with regard to their binding potency for HDAC1 and HDAC6. Two structures seemed especially promising since one showed unselectively very low IC₅₀ (**10h**) while the other was highly selective for HDAC6 (**10p**). **10h** bound to HDAC6 crystal structures could be established to investigate the binding mechanism. An initial screening of the whole library with three different leukemic cell lines revealed a correlation between HDAC1 affinity and antileukemic properties. A focused screening with established reference inhibitors and more cell lines validated the previous findings. The actual cellular binding of the inhibitor to HDAC was validated through western blot analysis. The paper's conclusion was an in-depth matrix synergy screening between the novel HDACi and methyl transferase inhibitor decitabine. Especially in AML cell lines, a potent effect was observable. In conclusion, the novel inhibitors did display several interesting anti-leukemic properties, which could make them valuable tools in treating leukemia.

Contributions: In general, writing of the manuscript and experimental design, execution, evaluation, and visualization of the following biological experiments:

- Initial screening of the compound library (see Table 2)
- Focused screening with an extended number of leukemia cell lines (see Figure 5a)
- Western blot analysis of downstream HDAC deacetylation targets after HDACi treatment (see Figure 5c)
- Matrix synergy screening of **10h** with decitabine in four different leukemia cell lines (see Figure 6a)
- Western blot analysis of the increased H3/ α -tubulin acetylation upon combined treatment (see Figure 6b)
- Matrix synergy screening of **10p** with decitabine in two AML leukemia cell lines (see Figure 7)

Development of Fluorinated Peptoid-Based Histone Deacetylase (HDAC) Inhibitors for Therapy-Resistant Acute Leukemia

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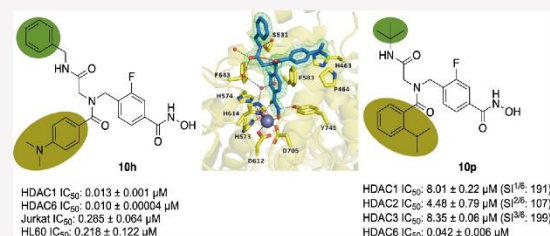
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ABSTRACT: Using a microwave-assisted protocol, we synthesized 16 peptoid-capped HDAC inhibitors (HDACi) with fluorinated linkers and identified two hit compounds. In biochemical and cellular assays, **10h** stood out as a potent unselective HDACi with remarkable cytotoxic potential against different therapy-resistant leukemia cell lines. **10h** demonstrated prominent antileukemic activity with low cytotoxic activity toward healthy cells. Moreover, **10h** exhibited synergistic interactions with the DNA methyltransferase inhibitor decitabine in AML cell lines. The comparison of crystal structures of HDAC6 complexes with **10h** and its nonfluorinated counterpart revealed a similar occupation of the L1 loop pocket but slight differences in zinc coordination. The substitution pattern of the acyl residue turned out to be crucial in terms of isoform selectivity. The introduction of an isopropyl group onto the phenyl ring provided the highly HDAC6-selective inhibitor **10p**, which demonstrated moderate synergy with decitabine and exceeded the HDAC6 selectivity of tubastatin A.



INTRODUCTION

Epigenetic events are largely induced by environmental factors and determine the post-translational alteration of a cell phenotype.¹ In today's drug discovery, controlling epigenetic mechanisms by regulating histone modifications such as histone acetylation, methylation, or phosphorylation is increasingly important.^{1,2} One group of enzymes involved in such processes consists of histone deacetylases (HDACs), which have attracted attention as promising targets for cancer therapy. Consisting of 11 zinc-dependent isoforms and the NAD⁺-dependent sirtuins, the HDAC family is divided into classes I–IV.³ Among those, only class I isoforms HDAC1–3 control the acetylation levels of histones, thus modifying the chromatin structure and its accessibility for gene transcription.³ Other isoforms participate in a variety of biological processes such as the formation of protein–protein complexes, the mediation of immunosuppression via Forkhead box P3 (Foxp3) deacetylation, and the regulation of transcription factors.^{4–7} HDAC inhibitors (HDACi) typically comprise a capping group occupying the outside of the enzyme's active site, a zinc-binding group chelating the catalytically active zinc ion, and a linker chain connecting the two parts. Because of the highly conserved nature of the enzymes, this pharmacophore model generally applies to all isoforms, including the class I enzymes.³ Being particularly cytotoxic due to their interference

with cell cycle progression and proliferation, selective class I HDACi as well as pan-inhibitors are considered promising tools for the therapy of both hematological and solid tumors.⁸ Since their discovery, four HDACi, e.g., vorinostat, belinostat, panobinostat, and the class I-selective natural compound romidepsin, have been approved by the FDA for the treatment of hematological cancers, including multiple myeloma and T-cell lymphoma.^{6,8,9} The class I-selective tucidinostat has recently become the first HDACi to be approved for the therapy of solid tumors by the NMPA in China.¹⁰ Another field of interest concerning class I HDACi on the clinical level is their presumed ability to eradicate latent HIV reservoirs.¹¹

Despite their potential in cancer therapy, pan-HDACi are typically associated with adverse effects. To explore the scope beyond oncology, the research on isoform-specific HDACi with weakened off-target interactions and higher tolerability is quickly evolving.⁸ The most promising target for isoform-selective inhibition with improved safety profiles is HDAC6,

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which appears to play a role in cancer, neurodegenerative diseases, inflammation, rheumatoid arthritis, and neurological disorders.^{12–17} The first HDAC6-preferential drug candidate, ricolinostat (phase II), its chlorinated analogue, citarinostat (phase I), and several yet undisclosed HDAC6i considered for non-oncological conditions (phase II) are currently being assessed in clinical trials.¹⁸ Unlike other isoforms, the class IIb isoform HDAC6 is located in the cytoplasm instead of the nucleus and comprises two catalytic domains (CDs).^{19,20} One of them, CD1, is considered inactive toward histones, and its biological role remains unclear.²¹ Confirmed substrates of the second catalytic domain (CD2) include α -tubulin and cortactin, which are both required for cytoskeleton formation; tau; and chaperone protein Hsp90.^{21–26} HDAC6 is furthermore unique in comprising a ubiquitin-binding domain that regulates the aggresome–autophagy pathway as an important protein degradation system in addition to the 26S proteasome.^{27,28} Although recent studies imply only moderate cytotoxicity induced by selective HDAC6 inhibition,²⁹ the enzyme remains a relevant drug target with high eligibility for combination therapies.³⁰

Supposedly triggering a beneficial alteration in gene expression patterns, the combination of HDACi and DNA-hypomethylating agents is another field of interest.³¹ The concurrent application of HDACi and decitabine in diffuse large B-cell lymphoma cell lines appeared to induce time-dependent synergistic effects resulting in tumor growth inhibition and increased apoptosis levels that exceeded the effect of HDACi alone.³¹ Other preclinical experiments hinted at synergistic effects activated by vorinostat and decitabine in mantle cell lymphoma and pancreatic cancer cell lines.^{31–33} Succeeding prior clinical trials on a pan-HDACi as an additive to decitabine, current studies are focusing on the class I-selective HDACi tucidinostat in lymphoma patients.^{34–36}

A feature with increasing prevalence in modern pharmaceuticals is fluorine. Although rarely occurring in natural compounds, fluorination has become an important means of improving the bioavailability and pharmacokinetic properties of drugs.³⁷ After adding up to 20–25% of all pharmaceuticals a decade ago, fluorinated drugs now account for one-half of all blockbuster drugs.^{37,38} Compared to that of nonfluorinated analogues, the low degree of polarization of the C–F bond and the high electron-withdrawing capabilities of fluorine substituents may significantly alter the pK_a, lipophilicity, and metabolic stability of drugs.^{37,39,40} Conformational changes due to the characteristic steric demands of fluorinated groups could have an additional impact on the binding mode and target affinity.^{37,39,40} Indeed, another notable benefit of drug fluorination is the possibility of ¹⁸F radiolabeling. Apart from diagnostic measures, PET tracing of ¹⁸F-labeled compounds can be useful for biodistribution studies of early stage drug candidates, thus facilitating the drug development process.³⁷ Despite such advantages, fluorinated drugs bear the risk of high intrinsic toxicity owing to the release of fluorine-containing metabolites.^{41,42} A careful investigation of the metabolic behavior of each fluorinated drug candidate is therefore inevitable.⁴¹

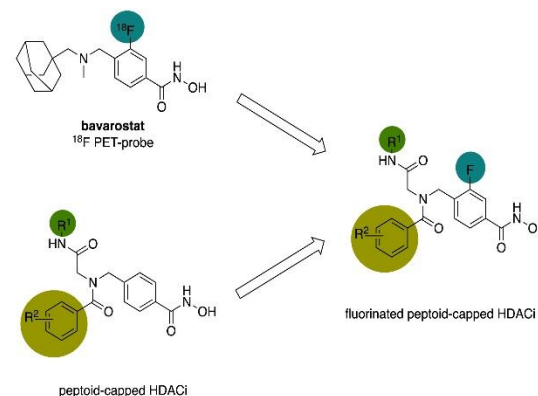
In 2016, our group published results on selective HDAC6 inhibition by peptoid-based compounds obtained by the Ugi four-component reaction (U4CR).⁴³ Follow-up studies using co-crystals of selected peptoid-capped inhibitors in complex with CD2 of *Danio rerio* (zebrafish) HDAC6 (zCD2) revealed that the compounds adopt binding modes different from those

of the highly HDAC6-selective PET probe bavarostat that carries a fluorine substituent in the *meta* position to the hydroxamate in the otherwise identical linker moiety.^{44,45} Given that bavarostat and the unselective HDACi [¹¹C]-martinostat, a cinnamoyl-based PET probe in clinical phase I, share identical cap groups, we concluded that the remarkable HDAC6 selectivity of bavarostat is likely to derive from the unique binding mode of the fluorinated linker moiety.⁴⁶ Moreover, a recent study of inhibitors similarly fluorinated and difluorinated at the *meta* positions of phenyl linker groups found that the compounds also exhibit HDAC6 selectivity, possibly due to interactions between the fluorine atoms and His361, Gly582, and Ser531 in loop pockets L1 and L2.⁴⁷ On the basis of the observation that selectivity profiles can be steered through substitution patterns in the cap group area, we decided to investigate the effect of linker fluorination on scaffolds that had previously turned out to favor either HDAC6 inhibition or pan-inhibition. The resulting set of highly potent, fluorinated HDACi was then assessed with respect to their antileukemic properties.

RESULTS AND DISCUSSION

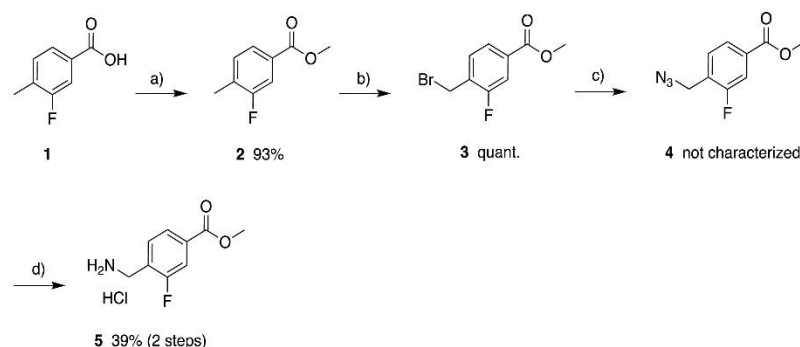
Design and Synthesis. Aiming to determine whether the bavarostat-inspired linker fluorination would improve the inhibitory qualities of our previously reported peptoids, we designed a new set of compounds that includes fluorinated analogues of nonselective compounds DDK115 and DDK137 as well as the HDAC6-preferential inhibitors IIe and IIf (Scheme 1).^{45,48} In a recent study of tetrazole-capped HDACi

Scheme 1. Design of Fluorinated Peptoid-Capped HDACi Inspired by the Preclinical PET Probe Bavarostat



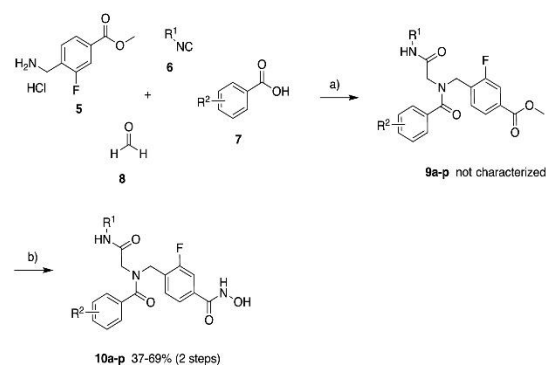
derived from the peptoid scaffold, we moreover identified the 2-trifluoromethyl substituent of the acyl moiety as a source of excellent selectivity.⁴⁹ An additional structure–activity relationship (SAR) study therefore featured several analogues with differently shaped substituents in the same position.

The synthesis of the 2-fluorophenyl linker was performed in accordance with a literature protocol (Scheme 2).⁵⁰ Following the esterification of 1, NBS was used to introduce a bromine substituent (3) that was then substituted to give the corresponding azide 4. Next, the Staudinger reaction using triphenylphosphine was carried out to afford the desired amine, which was easily isolated as hydrochloride salt 5. The U4CR of 5, the respective isonitriles 6, and paraformaldehyde 8 built peptoid

Scheme 2. Synthesis of Fluorinated Linker 5^a

^a(a) SOCl₂, MeOH, 0 °C to rt, 16 h; (b) NBS, AIBN, DCM, reflux, 16 h; (c) NaN₃, DMF, 80 °C, 16 h; (d) PPh₃, HCl, THF, rt, 24 h.

scaffolds **9** within <4 h of microwave irradiation (Scheme 3). After aqueous workup and recrystallization of the crude

Scheme 3. Synthesis of Fluorinated Peptoid-Capped HDACi 10a–p^a

^a(a) Et₃N, 4 Å MS, MeOH, microwave, 45 °C, 150 W, 60–210 min; (b) H₂NOH, NaOH, MeOH, DCM, 0 °C, 15 min.

products, the resulting esters **9** were treated with a mixture of sodium hydroxide and aqueous hydroxylamine to yield the hydroxamates **10** within 15 min of stirring at 0 °C. All final compounds were isolated by precipitation from the aqueous crude mixture, and their purity exceeded 95% without further purification.

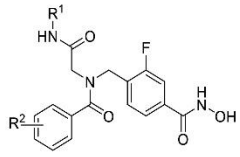
HDAC Inhibition. Biochemical assays were performed to evaluate the inhibitory activities of all final compounds against HDAC1 and HDAC6. The resulting IC₅₀ values (Table 1) indicate that minor structural modifications suffice to steer between unselective inhibition and HDAC6 selectivity without affecting the overall high inhibitory activities against HDAC6 in the low nanomolar concentration range. In comparison to compounds featuring a cyclohexyl group in position R¹ [**10c–f**; IC₅₀ HDAC6 = 0.018–0.020 μM, selectivity indices (SI^{HDAC1/6}) from 43 to 75], it is apparent that *tert*-butyl derivatives **10k–n** with an identical substitution pattern at the R² moiety display more promising selectivity profiles (SI^{HDAC1/6} = 45–103) and similar potencies (IC₅₀ HDAC6 = 0.017–0.027 μM). The selectivity profile of the voluminous and branched isopropyl derivative **10p** (SI^{HDAC1/6} = 191) exceeded those of all other analogues as well as that of the control compound tubastatin A (SI^{HDAC1/6} = 178), in spite of

the slightly impaired inhibition of HDAC6 (0.042 μM). Hence, **10p** was singled out as the most selective inhibitor of this set. The equally bulky 2-chloro analogue **10o** was found to be twice as potent (IC₅₀ HDAC6 = 0.021 μM) but less selective (SI^{HDAC1/6} = 165). Additional assays using HDAC2 and HDAC3 confirmed the low activity of **10p** against other class I isoforms (IC₅₀ HDAC2 = 4.48 μM, and SI^{HDAC2/6} = 107; HDAC3 = 8.35 μM, and SI^{HDAC3/6} = 199). Furthermore, we screened **10p** against HDAC4 as a representative class IIa isoform. As expected, the compound turned out to be inactive [IC₅₀ HDAC4 > 10 μM, and SI^{HDAC4/6} > 238 (Figure 1)].

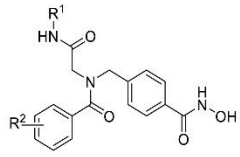
In accordance with previous SAR data, all inhibitors featuring 3,5-dimethyl residues in the acyl ring (**10a**, **10g**, and **10i**) exhibited only low to moderate HDAC6 selectivity (SI^{HDAC1/6} = 6–24) but high inhibitory qualities ranging from 0.013 to 0.024 μM against HDAC6.^{48,51} The two 4-dimethylamino derivatives **10b** and **10h** met the expectations by turning out to be strong unselective inhibitors (SI = 1.4–8); **10h** proved to be the most potent ligand against both isoforms (IC₅₀ HDAC1 = 0.013 μM; IC₅₀ HDAC6 = 0.010 μM) and other class I enzymes [IC₅₀ HDAC2 = 0.014 μM, and SI^{HDAC2/6} = 1.4; IC₅₀ HDAC3 = 0.021 μM, and SI^{HDAC3/6} = 2.1 (Figure 1)]. Compound **10h** also displayed some inhibitory activity against HDAC4 [IC₅₀ HDAC4 = 1.88 μM, and SI^{HDAC4/6} = 188 (Figure 1)]. The *tert*-butyl-capped analogue **10j** (IC₅₀ HDAC1 = 0.158 μM; IC₅₀ HDAC6 = 0.020 μM, and SI = 7.9) exhibited inhibition qualities similar to those of ricolinostat. In summary, those results indicate that the substitution pattern of the acyl ring is crucial in terms of isoform selectivity. Depending on the size of the residue, the sole occupation of position 2 of the aromatic ring promises a strong preference for HDAC6. The introduction of small residues at positions 3–5, in turn, effectuates equal inhibition of HDAC1 and HDAC6 or a marginal preference for the latter. The HDAC6 selectivity achieved by appropriate R² residues may moreover be boosted by choosing the *tert*-butyl motif over a cyclohexyl group in position R¹. Overall, all new ligands, except for the most selective inhibitor **10p**, exceeded the HDAC6 inhibition of the control compound vorinostat and were as potent as tubastatin A and ricolinostat.^{52,53}

The comparison of the unselective inhibitors **10a** and **10h** with their nonfluorinated analogues DDK115 and DDK137 implies that linker fluorination results in a decreased level of HDAC1 inhibition (DDK115 vs **10a**, 0.249 μM/0.310 μM; DDK137 vs **10h**, 0.005 μM/0.013 μM) accompanied by

Table 1. IC₅₀ Values of Fluorinated HDACi 10a–p and Nonfluorinated Analogues DDK115, DDK137, Ile, and IIf in Comparison to Those of Vorinostat, Ricolinostat, HPOB, and Tubastatin A (Tub A) against HDAC1 and HDAC6



10a-p



Ile-f, DDK115, DDK137

compound	R ¹	R ²	IC ₅₀ HDAC1 (μM)	IC ₅₀ HDAC6 (μM)	SI ^a
10a	c-hexyl	3,5-Me	0.310 ± 0.004	0.020 ± 0.002	16
10b	c-hexyl	4-NMe ₂	0.066 ± 0.008	0.016 ± 0.004	4
10c	c-hexyl	2-F	0.771 ± 0.006	0.018 ± 0.002	43
10d	c-hexyl	2-Me	0.938 ± 0.013	0.018 ± 0.002	52
10e	c-hexyl	2-OMe	1.50 ± 0.10	0.020 ± 0.004	75
10f	c-hexyl	2-CF ₃	1.45 ± 0.05	0.027 ± 0.002	54
10g	Bn	3,5-Me	0.077 ± 0.003	0.013 ± 0.004	6
10h	Bn	4-NMe ₂	0.013 ± 0.001	0.010 ± 0.00004	1.3
10i	<i>t</i> -Bu	3,5-Me	0.573 ± 0.011	0.024 ± 0.002	24
10j	<i>t</i> -Bu	4-NMe ₂	0.158 ± 0.056	0.020 ± 0.006	8
10k	<i>t</i> -Bu	2-F	1.18 ± 0.15	0.026 ± 0.002	45
10l	<i>t</i> -Bu	2-Me	1.75 ± 0.13	0.027 ± 0.005	65
10m	<i>t</i> -Bu	2-OMe	1.75 ± 0.05	0.017 ± 0.0001	103
10n	<i>t</i> -Bu	2-CF ₃	2.33 ± 0.43	0.027 ± 0.005	86
10o	<i>t</i> -Bu	2-Cl	3.47 ± 0.14	0.021 ± 0.001	165
10p	<i>t</i> -Bu	2- ⁱ Pr	8.01 ± 0.22	0.042 ± 0.006	191
DDK115	c-hexyl	3,5-Me	0.249 ± 0.028	0.040 ± 0.008	6
DDK137	Bn	4-NMe ₂	0.005 ± 0.0006	0.011 ± 0.003	0.5
Ile ^b	<i>t</i> -Bu	2-Me	2.41 ± 0.086	0.051 ± 0.004	47
IIf ^b	<i>t</i> -Bu	2-OMe	3.11 ± 0.308	0.038 ± 0.004	82
vorinostat	—	—	0.099 ± 0.005	0.042 ± 0.005	2
givinostat	—	—	0.035 ± 0.002	0.014 ± 0.0002	2.5
givinostat ^c	—	—	0.070 ± 0.012	0.023 ± 0.004	3
ricolinostat ^d	—	—	0.19 ± 0.022	0.018 ± 0.003	11
Tub A ^e	—	—	2.49 ± 0.14	0.014 ± 0.0006	178
HPOB ^e	—	—	2.10 ± 0.23	0.085 ± 0.009	25

^aSelectivity index [SI = (IC₅₀ HDAC1)/(IC₅₀ HDAC6)]. ^bData taken from ref 48. ^cData taken from ref 47. ^dData taken from ref 52. ^eData taken from ref 53.

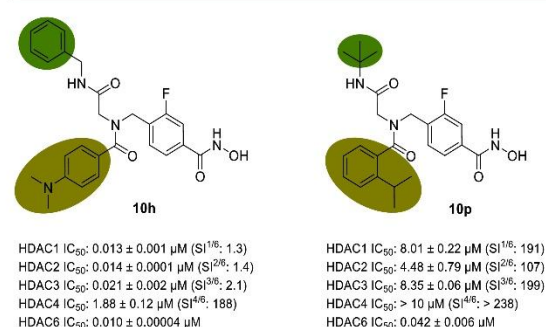


Figure 1. Inhibition of HDAC1–3 and HDAC6 by selected hit compounds 10h and 10p. Selectivity index [SI = (IC₅₀ HDAC1, -2, -3, or -4)/(IC₅₀ HDAC6)].

higher or similar inhibitory activity against HDAC6 (DDK115 vs 10a, 0.040 μM/0.020 μM; DDK137 vs 10h, 0.011 μM/0.010 μM), thus almost tripling the selectivity for HDAC6 in both cases.

In terms of HDAC6-selective compounds Ile and IIf, linker fluorination increased the inhibitory activity toward HDAC1

(Ile vs 10l, 2.41 μM/1.75 μM; IIf vs 10m, 3.11 μM/1.75 μM) and nearly doubled the inhibitory potency against HDAC6 (Ile vs 10l, 0.051 μM/0.027 μM; IIf vs 10m, 0.038 μM/0.017 μM), thereby improving the selectivity profiles. Those results are generally in line with the observations reported by Sandrone et al., who concluded that the increased HDAC6 selectivity of HDACi with mono- or difluorinated linkers in the *meta* position to the hydroxamic acid results from an increased level of HDAC6 inhibition and/or decreased inhibitory activity toward HDAC1.⁴⁷ Differences in the assay setups (in particular different substrates) prohibit a direct comparison of the results published by Sandrone et al. and our results summarized in Table 1, so we chose to include givinostat as an additional control compound. Although we (IC₅₀ HDAC1 = 0.035 μM; IC₅₀ HDAC6 = 0.014 μM, and SI = 2.5) measured different IC₅₀ values for givinostat compared to those of Sandrone et al. (IC₅₀ HDAC1 = 0.070 μM; IC₅₀ HDAC6 = 0.023 μM, and SI = 3),⁴⁷ the resulting selectivity indices were comparable (Table 1). In general, Sandrone et al. observed different reasons for the increased HDAC6 selectivity depending on whether small cap-less inhibitors or more complex larger HDACi with (hetero)aromatic caps were investigated. In the case of small

cap-less HDACi, the increased HDAC6 selectivity is mainly driven by improved HDAC6 inhibition. This was exemplified by the comparison of *N*-hydroxybenzamide (IC_{50} HDAC1 = 25.4 μ M; IC_{50} HDAC6 = 1.34 μ M, and SI = 19) and 3-fluoro-*N*-hydroxybenzamide (IC_{50} HDAC1 = 29.4 μ M; IC_{50} HDAC6 = 0.453 μ M, and SI = 65).⁴⁷ The improvement in the HDAC6 selectivity of larger HDACi with complex (hetero)aromatic caps, in contrast, originates mainly from a decreased level of HDAC1 inhibition.⁴⁷ This can be explained by differences in the L1 loops of HDAC1 and HDAC6: the active site of HDAC1 is more restricted and thus unable to accommodate larger cap groups, whereas HDAC6 features a wider entrance area to the catalytic tunnel.⁴⁷ Moreover, unlike most other HDACs that feature an aspartate in the respective position, HDAC6 possesses a serine (Ser531) on loop L2 at the entrance of the catalytic channel.⁴⁷ In the case of larger HDACi with (hetero)aromatic caps, a fluorine atom in the *meta* position of the linker is oriented toward loop L2, which might result in steric repulsion in HDACs featuring an aspartate at the tunnel entrance. This could explain the results observed by Sandrone et al., indicating that fluorinated phenyl linkers with a bulky cap group are more likely to reduce the inhibitory activity toward HDAC1 rather than increasing the potency against HDAC6, thus boosting the selectivity.⁴⁷ Considering our data and the results published by Sandrone et al.,⁴⁷ we can conclude that linker fluorination has a distinct beneficial effect on HDAC6 inhibition and/or selectivity.

In addition to the effects on HDAC6 mentioned above, Sandrone et al.⁴⁷ found that the linker fluorination restores activity against class IIa HDACs. To investigate whether this is also the case for our peptoid-based HDACi, we screened DDK137, the direct nonfluorinated analogue of compound **10h**, against HDAC4. In good agreement with the results of Sandrone et al.,⁴⁷ DDK137 (IC_{50} HDAC4 = 2.67 μ M) demonstrated a reduced level of HDAC4 inhibition compared to that of **10h** [IC_{50} HDAC4 = 1.88 μ M (Figure 1)], thus indicating that linker fluorination can lead to improved inhibitory properties against class IIa HDACs.

Crystal Structure of HDAC6 in Complex with 10h. The 1.85 Å resolution structure of the HDAC6–**10h** complex contains four independent copies of the enzyme–inhibitor complex in the asymmetric unit of the crystal. Hydroxamate– Zn^{2+} coordination differs in each of the four structures. In chain A, hydroxamate– Zn^{2+} coordination is modeled with bidentate geometry but the electron density for the hydroxamate group is ambiguous. In chains B and C, exclusively bidentate hydroxamate– Zn^{2+} coordination geometry is observed, and in chain D, exclusively monodentate hydroxamate– Zn^{2+} coordination geometry is observed. Below, the structures of the enzyme–inhibitor complex in chain B and chain D are outlined in greater detail.

For chain B (Figure 2), the HDAC6–**10h** complex does not reveal any major structural rearrangements caused by inhibitor binding, and the root-mean-square deviation (rmsd) is 0.19 Å for 311 C α atoms between the inhibitor-bound and unliganded enzyme structures. In the bidentate hydroxamate– Zn^{2+} chelate complex, the Zn^{2+} –O distances are 2.2 and 2.3 Å for the N–O[−] and C=O groups, respectively. The side chain of Y745 additionally donates a hydrogen bond to the hydroxamate C=O group (O–O distance of 2.6 Å), and H574 accepts a hydrogen bond from the hydroxamate NH group (N–N distance of 2.8 Å).

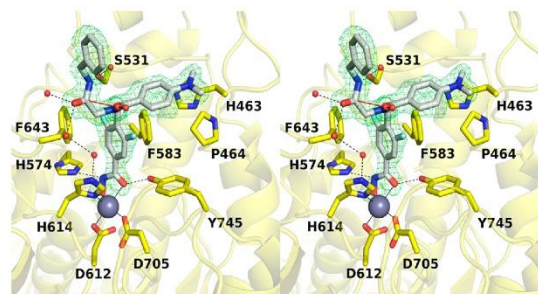


Figure 2. Stereoview of a Polder omit map (contoured at 4.0 σ) depicting the bidentate binding mode of **10h** (light gray) in the active site of HDAC6 chain B (yellow) (PDB entry 7U8Z). Two conformations of the aromatic ring are shown, one with the C–F group oriented into the F583–F643 aromatic crevice and the other with the C–F group oriented toward the solvent. The catalytic Zn^{2+} ion is shown as a gray sphere, and water molecules are shown as small red spheres. Metal coordination, hydrogen bond, and $n \rightarrow \pi^*$ interactions are shown as solid black, dashed black, and solid red lines, respectively.

The fluorophenyl linker is nestled in the aromatic crevice formed by F583 and F643; however, the aromatic ring adopts two mutually exclusive conformations (50% occupancy each). In one conformation, the C–F group is oriented into the crevice and the fluorine atom is 3.4 Å from the hydroxyl group of S531. In the other conformation, the C–F group is oriented toward the solvent. The peptoid carbonyl group is oriented toward the solvent and appears to form an $n \rightarrow \pi^*$ interaction with the adjacent benzylamide carbonyl group. The dimethylaniline group resides in the L1 loop pocket, as observed for the nonfluorinated inhibitor.⁴⁵ The benzylamide carbonyl forms hydrogen bonds with two solvent molecules, while the adjacent nitrogen forms a water-mediated hydrogen bond with the hydroxyl side chain of S531.

For chain D (Figure 3), the HDAC6–**10h** complex does not reveal any major structural rearrangements caused by inhibitor binding, and the rmsd is 0.16 Å for 302 C α atoms between the inhibitor-bound and unliganded enzyme structures. The hydroxamate moiety adopts monodentate Zn^{2+} coordination geometry in which the hydroxamate N–O[−] group coordinates to Zn^{2+} at a distance of 1.8 Å. The hydroxamate C=O group

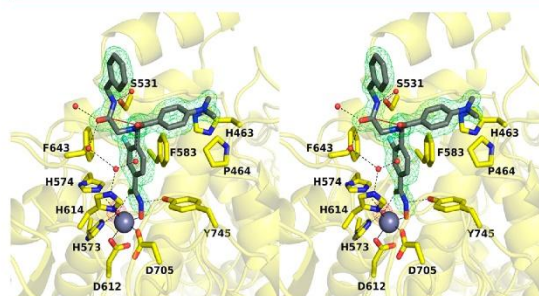


Figure 3. Stereoview of a Polder omit map (contoured at 4.0 σ) depicting the monodentate binding of **10h** (dark gray) in the active site of HDAC6 chain D (yellow) (PDB entry 7U8Z). The catalytic Zn^{2+} ion is shown as a gray sphere, and water molecules are shown as small red spheres. Metal coordination, hydrogen bond, and $n \rightarrow \pi^*$ interactions are shown as solid black, dashed black, and solid red lines, respectively.

accepts a short hydrogen bond from the Zn^{2+} -coordinated water molecule (O–O distance of 2.3 Å) that in turn forms hydrogen bonds with H573 and H574. The hydroxamate NH group additionally interacts with the side chain of Y745 (O–N distance of 2.5 Å).

The fluorophenyl aromatic linker is nestled in the F583–F643 aromatic crevice with the C–F group oriented exclusively into the crevice; the fluorine atom is 3.4 Å from the hydroxyl group of S531. Other aspects of inhibitor binding are similar to those observed in chain B described above. The peptoid carbonyl of the inhibitor capping group is oriented toward the solvent, and the carbonyl oxygen appears to form an $n \rightarrow \pi^*$ interaction with the adjacent benzylamide carbonyl group. The benzylamide carbonyl and peptoid carbonyl form a hydrogen bond network with four water molecules, and this network includes Zn^{2+} ligand H614. The benzylamide NH group forms a water-mediated hydrogen bond with the side chain of S531. Finally, the dimethylaniline group resides in the L1 loop pocket. The overall binding modes of **10h** and its non-fluorinated counterpart⁴⁴ are almost entirely identical, with the only major difference being variations in hydroxamate– Zn^{2+} coordination (Figure 4).

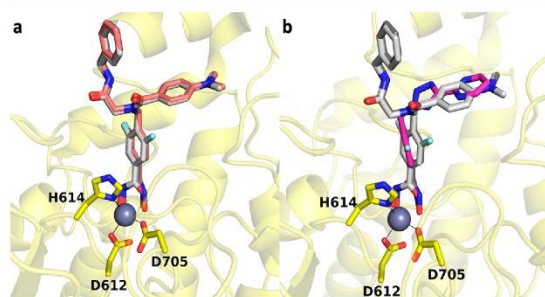


Figure 4. (a) Overlay of the HDAC6–**10h** complex (chain B, light gray; chain D, dark gray; PDB entry 7U8Z) with the HDAC6 complex with the nonfluorinated inhibitor (light pink; PDB entry 6DVN). The catalytic Zn^{2+} ion is shown as a gray sphere, and metal coordination is indicated by solid black lines. (b) Overlay of the HDAC6–**10h** complex (chain B, light gray; chain D, dark gray; PDB entry 7U8Z) with an inhibitor containing a *m*-difluorophenyl linker (PDB entry 7O2R).

Bavarostat is a selective inhibitor of HDAC6⁴⁴ and contains a *m*-fluorophenyl linker that, like the *m*-fluoro linker of **10h**, binds in the aromatic crevice defined by F583 and F643.⁴⁵ More recently, inhibitors have been developed that contain *m*-fluorophenyl and *m*-difluorophenyl linkers with different capping groups, and these inhibitors exhibit enhanced selectivity for inhibition of HDAC6 over HDAC1.⁴⁷ The X-ray crystal structure of one of these inhibitors complexed with HDAC6 reveals that the *m*-difluorophenyl linker makes favorable π – π interactions in the aromatic crevice as well as weak interaction between one of the C–F groups and S531. The fluoroaromatic ring of this inhibitor generally binds in a similar manner to the fluoroaromatic rings of bavarostat and **10h**, although the inhibitor-binding orientation is slightly tilted so that the rings are not coplanar (Figure 4b). Intriguingly, a key feature of isozyme selectivity derives from the reduction of inhibitory potency toward HDAC1, especially for inhibitors containing a *m*-difluorophenyl linker.

Cytotoxic Effects on Leukemia Cell Lines. The 16 new inhibitors (**10a–p**) were screened against three selected leukemia cell lines that originated from myeloid (HL60), B-cell (HAL01), and T-cell (Jurkat) lymphoid lineages (Table 2). The FDA-approved drug vorinostat, the HDAC6i HPOB, and the clinical candidate ricolinostat were used as controls. For selective HDAC6i, cytotoxicity levels are typically low, and therefore, it was not surprising that neither of the IC_{50} values measured in the presence of selective ligands lies below the micromolar concentration range.^{29,54} Compound **10j** was found to resemble ricolinostat in terms of both HDAC inhibition and isoform selectivity. Accordingly, it induced similar levels of cytotoxicity with IC_{50} values ranging from 1.46 to 2.47 μM in all three cell lines. Two other compounds, **10c** and **10d**, exceeded the cytotoxic effects of HPOB throughout all screenings. With respect to the unselective inhibitors, the most potent derivative, **10h**, proved to be similarly active against the B-cell acute lymphoblastic leukemia (B-ALL) cell line HAL01 and even more toxic against the T-ALL Jurkat cell line than vorinostat. On the basis of these results, compound **10h** was picked as the most promising candidate for additional experiments.

Extended Functional Analysis of **10h in Leukemia Cell Lines.** To study the inhibitory effects of hit compound **10h** (with highest cytotoxicity) on the growth and apoptosis induction of leukemia cells, further functional assays were carried out. First, we evaluated the effects of **10h** on the cellular viability of a broad range of leukemia cell lines that originated from therapy refractory subgroups; the class I-selective HDACi CI994 (tacedinaline; phase III) and the HDAC6i ricolinostat were used as controls (Figure 5A and Table S1). To exclude the general cytotoxicity effects (i.e., therapeutic window) of **10h** exposure, two healthy human fibroblast controls were also included in the assays. **10h** markedly outperformed CI994 and ricolinostat in inhibiting the cellular viability of most tested leukemia cell lines at a much lower IC_{50} concentration range than the healthy fibroblast controls (Table S1). Next, the induction of apoptosis following the treatment of HL60 cells with 1 or 5 μM **10h** (for 48 h) was determined (Figure 5B). The treated cells were stained with annexin V and propidium iodide to be analyzed by flow cytometry. The significant ($p < 0.0001$) increase in the level of apoptotic cells observed upon treatment with **10h** at 1 and 5 μM indicates that induction of apoptosis contributes to its antiproliferative potential. To further determine the intracellular target specificity of **10h**, HL60 cells were treated with the respective $\text{IC}_{12.5}$, IC_{25} , IC_{50} , and IC_{75} concentrations of **10h** and subsequently immunoblotted along with the reference HDAC6i ricolinostat at its IC_{50} concentration (Figure 5C). After incubation with **10h**, a dose-dependent increase in the level of acetylation of α -tubulin and histone 3 (H3) was observed. As expected, ricolinostat-treated cells showed prominent acetylation of α -tubulin and Ac-H3 levels lower than that observed for **10h**.

Resistance against a single use of HDACi is often reported;^{55,56} however, co-administration of HDACi with either chemotherapeutic agents or targeted drugs can maximize their efficacy by minimizing the incidence of resistance development and through reducing the toxicity by decreasing the doses. We have previously shown the synergistic effects of HDACi in combination with proteasome inhibitors.^{49,57–59} In the study presented here, we focused on finding clinically relevant synergistic partners of HDACi that are frequently used

Table 2. Cytotoxicities of 10a–p against Selected Leukemia Cell Lines^a

compound	R ¹	R ²	IC ₅₀ HAL01 (μM)	IC ₅₀ HL60 (μM)	IC ₅₀ Jurkat (μM)
10a	c-Hexyl	3,5-Me	3.10 ± 0.91	2.47 ± 2.05	3.91 ± 0.67
10b	c-hexyl	4-NMe ₂	1.05 ± 0.28	0.494 ± 0.126	0.946 ± 0.087
10c	c-hexyl	2-F	11.8 ± 5.14	6.79 ± 3.31	8.97 ± 1.73
10d	c-hexyl	2-Me	9.93 ± 1.55	7.72 ± 3.45	9.47 ± 3.55
10e	c-hexyl	2-OMe	>25	12.3 ± 9.70	18.6 ± 9.01
10f	c-hexyl	2-CF ₃	9.12 ± 4.44	2.00 ± 0.80	>25
10g	Bn	3,5-Me	2.75 ± 1.92	9.15 ± 0.28	6.23 ± 1.62
10h	Bn	4-NMe ₂	0.375 ± 0.162	0.218 ± 0.122	0.285 ± 0.064
10i	<i>t</i> -Bu	3,5-Me	4.93 ± 2.60	4.84 ± 2.78	5.47 ± 0.86
10j	<i>t</i> -Bu	4-NMe ₂	2.47 ± 0.74	1.46 ± 0.77	1.74 ± 0.26
10k	<i>t</i> -Bu	2-F	22.2 ± 3.8	15.7 ± 6.87	19.0 ± 4.58
10l	<i>t</i> -Bu	2-Me	23.9 ± 1.54	13.6 ± 8.19	23.9 ± 1.49
10m	<i>t</i> -Bu	2-OMe	>25	18.4 ± 9.34	24.5 ± 0.75
10n	<i>t</i> -Bu	2-CF ₃	>25	18.6 ± 9.05	>25
10o	<i>t</i> -Bu	2-Cl	22.9 ± 2.64	12.8 ± 8.66	21.6 ± 3.37
10p	<i>t</i> -Bu	2- ⁱ Pr	>25	12.2 ± 9.36	>25
vorinostat	—	—	0.299 ± 0.045	0.22 ± 0.05	0.470 ± 0.023
ricolinostat	—	—	2.04 ± 0.39	1.54 ± 0.16	2.42 ± 0.18
HPOB	—	—	13.9 ± 3.89	11.3 ± 7.2	16.1 ± 2.52

^aVorinostat, HPOB, and ricolinostat were used as controls.

in the therapy protocol for the treatment of leukemia. For instance, in previous preclinical reports, the combination of DNA hypomethylating agents along with HDACi was reported to be synergistic in different hematological malignancies, potentially by disrupting the transcriptional repressor complex involving methyl-CpG-binding proteins and HDACs.^{31,60,61} Therefore, we evaluated the synergistic effects of **10h** in combination with decitabine, a DNA methyltransferase inhibitor (DNMTi) used to treat myelodysplastic syndromes (Figure 6A). Interestingly, the combination of **10h** and decitabine was found to be differentially synergistic in the tested myeloid lineage-derived cell lines HL60 (ZIP synergy score of 25.08) and MOLM13 (27.44) but not in the tested B-cell or T-cell lymphoid lineage-originated leukemic cell lines HAL01 (1.18) and Jurkat (−0.87), respectively (Figure 6a). The observed synergism of **10h** with decitabine later prompted us to examine the underlying molecular mechanism. The co-administration of **10h** and decitabine revealed a strong induction of acetylation of H3 as compared to that with monotherapy, thus indicating that class I HDAC inhibition is required for the synergistic activity with decitabine and the resulting enhancement of anticancer activity (Figure 6C).

Extended Functional Analysis of 10p in Leukemia Cell Lines. In parallel, the inhibitory effects of the most selective HDAC6i **10p** on leukemia cell growth and apoptosis induction were determined. As expected, **10p** had a significantly weaker impact on the viability of tested leukemia cell lines, with IC₅₀ values in the high micromolar concentration range (Figure S4 and Table S1). As in the apoptosis assay, which was performed after treatment of HL60 cells with 1 or 5 μM **10p** for 48 h, a weaker (in comparison to **10h**) although significant (*p* < 0.05) apoptosis induction was noticed (Figure S5). Furthermore, to evaluate the intracellular specificity of **10p**, HL60 cells were treated with the respective IC_{12.5}, IC₂₅, IC₅₀, and IC₇₅ concentrations of **10p** and immunoblotted along with the reference HDAC6i ricolinostat at its IC₅₀ concentration. In agreement, treatment with a selective HDAC6i **10p** induced acetylation of α-tubulin but had no effect on Ac-H3 levels even at concentrations of ≤36

μM (Figure S5C). In a similar manner, we next analyzed the combinatorial potential of **10p** in combination with decitabine, which revealed a moderate (in comparison to that of **10h**) synergism in the tested AML cell lines HL60 (7.78) and MOLM13 (7.18) (Figure 7).

Overall, these data support the therapeutic potential of **10h** and **10p** with regard to leukemia, especially for AML when used in combination with decitabine.

CONCLUSIONS

Selected from a library of 16 compounds that were synthesized using a simple, microwave-assisted two-step protocol, **10h** stood out due to its notable antileukemic effects and, importantly, low cytotoxic activity toward healthy cells. Additional drug combinatorial drug screenings demonstrated a strong synergistic interaction of **10h** with decitabine, which was especially prominent against AML cells. Designed as a pan-inhibitor, **10h** proved to potently inhibit class I HDACs and HDAC6 in biochemical and cellular assays. The analysis of a crystal structure of CD2 from zebrafish HDAC6 complexed with **10h** disclosed differences in zinc coordination but similar occupation of the L1 loop pocket in comparison to those of its nonfluorinated analogue. The remarkable HDAC6 selectivity of our other hit compound **10p** exceeded that of tubastatin A in biochemical enzyme inhibition assays and was confirmed in Western blotting experiments. Despite moderate cytotoxicity on its own, the combination of **10p** and decitabine displayed moderate synergism in AML cell lines, thus indicating the potential of **10p** for combination therapies and targeted therapies involving HDAC6i. On the whole, our study provided valuable SAR data that can be used to fine-tune selectivity profiles toward HDAC6i or unselective but highly cytotoxic pan-HDACi.

EXPERIMENTAL SECTION

Chemistry. Dry MeOH was obtained from the MBraun MB SPS-800 solvent purification system. Except for DCM, which was purified by distillation prior to use, all reagents and solvents were purchased from commercial sources and used without further purification. Thin

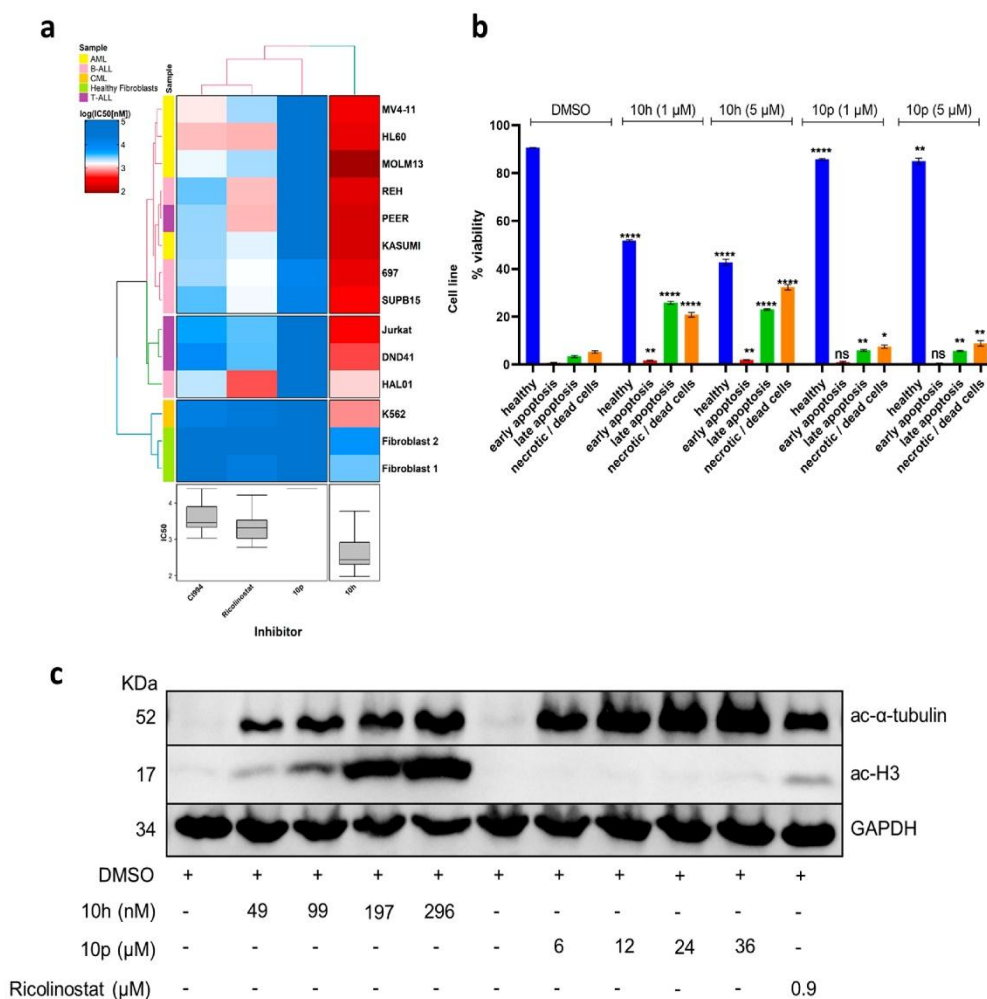


Figure 5. Cytotoxic and target specificity analysis of compounds **10h** and **10p**. (a) Comparative cellular viability (log IC₅₀ in nanomolar) of different subgroups of leukemic cell lines and two healthy human fibroblast controls after treatment with **10h** and **10p** in comparison to the commercially available selective HDAC6 inhibitors ricolinostat and the HDAC class I inhibitor CI994. The IC₅₀ data are plotted as a clustered heat map, followed by unsupervised hierarchical clustering. The horizontal axis of the dendrogram exemplifies the dissimilarity between clusters, whereas the color of the cell is related to its position along with a log IC₅₀ (nanomolar) gradient. (b) Annexin-P1 staining of HL60 cells exposed to **10h** and **10p** at 1 and 5 μM after 48 h (*n* = 3). (c) Immunoblot of HL60 cells after treatment with **10h**, **10p**, and ricolinostat at the indicated concentrations for 24 h.

layer chromatography was carried out using Macherey-Nagel precoated aluminum foil sheets, which were visualized using UV light (254 nm). Hydroxamic acids were further stained using a 1% solution of iron(III) chloride in MeOH. ¹H NMR and ¹³C NMR spectra were recorded at rt or, due to the occurrence of rotamers, at 60 °C using Bruker Avance III HD (400 MHz) and Varian/Agilent Mercury-plus (300 and 400 MHz) spectrometers. Chemical shifts (δ) are quoted in parts per million. All spectra were standardized in accordance with the signals of the deuterated solvents (DMSO-*d*₆, δ_H = 2.50 ppm, δ_C = 39.5 ppm; CDCl₃, δ_H = 7.26 ppm, δ_C = 77.0 ppm). Coupling constants (*J*) are reported in hertz. Mass spectra were recorded by the Leipzig University Mass Spectrometry Service via electrospray ionization (ESI) on Bruker Daltonics Impact II and Bruker Daltonics micrOTOF spectrometers. The uncorrected melting points were determined using a Barnstead Electrothermal 9100 apparatus. Analytical HPLC analyses were carried out using a Thermo Fisher Scientific UltiMate 3000 system equipped with an UltiMate HPG-3400SD pump, an UltiMate 3000 Dioden array detector, an UltiMate 3000 autosampler, and a TCC-3000SD standard thermo-

stated column compartment by Dionex. The system was operated using a Macherey-Nagel NUCLEODUR 100-5 C₁₈ ec column (250 mm × 4.6 mm). UV absorption was detected at 254 nm with a linear gradient from 5% to 95% B within 23 min. HPLC-grade water with 0.1% TFA (solvent A) and HPLC-grade acetonitrile with 0.1% TFA (solvent B) were used for elution at a flow rate of 1 mL/min. The purity of the final compounds was at least 95.0%.

Methyl 3-Fluoro-4-methylbenzoate (2). To a cooled (0 °C) solution of 3-fluoro-4-methylbenzoic acid (5.00 g, 32.5 mmol, 1.0 equiv) in MeOH (150 mL) was added thionyl chloride (3.05 mL, 42.5 mmol, 1.3 equiv), and the resulting mixture was stirred at rt for 16 h. Upon removal of the solvent under reduced pressure, the residue was dissolved in EtOAc (200 mL) and 10% HCl (50 mL) was added. The mixture was extracted with EtOAc (3 × 100 mL), and the combined organics were washed with saturated aqueous NaHCO₃ (2 × 20 mL) and water (1 × 20 mL). Drying over MgSO₄ and evaporation of the solvent afforded **2** as a pale yellow liquid (5.10 g, 30.4 mmol, 93%). Spectroscopic data matched those reported in the literature.⁶³

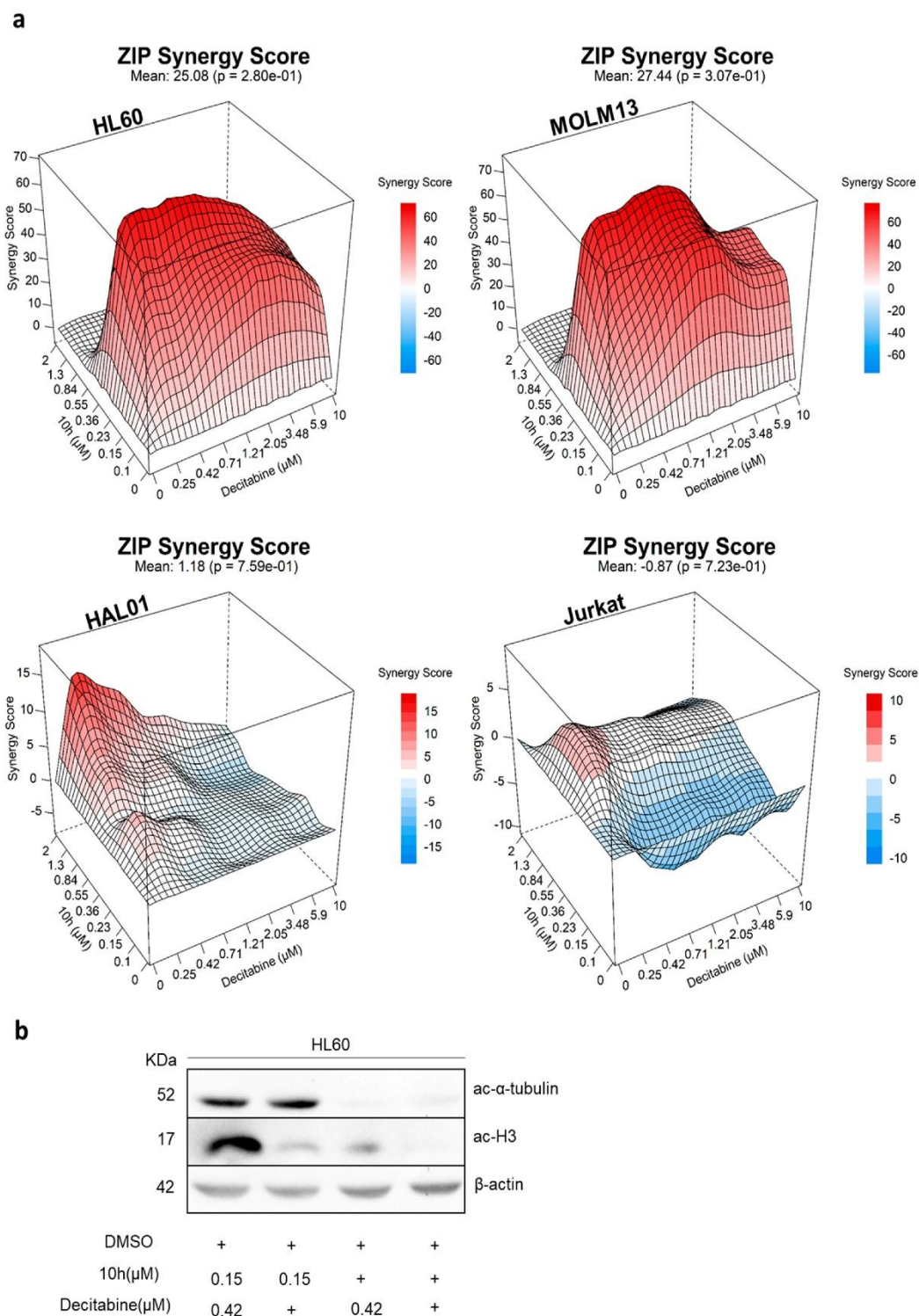


Figure 6. (a) Illustrative synergy plot of 10h after co-treatment of HL60, MOLM13 (AML), HAL01 (B-ALL), and Jurkat (T-ALL) leukemic cell lines with decitabine at the depicted concentrations for 72 h. The mean synergy score calculations were performed using the ZIP model; visualizations were carried out using the Synergy Finder webtool.⁶² (b) Immunoblotting of HL60 cells after treatment with 10h alone and in combination with decitabine at the indicated concentrations for 24 h.

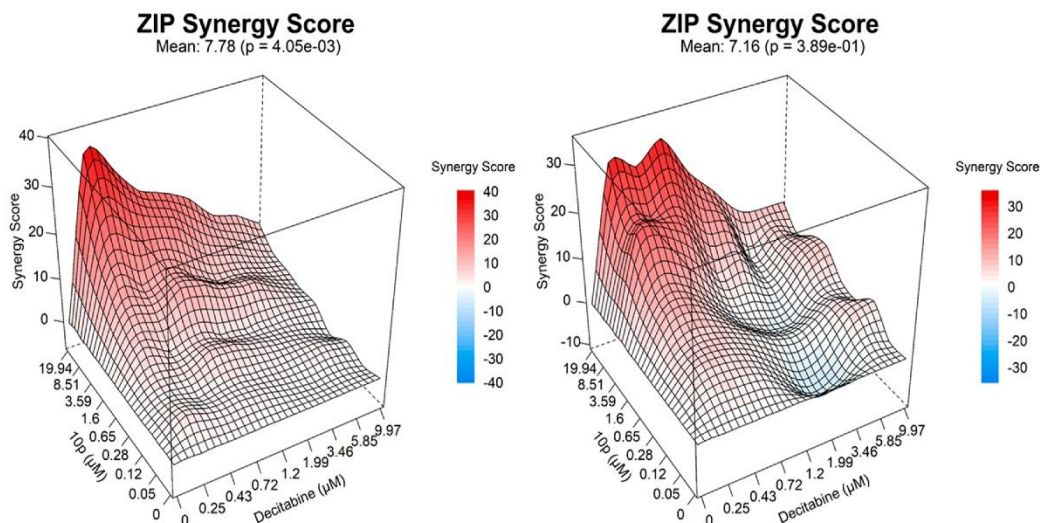


Figure 7. Illustrative synergy plot of **10p** after co-treatment of HL60 and MOLM13 (AML) leukemic cell lines with decitabine at the depicted concentrations for 72 h. The mean synergy score calculations were performed using the ZIP model; visualizations were carried out using the Synergy Finder webtool.⁶²

Methyl 4-(Bromomethyl)-3-fluorobenzoate (3). The title compound was synthesized according to the procedure reported in the literature.⁵⁰ To a solution of **2** (4.91 g, 29.0 mmol, 1.0 equiv) and NBS (7.83 g, 44.0 mmol, 1.5 equiv) in DCM (150 mL) was added AIBN (476 mg, 2.90 mmol, 0.1 equiv). The mixture was first refluxed for 10 h and then stirred at rt for an additional 16 h, after which DCM (100 mL) was added. The resulting solution was washed with 1 M NaOH (3 × 50 mL) and brine (2 × 50 mL) before it was dried over Na₂SO₄. Removal of the solvent under reduced pressure afforded crude product **3** (quant) as a colorless liquid that solidified upon being stored at −18 °C. The crude product was used without further purification.

Methyl 4-(Aminomethyl)-3-fluorobenzoate Hydrochloride (5). This compound was synthesized according to the procedure reported in the literature.⁵⁰ A solution of **3** (1.62 g, 6.50 mmol, 1.0 equiv) and NaN₃ (510 mg, 7.80 mmol, 1.2 equiv) in DMF (25 mL) was stirred at 80 °C for 12 h. After the mixture had cooled to rt, brine (25 mL) was added and the resulting solution was extracted with a mixture of Et₂O and cyclohexane (1:1; 3 × 100 mL). The combined organics were washed with brine (2 × 20 mL), dried over MgSO₄, and concentrated under reduced pressure to afford **4** as a pale yellow liquid. The crude azide thus obtained was subsequently dissolved in a mixture of THF (40 mL) and water (4 mL) to which triphenylphosphine (3.00 g, 11.4 mmol, 1.8 equiv) was added in small portions. After the mixture had been stirred at rt for 40 h, the solvent was evaporated. The residue was redissolved in DCM (200 mL) and extracted with 4 M HCl (2 × 50 mL). The combined aqueous layers were then basified using 2 M NaOH (pH 10) and extracted with DCM (3 × 100 mL). Drying of the combined organics over MgSO₄ and removal of the solvent under reduced pressure afforded the crude amine that was precipitated from MeOH (2 mL), 37% HCl (0.5 mL), and Et₂O (20 mL) to afford hydrochloride salt **5** as a white solid (553 mg, 2.50 mmol, 39% over two steps): mp 236–241 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.70 (s, 3H, NH₃⁺), 7.87–7.62 (m, 3H, arom), 4.12 (q, *J* = 5.8 Hz, 2H, CH₂), 3.88 (s, 3H, OCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 164.9, 161.2, 158.7, 132.0, 131.9, 131.7, 131.6, 126.6, 126.5, 125.2, 125.1, 115.9, 115.7, 52.6, 35.4; HRMS (*m/z*) MNa⁺ calcd for C₉H₁₀FNO₂ 184.0768, found 184.0771.

General Procedure A. Linker building block **5** (132 mg, 0.60 mmol, 1.2 equiv), paraformaldehyde (18.0 mg, 0.60 mmol, 1.2 equiv), and crushed 4 Å molecular sieves (50.0 mg) were suspended in MeOH (1.0 mL), and Et₃N (0.08 mL, 0.60 mmol, 1.2 equiv) was

added. The mixture was subjected to microwave irradiation at 150 W and 45 °C for 20 min before the respective isonitrile (0.50 mmol, 1.0 equiv) and the respective carboxylic acid (0.50 mmol, 1.0 equiv) were added. The resulting mixture was again subjected to microwave irradiation at the same settings for 60 min, and then the molecular sieves were removed by filtration and washed with DCM (10 mL). The filtrate was concentrated under reduced pressure, and the residue was redissolved in DCM (100 mL) and washed with 10% HCl (1 × 10 mL), water (1 × 10 mL), 1 M NaOH (2 × 5 mL), and brine (1 × 10 mL). The organic layer was dried over MgSO₄, and the solvent was removed under reduced pressure to afford crude product **9** that was recrystallized from EtOAc (1 mL) and petrol (20 mL). The ester thus obtained was allowed to dry before it was added to a mixture of NaOH (200 mg, 5.00 mmol, 10 equiv) and hydroxylamine (50% solution in water; 0.95 mL, 15.5 mmol, 31 equiv) in MeOH (4 mL) and DCM (1 mL). The resulting solution was stirred at 0 °C for approximately 15 min until TLC (9:1 DCM/MeOH) indicated full conversion; then, the solvents were evaporated, and the residue was dissolved in water (10 mL). Dropwise addition of 10% HCl (pH 8) induced precipitation of hydroxamic acid **10**, which was then isolated by filtration and washed with 5% HCl (2 × 3 mL) and chilled water (3 × 5 mL), successively. For compounds derived from *tert*-butyl isocyanide, precipitation of the product was often incomplete so that the aqueous layer was further extracted with EtOAc (3 × 30 mL). The combined organics were washed with brine (1 × 10 mL) and dried over Na₂SO₄. Removal of the solvent under reduced pressure yielded the remaining product.

General Procedure B. Linker building block **5** (109 mg, 0.50 mmol, 1.0 equiv), paraformaldehyde (15.0 mg, 0.50 mmol, 1.0 equiv), and crushed 4 Å molecular sieves (50.0 mg) were suspended in MeOH (1.0 mL), and Et₃N (0.08 mL, 0.60 mmol, 1.2 equiv) was added. The mixture was subjected to microwave irradiation at 150 W and 45 °C for 30 min before the respective isonitrile (0.50 mmol, 1.0 equiv) and 4-(dimethylamino)benzoic acid (83.0 mg, 0.50 mmol, 1.0 equiv) were added. The resulting mixture was again subjected to microwave irradiation at the same settings for 3 h, and then the molecular sieves were removed by filtration and washed with DCM (10 mL). The filtrate was concentrated under reduced pressure, and the residue was redissolved in DCM (100 mL) and washed with 1 M NaOH (2 × 5 mL), water (1 × 10 mL), and brine (1 × 10 mL). Drying over MgSO₄ and evaporation of the solvent afforded crude product **9** that was recrystallized from EtOAc (1 mL) and petrol (20 mL). The ester thus obtained was allowed to dry before it was added

to a mixture of NaOH (200 mg, 5.00 mmol, 10 equiv) and hydroxylamine (50% solution in water; 0.95 mL, 15.5 mmol, 31 equiv) in MeOH (4 mL) and DCM (1 mL). The resulting solution was stirred at 0 °C for approximately 15 min until TLC (9:1 DCM/MeOH) indicated full conversion. The organic solvents were removed under reduced pressure, and the residue was dissolved in water (10 mL). Dropwise addition of 10% HCl (pH 8) induced precipitation of hydroxamic acid **10**, which was then isolated by filtration and washed with chilled water (3 × 5 mL). For compound **10j** derived from *tert*-butyl isocyanide, precipitation of the product was incomplete so that the aqueous layer was extracted with EtOAc (3 × 30 mL). The combined organics were washed with brine (1 × 10 mL) and dried over Na₂SO₄. Removal of the solvent under reduced pressure yielded the remaining product.

4-((N-[(Cyclohexylcarbamoyl)methyl]-1-(3,5-dimethylphenyl)formamido)methyl)-3-fluoro-N-hydroxybenzamide (10a). Synthesis according to general procedure A using 3,5-dimethylbenzoic acid (75.0 mg) and cyclohexyl isocyanide (0.06 mL) afforded **10a** as a white solid (86.0 mg, 0.18 mmol, 38%); mp 177 °C; *t*_R 8.02 min, purity 99.7%; ¹H NMR (400 MHz, DMSO-*d*₆, 20 °C) δ 7.75 (d, *J* = 8.2 Hz, 1H, NH), 7.63–7.31 (m, 3H, arom), 7.10–6.93 (m, 3H, arom), 4.68/4.52 (2s, 2H, CH₂), 3.91/3.72 (2s, 2H, CH₂), 3.52 (s, 1H, CH), 2.26/1.80 (2s, 6H, 2 × CH₃), 1.74–0.97 (m, 10H, c-hexyl); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.6, 166.7, 162.4, 161.3, 158.9, 137.6, 135.9, 130.9, 129.8, 124.1, 122.9, 113.7, 113.4, 51.4, 47.7, 43.0, 32.4, 32.3, 25.2, 24.4, 20.8; HRMS (*m/z*) MNa⁺ calcd for C₂₅H₃₀FN₃O₄ 478.2113, found 478.2128.

4-((N-[(Cyclohexylcarbamoyl)methyl]-1-[4-(dimethylamino)phenyl]formamido)methyl)-3-fluoro-N-hydroxybenzamide (10b). Synthesis according to general procedure B using cyclohexyl isocyanide (0.06 mL) afforded **10b** as a white solid (162 mg, 0.34 mmol, 69%); mp 123 °C; *t*_R 6.38 min, purity 98.1%; ¹H NMR (400 MHz, DMSO-*d*₆, 20 °C) δ 11.31 (s, 1H, NH–OH), 9.17 (s, 1H, OH), 7.81 (s, 1H, NH), 7.70–7.26 (m, 5H, arom), 6.78–6.62 (m, 2H, arom), 4.64 (s, 2H, CH₂), 3.85 (s, 2H, CH₂), 3.55 (d, *J* = 9.8 Hz, 1H, CH), 2.93 (s, 6H, 2 × CH₃), 1.76–1.45 (m, 5H, c-hexyl), 1.31–1.02 (m, 5H, c-hexyl); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.7, 166.9, 162.5, 161.2, 158.9, 151.3, 133.9, 129.8, 128.7, 127.5, 127.4, 123.0, 121.9, 113.7, 113.5, 111.0, 47.6, 32.3, 25.2, 24.5; HRMS (*m/z*) MNa⁺ calcd for C₂₅H₃₁FN₃O₄ 493.2222, found 493.2214.

4-((N-[(Cyclohexylcarbamoyl)methyl]-1-(2-fluorophenyl)formamido)methyl)-3-fluoro-N-hydroxybenzamide (10c). Synthesis according to general procedure A using 2-fluorobenzoic acid (70.0 mg) and cyclohexyl isocyanide (0.06 mL) afforded **10c** as a pink solid (85.0 mg, 0.19 mmol, 38%); mp 179 °C; *t*_R 7.40 min, purity 96.6%; ¹H NMR (300 MHz, DMSO-*d*₆, 20 °C) δ 11.30 (d, *J* = 4.7 Hz, 1H, NH–OH), 9.14 (s, 1H, OH), 7.77–7.16 (m, 8H, arom, NH), 4.73/4.50 (2s, 2H, CH₂), 3.98/3.73 (2s, 2H, CH₂), 3.47–3.40 (m, 1H, CH), 1.81–1.43 (m, 5H, c-hexyl), 1.36–0.89 (m, 5H, c-hexyl); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 166.5, 166.0, 165.9, 162.6, 161.3, 161.1, 158.9, 158.8, 158.7, 156.5, 156.3, 133.9, 131.5, 131.4, 129.8, 129.7, 129.6, 128.72, 128.68, 128.4, 127.1, 126.9, 124.8, 124.7, 124.6, 124.0, 123.8, 122.9, 115.9, 115.7, 113.9, 113.6, 50.9, 47.62, 47.55, 47.1, 47.0, 43.2, 32.4, 32.1, 25.2, 25.1, 24.5, 24.3; HRMS (*m/z*) M[–] calcd for C₂₃H₂₅F₂N₃O₄ 444.1740, found 444.1741.

4-((N-[(Cyclohexylcarbamoyl)methyl]-1-(2-methylphenyl)formamido)methyl)-3-fluoro-N-hydroxybenzamide (10d). Synthesis according to general procedure A using 2-methylbenzoic acid (68.0 mg) and cyclohexyl isocyanide (0.06 mL) afforded **10d** as a pink solid (84.0 mg, 0.19 mmol, 38%); mp 115 °C; *t*_R 7.55 min, purity 95.1%; ¹H NMR (400 MHz, DMSO-*d*₆, 60 °C) δ 11.12 (s, 1H, NH–OH), 8.99 (s, 1H, OH), 7.71–7.37 (m, 4H, arom, NH), 7.37–7.06 (m, 4H, arom), 4.75/4.41 (2s, 2H, CH₂), 4.01/3.64 (2s, 2H, CH₂), 3.48 (d, *J* = 9.7 Hz, 1H, CH), 2.30/2.22 (2s, 3H, CH₃), 1.82–1.47 (m, 5H, c-hexyl), 1.36–0.95 (m, 5H, c-hexyl); ¹³C NMR (75 MHz, DMSO-*d*₆) δ 171.1, 166.2, 135.8, 134.3, 134.1, 130.3, 128.8, 125.7, 125.6, 123.0, 113.9, 113.6, 50.6, 47.7, 47.6, 42.5, 40.1, 32.4, 32.2, 25.2, 25.1, 24.5, 24.4, 18.5; HRMS (*m/z*) M[–] calcd for C₂₄H₂₈FN₃O₄ 440.1991, found 440.1988.

4-((N-[(Cyclohexylcarbamoyl)methyl]-1-(2-methoxyphenyl)formamido)methyl)-3-fluoro-N-hydroxybenzamide (10e). Synthesis according to general procedure A using 2-methoxybenzoic acid (76.0 mg) and cyclohexyl isocyanide (0.06 mL) afforded **10e** as a white solid (121 mg, 0.26 mmol, 53%); mp 130 °C; *t*_R 7.33 min, purity 95.1%; ¹H NMR (400 MHz, DMSO-*d*₆, 20 °C) δ 11.31 (d, *J* = 6.8 Hz, 1H, NH–OH), 9.14 (s, 1H, OH), 7.69–6.92 (m, 8H, arom, NH), 4.95 (s, 1H, CH₂), 4.46–3.99 (m, 2H, CH₂), 3.83–3.57 (m, 4H, OCH₃, CH₂), 3.47–3.39 (m, 1H, CH), 1.80–1.43 (m, 5H, c-hexyl), 1.32–0.90 (m, 5H, c-hexyl); ¹³C NMR (75 MHz, DMSO-*d*₆) δ 169.2, 169.1, 166.3, 166.2, 162.7, 161.6, 158.3, 154.9, 154.5, 133.61, 130.57, 130.4, 129.0, 127.7, 125.3, 125.1, 123.0, 120.5, 113.7, 113.4, 111.4, 55.6, 55.3, 50.7, 47.5, 42.7, 32.4, 32.2, 25.1, 24.5, 24.4; HRMS (*m/z*) MH⁺ calcd for C₂₄H₂₈FN₃O₅ 458.2086, found 458.2066.

4-((N-[(Cyclohexylcarbamoyl)methyl]-1-[2-(trifluoromethyl)phenyl]formamido)methyl)-3-fluoro-N-hydroxybenzamide (10f). Synthesis according to general procedure A using 2-(trifluoromethyl)benzoic acid (95.0 mg) and cyclohexyl isocyanide (0.06 mL) afforded **10f** as a white solid (142 mg, 0.29 mmol, 57%); mp 107 °C; *t*_R 7.84 min, purity 95.0%; ¹H NMR (400 MHz, DMSO-*d*₆, 20 °C) δ 11.31 (d, *J* = 8.4 Hz, 1H, NH–OH), 9.15 (s, 1H, OH), 7.95–7.23 (m, 8H, arom, NH), 5.27–5.07/4.60–4.24 (2m, 2H, CH₂), 3.70–3.43 (m, 3H, CH, CH₂), 1.81–1.43 (m, 5H, c-hexyl), 1.36–0.91 (m, 5H, c-hexyl); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 168.6, 168.5, 166.0, 162.5, 161.4, 159.0, 158.7, 134.2, 134.1, 134.0, 132.7, 132.0, 131.5, 131.4, 130.5, 130.0, 129.8, 129.7, 128.8, 128.7, 127.6, 126.7, 126.54, 126.49, 126.45, 125.5, 125.2, 125.0, 124.9, 122.8, 122.3, 113.8, 113.6, 50.6, 47.7, 47.5, 47.0, 42.5, 32.4, 32.1, 25.2, 25.1, 24.5, 24.3; HRMS (*m/z*) M[–] calcd for C₂₄H₂₅F₄N₃O₄ 494.1708, found 494.1718.

4-((N-[(Benzylcarbamoyl)methyl]-1-(3,5-dimethylphenyl)formamido)methyl)-3-fluoro-N-hydroxybenzamide (10g). Synthesis according to general procedure A using 3,5-dimethylbenzoic acid (75.0 mg) and benzyl isocyanide (0.06 mL) afforded **10g** as a dark brown solid (85.0 mg, 0.18 mmol, 37%); mp 215 °C; *t*_R 7.77 min, purity 95.2%; ¹H NMR (300 MHz, DMSO-*d*₆, 20 °C) δ 11.31 (s, 1H, NH–OH), 9.14 (s, 1H, OH), 8.42 (s, 1H, NH), 7.75–6.92 (m, 11H, arom), 4.71/4.59 (s, 2H, CH₂), 4.36–4.23 (m, 2H, CH₂), 4.02/3.87 (s, 2H, CH₂), 2.24 (s, 6H, 2 × CH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.6, 167.8, 139.4, 139.3, 139.1, 139.0, 137.6, 136.1, 135.8, 131.1, 130.9, 129.9, 129.8, 129.6, 129.51, 129.45, 128.3, 127.2, 126.9, 124.1, 123.1, 123.0, 113.9, 113.7, 51.5, 43.1, 42.2, 20.8; HRMS (*m/z*) M[–] calcd for C₂₆H₂₆FN₃O₄ 462.1835, found 462.1839.

4-((N-[(Benzylcarbamoyl)methyl]-1-[4-(dimethylamino)phenyl]formamido)methyl)-3-fluoro-N-hydroxybenzamide (10h). Synthesized according to general procedure B using benzyl isocyanide (0.06 mL). The reaction time for the ester formation was reduced to 60 min (imine formation, 20 min; U4CR, 40 min). **10h** was obtained as an off-white solid (162 mg, 0.34 mmol, 68%); mp 205 °C; *t*_R 6.35 min, purity 97.2%; ¹H NMR (400 MHz, DMSO-*d*₆, 20 °C) δ 11.31 (s, 1H, NH–OH), 9.16 (s, 1H, OH), 8.47 (t, *J* = 6.0 Hz, 1H, NH), 7.68–7.43 (m, 3H, arom), 7.39–7.16 (m, 7H, arom), 6.74–6.58 (m, 2H, arom), 4.69 (s, 2H, CH₂), 4.30 (d, *J* = 5.8 Hz, 2H, CH₂), 3.96 (s, 2H, CH₂), 2.93 (s, 6H, NMe₂); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.8, 168.2, 162.5, 158.7, 151.3, 139.2, 133.8, 128.6, 128.3, 127.2, 126.8, 123.0, 113.8, 110.9, 42.1; HRMS (*m/z*) MNa⁺ calcd for C₂₆H₂₇FN₃O₄ 501.1909, found 501.1910.

4-((N-[(*tert*-Butylcarbamoyl)methyl]-1-(3,5-dimethylphenyl)formamido)methyl)-3-fluoro-N-hydroxybenzamide (10i). Synthesis according to general procedure A using 3,5-dimethylbenzoic acid (75.0 mg) and *tert*-butyl isocyanide (0.06 mL) afforded **10i** as a white solid (125 mg, 0.29 mmol, 58%); mp 162 °C; *t*_R 7.72 min, purity 97.9%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.31 (s, 1H, NH–OH), 9.14 (s, 1H, OH), 7.69–7.28 (m, 4H, arom, NH), 7.12–6.87 (m, 3H, arom), 4.67/4.51 (2s, 2H, CH₂), 3.88/3.69 (2s, 2H, CH₂), 2.27 (s, 6H, 2 × CH₃), 1.42/1.24/1.21 (s, 9H, *t*-Bu); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.6, 166.6, 162.6, 161.3, 161.1, 158.9, 137.6, 137.5, 136.0, 133.8, 131.5, 131.4, 130.8, 129.9, 128.8, 128.7, 127.4, 127.3, 124.1, 123.5, 123.0, 113.8, 113.6, 51.6, 50.2, 43.1, 28.5, 28.4, 20.8; HRMS (*m/z*) M[–] calcd for C₂₃H₂₈FN₃O₄ 428.1991, found 428.1992.

4-([N-[(*tert*-Butylcarbamoyl)methyl]-1-[4-(dimethylamino)phenyl]formamido)methyl]-3-fluoro-*N*-hydroxybenzamide (10j). Synthesis according to general procedure B using *tert*-butyl isocyanide (0.06 mL) afforded **10j** as an off-white solid (85.0 mg, 0.19 mmol, 38%): mp 132 °C; t_R 6.10 min, purity 95.5%; 1H NMR (400 MHz, DMSO- d_6 , 20 °C) δ 11.22 (s, 1H, NH–OH), 9.14 (s, 1H, OH), 7.71–7.23 (m, 6H, arom, NH), 6.78–6.62 (m, 2H, arom), 4.63 (s, 2H, CH₂), 3.82 (s, 2H, CH₂), 2.93 (s, 6H, NMe₂), 1.22 (s, 9H, *t*-Bu); ^{13}C NMR (101 MHz, DMSO- d_6) δ 171.7, 169.3, 167.3, 162.5, 161.3, 151.3, 133.7, 131.5, 131.4, 129.7, 128.8, 128.7, 127.7, 127.5, 123.0, 122.0, 113.8, 113.6, 111.0, 58.4, 50.3, 49.9, 28.43, 28.38; HRMS (m/z) M⁺ calcd for C₂₃H₂₉FN₄O₄ 467.2065, found 467.2071.

4-([N-[(*tert*-Butylcarbamoyl)methyl]-1-(2-fluorophenyl)-formamido)methyl]-3-fluoro-*N*-hydroxybenzamide (10k). Synthesis according to general procedure A using 2-fluorobenzoic acid (70.0 mg) and *tert*-butyl isocyanide (0.06 mL) afforded **10k** as an orange solid (133 mg, 0.32 mmol, 63%): mp 111 °C; t_R 7.10 min, purity 96.8%; 1H NMR (400 MHz, DMSO- d_6 , 20 °C) δ 9.21 (s, 1H, OH), 7.73–7.12 (m, 8H, arom, NH), 4.72/4.47 (s, 2H, CH₂), 3.95/3.75 (2s, 2H, CH₂), 1.44/1.24/1.17/1.14 (4s, 9H, *t*-Bu); ^{13}C NMR (101 MHz, DMSO- d_6) δ 166.9, 166.4, 166.1, 162.5, 161.3, 161.2, 158.9, 158.7, 134.4, 134.0, 133.9, 131.5, 131.4, 129.9, 129.6, 128.8, 128.7, 128.5, 127.1, 126.9, 126.1, 124.8, 124.6, 124.0, 123.9, 122.9, 115.9, 115.7, 113.8, 113.6, 51.2, 50.2, 47.3, 46.9, 43.3, 28.5, 28.3, 27.9; HRMS (m/z) M⁺ calcd for C₂₁H₂₃F₂N₃O₄ 418.1584, found 418.1584.

4-([N-[(*tert*-Butylcarbamoyl)methyl]-1-(2-methylphenyl)-formamido)methyl]-3-fluoro-*N*-hydroxybenzamide (10l). Synthesis according to general procedure A using 2-methylbenzoic acid (68.0 mg) and *tert*-butyl isocyanide (0.06 mL) afforded **10l** as an off-white solid (105 mg, 0.25 mmol, 50%): mp 109 °C; t_R 7.24 min, purity 96.4%; 1H NMR (300 MHz, DMSO- d_6 , 60 °C) δ 11.05 (s, 1H, NH–OH), 8.98 (s, 1H, OH), 7.70–7.12 (m, 8H, arom, NH), 4.74/4.41 (2s, 2H, CH₂), 3.93/3.60 (2 × s, 2H, CH₂), 2.30/2.22 (2s, 3H, CH₃), 1.47/1.27/1.18/1.17 (s, 9H, *t*-Bu); ^{13}C NMR (101 MHz, DMSO- d_6) δ 171.2, 171.0, 166.5, 162.5, 162.3, 135.9, 134.3, 134.0, 133.94, 133.86, 132.0, 131.5, 131.4, 130.3, 129.8, 128.83, 128.78, 128.7, 127.4, 127.2, 125.7, 125.6, 125.5, 123.0, 113.9, 113.6, 51.0, 50.2, 46.7, 46.5, 42.7, 28.5, 28.3, 18.5; HRMS (m/z) M⁺ calcd for C₂₂H₂₆FN₃O₄ 414.1835, found 414.1835.

4-([N-[(*tert*-Butylcarbamoyl)methyl]-1-(2-methoxyphenyl)-formamido)methyl]-3-fluoro-*N*-hydroxybenzamide (10m). Synthesis according to general procedure A using 2-methoxybenzoic acid (76.0 mg) and *tert*-butyl isocyanide (0.06 mL) afforded **10m** as a white solid (83.0 mg, 0.19 mmol, 38%): mp 126 °C; t_R 7.54 min, purity 95.6%; 1H NMR (300 MHz, DMSO- d_6 , 60 °C) δ 9.46–8.85 (br s, 1H, OH), 7.71–6.80 (m, 8H, arom, NH), 4.42 (s, 1H, CH₂), 4.01–3.40 (m, 6H, OCH₃, 2 × CH₂), 1.47–1.09 (m, 9H, *t*-Bu); ^{13}C NMR (101 MHz, DMSO- d_6) δ 169.4, 169.3, 169.1, 166.7, 166.5, 162.6, 161.2, 158.7, 154.9, 154.7, 154.5, 134.0, 133.6, 133.5, 132.1, 131.5, 131.4, 130.7, 130.6, 130.4, 129.1, 129.0, 128.8, 128.7, 127.8, 127.7, 127.5, 127.4, 125.1, 123.0, 122.8, 122.7, 120.6, 120.5, 113.7, 113.4, 111.5, 111.4, 55.6, 55.3, 51.0, 50.2, 47.7, 46.5, 42.6, 28.5, 28.3; HRMS (m/z) M⁺ calcd for C₂₂H₂₆FN₃O₅ 430.1784, found 430.1784.

4-([N-[(*tert*-Butylcarbamoyl)methyl]-1-[2-(trifluoromethyl)phenyl]formamido)methyl]-3-fluoro-*N*-hydroxybenzamide (10n). Synthesis according to general procedure A using 2-(trifluoromethyl)benzoic acid (95.0 mg) and *tert*-butyl isocyanide (0.06 mL) afforded **10n** as a yellow solid (103 mg, 0.22 mmol, 44%): mp 137 °C; t_R 7.03 min, purity 95.1%; 1H NMR (300 MHz, DMSO- d_6 , 60 °C) δ 10.91 (s, 1H, NH–OH), 9.00 (s, 1H, OH), 7.89–7.17 (m, 8H, arom, NH), 5.11/4.47 (2s, 2H, CH₂), 3.90/3.57 (2s, 2H, CH₂), 1.46/1.26/1.18 (3s, 9H, *t*-Bu); ^{13}C NMR (75 MHz, DMSO- d_6) δ 168.5, 166.4, 166.2, 162.5, 162.4, 161.8, 134.4, 134.32, 134.29, 134.1, 134.04, 133.99, 132.71, 132.68, 132.6, 132.06, 132.05, 132.0, 131.5, 131.4, 130.52, 130.45, 130.4, 129.8, 129.7, 128.8, 128.7, 127.73, 127.67, 127.6, 126.8, 126.6, 126.51, 126.49, 126.4, 125.2, 124.8, 122.9, 122.81, 122.79, 121.8, 113.91, 113.87, 113.6, 50.8, 50.3, 42.7, 42.6,

28.5, 28.3; HRMS (m/z) M⁺ calcd for C₂₂H₂₃F₄N₃O₄ 468.1552, found 468.1548.

4-([N-[(*tert*-Butylcarbamoyl)methyl]-1-(2-chlorophenyl)-formamido)methyl]-3-fluoro-*N*-hydroxybenzamide (10o). Synthesis according to general procedure A using 2-chlorobenzoic acid (78.0 mg) and *tert*-butyl isocyanide (0.06 mL) afforded **10o** as a white solid (150 mg, 0.34 mmol, 69%): mp 145 °C; t_R 7.27 min, purity 98.6%; 1H NMR (400 MHz, DMSO- d_6 , 20 °C) δ 11.27 (s, 1H, NH–OH), 9.15 (s, 1H, OH), 7.71–7.24 (m, 8H, arom, NH), 5.27–5.05/4.73–4.22 (2m, 2H, CH₂), 4.05–3.79/3.72–3.52 (2m, 2H, CH₂), 1.24/1.14 (2s, 9H, *t*-Bu); ^{13}C NMR (101 MHz, DMSO- d_6) δ 168.3, 168.0, 166.3, 166.1, 162.5, 161.3, 161.2, 158.8, 158.7, 135.30, 135.27, 134.3, 134.2, 133.9, 133.9, 131.5, 131.4, 130.7, 130.6, 130.1, 130.04, 129.97, 129.9, 129.6, 129.4, 128.9, 128.8, 128.7, 128.3, 128.1, 127.4, 127.3, 127.0, 126.9, 50.8, 50.3, 47.3, 46.6, 42.9, 42.9, 28.5, 28.3; HRMS (m/z) M⁺ calcd for C₂₁H₂₃ClFN₃O₄ 434.1288, found 434.1273.

4-([N-[(*tert*-Butylcarbamoyl)methyl]-1-[2-(propan-2-yl)phenyl]formamido)methyl]-3-fluoro-*N*-hydroxybenzamide (10p). Synthesis according to general procedure A using 2-isopropylbenzoic acid (82.0 mg) and *tert*-butyl isocyanide (0.06 mL) afforded **10p** as a white solid (132 mg, 0.30 mmol, 60%): mp 130 °C; t_R 7.96 min, purity 96.4%; 1H NMR (400 MHz, DMSO- d_6 , 20 °C) δ 11.31 (s, 1H, NH–OH), 9.116 (s, 1H, OH), 7.68–7.09 (m, 8H, arom, NH), 4.98–4.82/4.64–4.08 (2m, 2H, CH₂), 3.81–3.70/3.66–3.48 (2m, 2H, CH₂), 3.12 (2p, J = 6.7/6.9 Hz, 1H, *i*-Pr-CH), 1.38–1.04 (m, 15H, *i*-Pr, *t*-Bu); ^{13}C NMR (101 MHz, DMSO- d_6) δ 171.1, 166.6, 166.5, 162.5, 161.4, 161.0, 159.0, 158.6, 145.1, 144.9, 135.0, 134.9, 134.0, 133.9, 131.5, 131.4, 129.6, 129.3, 129.2, 128.8, 128.7, 127.3, 127.2, 125.8, 125.7, 125.6, 125.5, 123.1, 123.0, 113.9, 113.7, 51.0, 50.3, 46.9, 42.3, 29.9, 29.8, 28.5, 28.3, 24.9, 24.2, 23.2, 22.8; HRMS (m/z) M⁺ calcd for C₂₄H₃₀FN₃O₄ 466.2113, found 466.2128.

Crystallization. Catalytic domain 2 of HDAC6 from *D. rerio* (hereafter noted simply “HDAC6”) was recombinantly expressed in *Escherichia coli* BL21 (DE3) cells via inoculation with the His-MBP-TEV-HDAC6-pET28a(+) vector. The protein was purified using techniques previously described.⁶⁴

The HDAC6–**10h** complex was crystallized via sitting drop vapor diffusion at 4 °C. Briefly, a 100 nL drop of a protein solution [10 mg/mL HDAC6, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM tris(2-carboxyethyl)phosphine (TCEP), **10h** (2 mM), and 5% DMSO (v/v)] was added to a 100 nL drop of the precipitant solution from the PEG/ION screen [2% (v/v) Tacsimate (pH 8.0), 0.1 M Tris (pH 8.5), and 16% (w/v) PEG 3,350] and equilibrated against 80 μ L of the precipitant solution. Crystals appeared after 2 weeks and were soaked in a cryoprotectant solution comprised of the mother liquor solution and 20% ethylene glycol prior to looping and flash cooling in liquid nitrogen.

Data Collection and Structure Determination. X-ray diffraction data for the HDAC6–**10h** complex were collected on the NSLS-II AMX beamline at Brookhaven National Laboratory. Data were indexed and integrated using iMosflm;⁶⁵ calculation of the Patterson map and self-rotation function indicated the presence of pseudotranslation with a peak height that was ~28% of that of the origin peak. Weak reflections resulting from the pseudotranslation were removed during phasing and refinement. The structure was phased by molecular replacement using the atomic coordinates of unliganded HDAC6 (PDB entry SEEM)¹⁹ as a search probe in Phaser.⁶⁶ The atomic structures were then modeled and refined using Coot⁶⁷ and Phenix,⁶⁸ and the **10h** inhibitor was built into well-defined electron density in the final stages of refinement. The quality of the final model was evaluated with MolProbity.⁶⁹ Data collection and refinement statistics are listed in Table S2. Atomic coordinates and crystallographic structure factors have been deposited in the Protein Data Bank as entry 7U8Z.

Biological Experiments. Cell Culture. Leukemia cell lines HL60, MOLM13, MV4–11 (acute myeloid leukemia or AML), HAL01, REH, 697 (B-cell acute lymphoblastic leukemia or B-ALL), K562

(chronic myeloid leukemia), and Jurkat (T-cell acute lymphoblastic leukemia) (DSMZ, Braunschweig, Germany) and two healthy fibroblast control cells (ATCC, Manassas, VA) were cultured in RPMI 1640 GlutaMax medium supplemented with 10–20% FCS. All cell lines were grown at 37 °C under humidified air supplemented with 5% CO₂.

Cell Viability Assay. The CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI) was used to determine the IC₅₀ values of the tested compounds for every cell line. The different compounds were printed on white 384-well plates (Thermo Fisher Scientific, Waltham, MA) with at least five different, increasing concentrations (5 nM to 25 μM) using a digital dispenser (D300e, Tecan, Männedorf, Switzerland). The cell viability was monitored after 72 h using the CellTiter-Glo luminescent assay using a microplate reader (Spark, Tecan). The IC₅₀ values for the tested compounds were determined by plotting raw data (normalized to controls) using a sigmoid dose curve and nonlinear regression (GraphPad Prism Inc., San Diego, CA).

Combinatorial Drug Screening. 10h and decitabine were printed on white 384-well plates (Thermo Fisher Scientific) with increasing concentrations in dose–response 8 × 8 matrices using a digital dispenser (D300e, Tecan). 10h was printed in the concentration range from 0.1 to 2 μM and for decitabine from 0.25 to 10 μM, in a logarithmic distribution. The viability was monitored after 72 h using the CellTiter-Glo luminescent assay (as described above), using a microplate reader (Spark, Tecan). The ZIP synergy score calculations were based on the ZIP model⁷⁰ using the Synergy Finder tool (<https://synergyfinder.fimm.fi/>).

Annexin V Staining. To evaluate apoptosis, cells treated with the respective compounds or the control at the indicated concentrations for 48 h were stained with annexin V and PI and later subjected to FACS analysis, following the supplier's guidelines (Invitrogen, Carlsbad, CA).

Immunoblotting. Whole-cell lysates were produced after treatment with the respective compounds under standard culture conditions for 24 h. Then, whole-cell lysates were immunoblotted using anti-acetyl-α-tubulin (catalog no. 5335), anti-acetyl-histone H3 (catalog no. 9677S), and anti-β-actin (catalog no. 5125S) or anti-GAPDH (catalog no. 97166), following the supplier's guidelines (Cell Signaling Technology, Danvers, MA).

Inhibition Assay for HDAC1–3 and HDAC6. *In vitro* inhibitory activities against human HDAC1, HDAC2, HDAC3/NcoR2, and HDAC6 were measured using a previously published protocol.⁷¹ In short, OptiPlate-96 black microplates (PerkinElmer) were used with an assay volume of 50 μL. Five microliters of the test compound or control, diluted in assay buffer [50 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, and 0.1 mg/mL BSA], was incubated with 35 μL of the fluorogenic substrate ZMAL [Z-Lys(Ac)-AMC]⁷² (21.43 μM in assay buffer) and 10 μL of human recombinant HDAC1 (BPS Bioscience, catalog no. 50051), HDAC2 (BPS Bioscience, catalog no. 50052), HDAC3/NcoR2 (BPS Bioscience, catalog no. 50003), or full length HDAC6 (BPS Bioscience, catalog no. 50006) at 37 °C. After an incubation time of 90 min, 50 μL of 0.4 mg/mL trypsin in trypsin buffer [50 mM Tris-HCl (pH 8.0) and 100 mM NaCl] was added, followed by further incubation at 37 °C for 30 min. Fluorescence was measured with an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Fluoroskan Ascent microplate reader (Thermo Scientific). All compounds were evaluated in duplicate in at least two independent experiments.

HDAC4 Inhibition Assay. *In vitro* inhibitory activities against HDAC4 were determined using a HDAC4 assay protocol adapted from ref 73. For compounds and controls, 3-fold serial dilutions of the respective DMSO stock solutions were prepared in assay buffer [50 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂·6H₂O, and 0.1 mg/mL BSA]. These serial dilutions (5.0 μL) were transferred into 96-well microplates (OptiPlate-96 Black, PerkinElmer), and 35 μL of the fluorogenic substrate Boc-Lys(Tfa)-AMC (Bachem, catalog no. 4060676, 42.86 μM in assay buffer) and 10 μL of HDAC4 (BPS Bioscience, catalog no. 50004) enzyme solution were added. The total assay volume (50 μL, maximum 1% DMSO)

was incubated at 37 °C for 90 min. Subsequently, 50 μL of trypsin (1.0 mg/mL) in trypsin buffer [50 mM Tris-HCl (pH 8.0) and 100 mM NaCl] was added, followed by incubation for an additional 30 min at 37 °C. Fluorescence (excitation, 355 nm; emission, 460 nm) was measured using a FLUOstar OPTIMA microplate reader (BMG LABTECH). IC₅₀ values were determined by generating normalized dose–response curves using the built-in “log(inhibitor) vs response (three parameters)” equation provided by GraphPad Prism (GraphPad Prism 9.0). All compounds were tested in duplicate, and reported mean IC₅₀ values, including the standard deviation, were calculated from at least two independent experiments. TMP269⁷⁴ (Selleck Chemicals, catalog no. S7324) was used as the control compound (IC₅₀ = 0.488 ± 0.049 μM).

PAINS Analysis. We filtered all compounds for pan-assay interference compounds (PAINS) using the online filter <http://zinc15.docking.org/patterns/home/>.⁷⁵ No compound was flagged as PAINS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.2c01418>.

Supplementary tables, NMR spectra, and HPLC traces of newly synthesized compounds (PDF)

Molecular formula strings and some data (CSV)

Protein Data Bank information (PDB)

Accession Codes

The atomic coordinates and crystallographic structure factors of the HDAC6–10h complex have been deposited in the Protein Data Bank as entry 7U8Z.

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Author Contributions

[#]N.R. and J.S.-D. share first authorship. S.B. and F.K.H. contributed equally to this work as senior authors. N.R. synthesized all intermediates and target compounds. P.R.W., A.D.C., and D.W.C. determined the crystal structure of the HDAC6–10h complex. J.S.-D., M.S., A.S., J.Y., and L.S.-H. performed the biological evaluation. A.B., D.W.C., S.B., and F.K.H. acquired funding and provided resources. N.R., S.B., and F.K.H. designed the study. The manuscript was written through contributions of all authors. All authors have given approval to the final version.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AIBN, azobis(isobutyronitrile); CDCl₃, chloroform-*d*; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DCM, dichloromethane; Et₂O, diethyl ether; EtOAc, ethyl acetate; MeOH, methanol; NBS, *N*-bromosuccinimide; PDB, Protein Data Bank; petrol, petroleum ether; rt, room temperature; TFA, trifluoroacetic acid; THF, tetrahydrofuran; U4CR, Ugi four-component reaction

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NOTE ADDED AFTER ASAP PUBLICATION

After this paper was published ASAP on November 9, 2022, a correction was made to the TOC and abstract graphics. The corrected version was reposted November 14, 2022.

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3.2 - Preferential HDAC6 inhibitors derived from HPOB exhibit synergistic antileukemia activity in combination with decitabine

In this publication, a library of HPOB-derived HDACi was synthesized and investigated regarding synergistic potency with decitabine against leukemia. The preferential HDAC6i was used as a platform structure. Chemically, a microwave-assisted Ubi-four-component reaction with an aniline, a carboxylic acid, an isocyanide, and formaldehyde was performed. After an initial characterization regarding their ability to bind to HDAC1 and HDAC6. While the selectivity of the novel compounds was comparable to HPOB, they gained increased binding potency. This correlated with lower IC₅₀ concentrations in three leukemia cell lines throughout all synthesized compounds. The inhibitors with the lowest selectivity but highest potency (**1g**) and those with the highest selectivity (**2b**) for HDAC6 were further characterized. An in-depth HDAC inhibition profiling revealed that **1g** targets HDAC1, HDAC2, HDAC3, and HDAC6 unselectively, while **2b** is much more specific to HDAC6. Further analysis of the antileukemic properties revealed that **1g** had a significantly lower IC₅₀ than CI994 and ricolinostat, while **2b** was comparable. A western blot analysis validated that the inhibitors bound to their targets. In an extended matrix synergy screening approach, the **2b** showed very promising synergistic potency in AML specifically, while **1g** induced, in comparison, low levels of synergistic toxicity. This leads to the conclusion that especially HDAC6 selective inhibitors could benefit from when combined with decitabine in AML.

Contributions: In general, writing of the manuscript and experimental design, execution, evaluation, and visualization of the following biological experiments:

- Initial screening of the compound library (see Table 2)
- Focused screening with an extended number of leukemia cell lines (see Figure 4A)
- Western blot analysis of downstream HDAC deacetylation targets after HDACi treatment (see Figure 4B)
- Matrix synergy screening of **2b** with decitabine in four different leukemia cell lines (see Figure 5A-5D)
- Matrix synergy screening of **1g** with decitabine in two AML leukemia cell lines (see Figure 6E-6F)



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Research paper

Preferential HDAC6 inhibitors derived from HPOB exhibit synergistic antileukemia activity in combination with decitabine

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ABSTRACT

Histone deacetylase 6 (HDAC6) is an emerging drug target to treat oncological and non-oncological conditions. Since highly selective HDAC6 inhibitors display limited anticancer activity when used as single agent, they usually require combination therapies with other chemotherapeutics. In this work, we synthesized a mini library of analogues of the preferential HDAC6 inhibitor HPOB in only two steps via an Ugi four-component reaction as the key step. Biochemical HDAC inhibition and cell viability assays led to the identification of **1g** (highest antileukemic activity) and **2b** (highest HDAC6 inhibition) as hit compounds. In subsequent combination screens, both **1g** and especially **2b** showed synergy with DNA methyltransferase inhibitor decitabine in acute myeloid leukemia (AML). Our findings highlight the potential of combining HDAC6 inhibitors with DNA methyltransferase inhibitors as a strategy to improve AML treatment outcomes.

1. Introduction

Histone deacetylases (HDACs), a class of enzymes, remove acetyl residues from the terminal *N*-acetyl lysine of histones, causing the DNA to wrap more tightly around its histone core [1]. Along with their counterpart, the histone acetyltransferases (HATs), they are essential for the regulation gene expression, playing a critical role in cellular functions such as cell cycle progression, cell differentiation, migration, protein activity and stability, and tumorigenesis [2,3]. Furthermore, dysregulation of histone (de)acetylation, one of the most common epigenetic processes in cancer, can impact cell death pathways and DNA damage repair [4].

HDACs are classified into different groups: class I (HDAC1, 2, 3, and 8), class IIa (HDAC4, 5, 7, and 9), class IIb (HDAC6 and 10), class III (Sirt1-7), and class IV (HDAC11) [5]. While class I, IIa, IIb, and IV HDACs are zinc-dependent, class III isoforms, the so-called sirtuins, are

nicotinamide adenine dinucleotide (NAD⁺)-dependent [6]. Abnormal expression of HDACs has been linked to the occurrence of different malignant tumors, making HDAC inhibitors (HDACi) a focus of intense research [7]. Currently, the FDA has approved four HDACi (Fig. 1A) for the treatment of multiple myeloma and T-cell lymphoma: romidepsin, panobinostat, belinostat, and vorinostat [7]. Furthermore, givinostat (Fig. 1A) received FDA approval in 2024 for treating Duchenne muscular dystrophy (DMD) in patients aged six and older, marking the first time an HDACi has been approved for a non-oncological condition [8]. These drugs share a common three-motif pharmacophore model, featuring a surface recognition cap group, a hydroxamic acid moiety as a zinc-binding group (ZBG), and a linker that connects the two parts [9, 10]. The ZBG is essential for chelation of Zn²⁺ in the enzyme's active site, while the linker contains a non-polar chain or an aromatic ring that allows the cap group to occupy the entrance to the pocket [11].

HDACs 1–3 and 6 are of particular interest in cancer drug development due to their unique mechanisms of action. HDAC1–3 are often

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Abbreviations		HDAC(s)	histone deacetylase(s)
ACN	acetonitrile	HDACi	histone deacetylase inhibitor(s)
AML	acute myeloid leukemia	HAT(s)	histone acetyltransferase(s)
CDCl ₃	chloroform-d	HPOB	N-Hydroxy-4-(2-[(2-hydroxyethyl)(phenyl)amino]-2-oxoethyl)benzamide
CH	cyclohexane	HSP90	heat shock protein 90
DMD	Duchenne muscular dystrophy	NAD ⁺	nicotinamide adenine dinucleotide
DMSO	dimethylsulfoxide	SI	selectivity index
DNMT	DNA methyltransferase	TFA	trifluoroacetic acid
DNMTi(s)	DNA methyltransferase inhibitor(s)	TLC	thin layer chromatography
EtOAc	ethyl acetate	U-4CR	Ugi four-component reaction
FDA	U.S. Food and Drug Administration	ZBG(s)	zinc-binding group(s)
h	hour(s)		

overexpressed in different cancer types, leading to aberrant gene expression patterns that promote cell survival, proliferation, and resistance to cell death. Inhibitors of HDAC1-3 have shown promising results in preclinical and clinical trials for the treatment of various types of cancer, including lymphoma, leukemia, breast cancer, and lung cancer [15].

HDAC6 differs from HDAC1-3 in that it predominantly targets non-histone proteins, including α -tubulin, cortactin, STAT3, and HSP90, which are all implicated in cancer development, progression, and metastasis [16,17]. While selective HDAC6 inhibitors have shown promising activity in combination with other therapies like

chemotherapy and immunotherapy, their anticancer activity is limited when used alone [18,19]. However, highly selective HDAC6 inhibitors are currently investigated for the treatment of a range of non-oncological disorder including Charcot-Marie-Tooth disease, cardiovascular diseases, obesity, idiopathic pulmonary fibrosis, and inflammation [20–24]. To this end, it will be crucial to replace the mutagenic and genotoxic hydroxamic acid ZBG [25]. Fortunately, recent progress with alternative ZBG such as 2-(difluoromethyl)-1,3,4-oxadiazoles (DFMOs) and mercaptoacetamides indicate that it is possible to develop highly efficient non-hydroxamic acid HDAC6 inhibitors [17,26, 27].

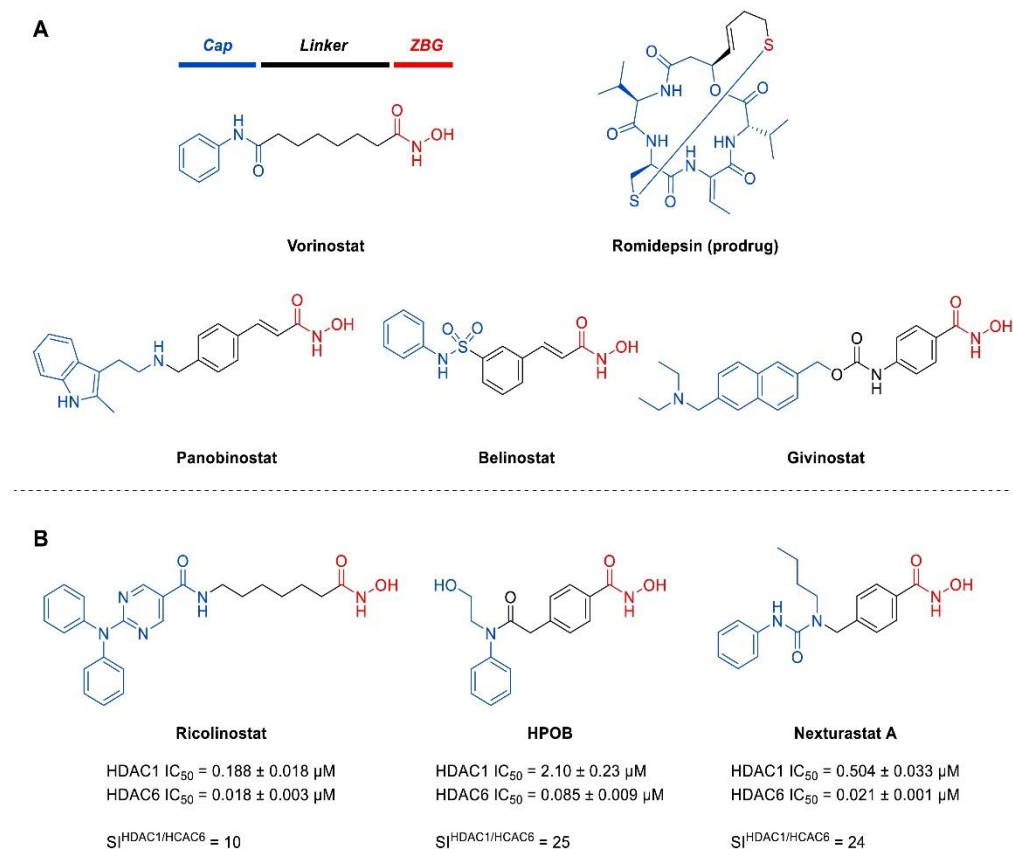


Fig. 1. A) Structures of FDA-approved HDACi: vorinostat, romidepsin, belinostat, panobinostat, and givinostat. B) Selected HDAC6 preferential HDACi with IC₅₀ values against HDAC1 and 6. SI = selectivity index. Data from Refs. [12–14].

In regards to cancer, there is growing evidence suggesting that the anticancer effects of certain HDAC6 inhibitors with anticancer activity are achieved by inhibiting multiple HDAC isozymes including HDAC1-3 [18]. As a result, HDAC6 preferential inhibitors like ricolinostat (Fig. 1B) and citarinostat, which retain some ability to inhibit HDAC1-3, have shown the most promising activities at the preclinical stage and in clinical trials. *N*-Hydroxy-4-(2-[(2-hydroxyethyl)(phenyl)amino]-2-oxoethyl)benzamide (HPOB, Fig. 1B) is an HDAC6 preferential inhibitor that exhibits 25-fold greater selectivity for HDAC6 over HDAC1 in biochemical HDAC inhibition assays [12]. While it shows inhibitory activity against HDAC6 (IC_{50} : 0.085 μ M), it is somewhat less potent than other preferential HDAC6 inhibitors such as ricolinostat or nexturastat A (see Fig. 1B).

Here we aimed to optimize the HDAC inhibitory properties and anticancer activity of HPOB. To expand the structure-activity relationships of this type of HDACi, we synthesized a series of compounds using the Ugi four-component reaction (U-4CR) as a key step, followed by post-Ugi transformations to introduce the hydroxamic acid ZBG. We then evaluated all synthesized compounds for inhibitory properties against HDAC1 and HDAC6, as well as their antiproliferative activity in three leukemia cell lines. The most promising compounds were then screened for their ability to induce apoptosis and synergistic anticancer activity with the DNA methyltransferase inhibitor decitabine.

2. Results and discussion

2.1. Design and multicomponent synthesis of HPOB analogues

HPOB is a preferential HDAC6 inhibitor utilizing an aromatic linker and a branched cap group. This design principle is well-established to achieve HDAC6 preferential or even selective inhibition and was similarly utilized in frequently used tool compounds such as nexturastat A [28,29]. However, compared to nexturastat A, HPOB demonstrates a reduced inhibitory potency against HDAC6. Consequently, our aim was to improve the HDAC6 inhibition. To this end, we replaced the hydroxyethyl substituent by a substituted acetamide group while retaining the anilide moiety (type 1, Fig. 2). Compounds of this type should be easily accessible by the U-4CR as the key step, thereby enabling the rapid access to a focused library of HPOB analogues. Recently, we and others reported the beneficial effect of linker fluorination in the *meta*-position to the hydroxamic acid on the HDAC6 selectivity profile [16,30–32]. Thus, we also included a series of fluorinated derivatives in our compound design (type 2, Fig. 2).

The designed compounds were synthesized in only two steps via an U-4CR as key step followed by a hydroxylaminolysis reaction to install the hydroxamic acid as ZBG. In detail, the U-4CR of the respective aniline of type 3, formaldehyde (4) (obtained from paraformaldehyde), the respective carboxylic acid 5 or 6, and the respective isocyanide 7, generated the intermediates **8a-i** and **9a-d** within less than 2 hours of microwave irradiation (Scheme 1). After purification of the intermediates, the esters **8a-i** and **9a-d** were treated with a mixture of sodium hydroxide and aqueous hydroxylamine to afford the HPOB analogues **1a-i** and **2a-d**. All final compounds were purified by precipitation from the aqueous crude mixture and subsequent washing steps,

column chromatography, or preparative HPLC and exceeded 95 % purity.

2.2. Inhibition of HDAC1 and HDAC6

The synthesized HPOB analogues **1a-i** and **2a-d** were first tested in fluorogenic assays for their inhibitory potencies against HDAC1 and HDAC6 using HPOB and vorinostat as control compounds. The results are presented in Table 1. HPOB displayed an IC_{50} value of 0.085 μ M in combination with a 25-fold selectivity in comparison to HDAC1 ($SI^{HDAC1/6}$ = 25). As expected, vorinostat demonstrated a slightly more potent, but unselective inhibition of HDAC6 (HDAC6 IC_{50} = 0.048 μ M; $SI^{HDAC1/6}$ = 2).

In a first set of compounds (**1a-d**) we utilized aniline as amine component and four commercially available isocyanides (cyclohexyl, benzyl, *n*-butyl, and *tert*-butyl) in the U-4CR. Compound **1a** containing a cyclohexyl moiety in the isocyanide region emerged as the most promising compound (HDAC6 IC_{50} = 0.035 μ M; $SI^{HDAC1/6}$ = 8). Consequently, we focused on compounds derived from cyclohexyl isocyanide and modified the aniline part of the HDACi by introducing a methyl group in the *ortho*-, *meta*-, or *para*-position (compounds **1e-g**). The introduction of a methyl group in the *ortho*-position (**1e**) led to a 2-fold increase in HDAC6 inhibitory potency compared to **1a** while the selectivity profile was retained (HDAC6 IC_{50} = 0.017 μ M; $SI^{HDAC1/6}$ = 8). In contrast, when the methyl group was inserted into the *meta*-position (**1f**), we noticed a slightly reduced HDAC6 inhibition and selectivity (HDAC6 IC_{50} = 0.050 μ M; $SI^{HDAC1/6}$ = 6). In the case of the *para*-substituted derivative **1g**, a reduced HDAC6 preference was observed (HDAC6 IC_{50} = 0.041 μ M; $SI^{HDAC1/6}$ = 2), leading to a vorinostat-like HDAC inhibition profile.

Due to the promising HDAC6 inhibitory activity of **1e**, we prepared additional compounds with altered residues in the *ortho*-position. The introduction of an ethyl substitution led to a reduced HDAC6 inhibition (compound **1h**, HDAC6 IC_{50} = 0.035 μ M; $SI^{HDAC1/6}$ = 10), while the replacement of the methyl in **1e** by a trifluoromethyl group resulted in similar HDAC6 inhibition in combination with slightly increased selectivity (compound **1i**, HDAC6 IC_{50} = 0.021 μ M; $SI^{HDAC1/6}$ = 11).

Next, we investigated a series of fluorinated analogues of **1a**, **1e**, **1h**, and **1i**. The unsubstituted aniline derivative **2a** (HDAC6 IC_{50} = 0.016 μ M; $SI^{HDAC1/6}$ = 22) displayed a 2-fold increased HDAC6 inhibition and ~3-fold improved selectivity compared to its non-fluorinated counterpart **1a**. Since the introduction of a methyl group in the *ortho*-position turned out to be beneficial in the nonfluorinated series, we also studied methyl (**2b**), ethyl (**2c**), and trifluoromethyl (**2d**) derivatives of **2a**. All three compounds showed potent (IC_{50} values ranging from 0.009 to 0.018 μ M) and preferential HDAC6 inhibition. Overall, compound **2b** (HDAC6 IC_{50} = 0.009 μ M; $SI^{HDAC1/6}$ = 25) emerged as the most potent and selective HDAC6 inhibitor from this series. To conclude, all synthesized HPOB analogues exceeded the HDAC6 inhibitory capacity of HPOB with IC_{50} values ranging from 0.009 to 0.060 μ M, albeit with different selectivity profiles ranging from vorinostat-like to HPOB-like.

2.3. Docking studies

Since compound **2b** was found to be the most potent and selective HDAC6 inhibitor in this series, docking studies were performed to investigate a potential binding mode of the inhibitor in the active site of HDAC6 (Fig. 3). Co-crystallization of the HDACi HPOB in complex with *Danio rerio* HDAC6 (PDB ID: 5EF7) revealed a coordination of the zinc ion with a monodentate geometry. Only the deprotonated hydroxyl group of the hydroxamate coordinates the zinc without displacing the zinc-bound water molecule. Additionally, many selective HDAC6 inhibitors bearing bulky and rigid capping groups exhibit this unusual binding mode [29,33,34]. Due to the structural similarity of compound **2b** with HPOB and a comparable selectivity for HDAC6, we assumed a similar binding mode of the hydroxamic acid within the active site of

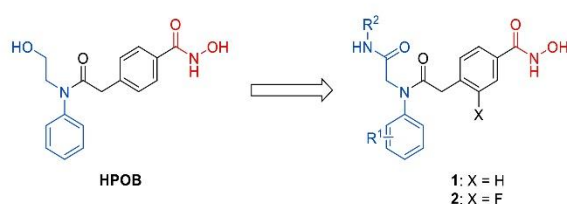
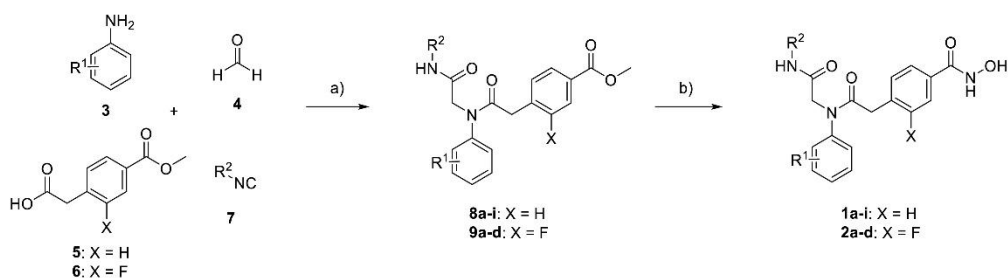


Fig. 2. Design of HPOB analogues.



Scheme 1. Microwave-assisted Ugi four-component reaction to synthesize the HPOB analogues **1a-i** and **2a-d**. *Reagents and conditions:* a) (i) **3**, paraformaldehyde, MeOH, Et₃N, 150 W, 45 °C, 30 min; (ii) **5** or **6**, R²-NC (**7**), 150 W, 45 °C, 1–2 h; b) MeOH, CH₂Cl₂, aq. H₂NOH, NaOH, rt, 2–4 h.

Table 1

IC₅₀ values of nonfluorinated HDACi **1a-i** and fluorinated analogues **2a-d** in comparison to those of HPOB and vorinostat against HDAC1 and HDAC6.

	R ¹	R ²	IC ₅₀ [μM]		SI ^a
			HDAC1	HDAC6	
1a	H	c-Hex	0.293 ± 0.033	0.035 ± 0.001	8
1b	H	Bn	0.283 ± 0.001	0.043 ± 0.005	7
1c	H	n-Bu	0.450 ± 0.062	0.060 ± 0.006	7
1d	H	t-Bu	0.407 ± 0.063	0.059 ± 0.006	7
1e	2-Me	c-Hex	0.138 ± 0.008	0.017 ± 0.0004	8
1f	3-Me	c-Hex	0.313 ± 0.031	0.050 ± 0.004	6
1g	4-Me	c-Hex	0.083 ± 0.001	0.041 ± 0.002	2
1h	2-Et	c-Hex	0.363 ± 0.056	0.035 ± 0.004	10
1i	2-CF ₃	c-Hex	0.230 ± 0.029	0.021 ± 0.004	11
2a	H	c-Hex	0.357 ± 0.004	0.016 ± 0.003	22
2b	2-Me	c-Hex	0.228 ± 0.035	0.009 ± 0.003	25
2c	2-Et	c-Hex	0.275 ± 0.014	0.018 ± 0.002	16
2d	2-CF ₃	c-Hex	0.265 ± 0.024	0.013 ± 0.001	21
HPOB			2.100 ± 0.231	0.085 ± 0.009	25
vorinostat			0.088 ± 0.008	0.048 ± 0.004	2

^a Selectivity index [SI = (IC₅₀ HDAC1)/(IC₅₀ HDAC6)].

HDAC6. Hence, the hydroxamic acid substructure of HPOB from the crystal structure 5EF7 was used as a template for the docking studies to provide a reasonable complexation of the zinc ion. In order to gain an insight into the putative binding mode of **2b** in human HDAC6, the hydroxamic acid scaffold as well as the zinc-bound water molecule was placed into the crystal structure of human HDAC6 (PDB ID: 5EDU) by structural alignment. The docking solution revealed a pose within HDAC6's active site that partially resembles the binding mode of HPOB. Due to the scaffold constraint, a monodentate coordination of the zinc ion was determined. In addition, the phenyl linker adjacent to the hydroxamate is placed between two aromatic residues F⁶⁸⁰ and F⁶²⁰ that could enable π - π interactions within the active site tunnel. For the two parts of the bifurcated cap group a binding mode was predicted that suggest the occupation of the L1 and L2 pockets. The aromatic 2-methylphenyl cap group is orientated towards L1 loop and fits in the hydrophobic pocket, which is formed by the side chains of H⁵⁰⁰ and P⁵⁰¹. Compared to HPOB (see Fig. S1, Supporting Information), a slightly different direction of the aromatic cap moiety of compound **2b** was predicted, thus leading to a better steric complementarity to the L1 pocket. The other longer hydrophobic cyclohexyl branch lies in the L2 pocket and is surrounded by the amino acids F⁶⁷⁹ and L⁷⁴⁹. Both amino acids create a favorable hydrophobic environment for the cyclohexane moiety. Although no direct interaction of the branched groups, i.e. acetamide and anilide moiety, could be observed, the high potency of **2b** against HDAC6 could arise from the optimal positioning of the cap groups that fill out the respective L1 and L2 pocket.

2.4. Cytotoxicity against leukemia cell lines

All synthesized compounds were next screened for their anti-proliferative activity against the three leukemia cell lines HALO1 (B-cell acute lymphoblastic leukemia or B-ALL), HL60 (acute myeloid leukemia

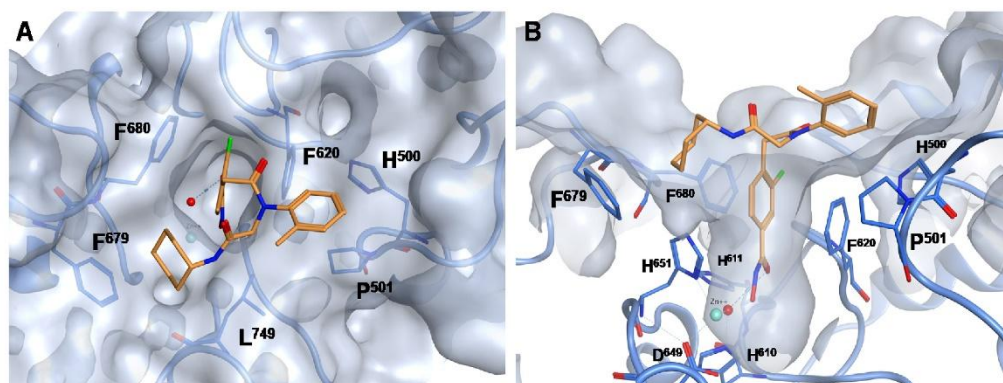


Fig. 3. Predicted binding mode of compound **2b** (orange colored) in crystal structure of HDAC6 (PDB ID: 5EDU). A) Top view of the L1 and L2 pocket. B) Side view of the active site. The zinc ion is represented as a cyan sphere. The water molecule is depicted as red sphere.

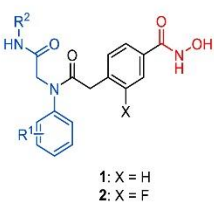
or AML), and Jurkat (T-cell acute lymphoblastic leukemia or T-ALL). Vorinostat and HPOB were used as controls; the results are presented in Table 2. Most of the synthesized HPOB analogues exhibited antiproliferative activities in the single digit micromolar range against all three cell lines and thus exceeded the activity of HPOB, which showed double-digit micromolar IC_{50} values. The only exception in this series was compound **1g**, which turned out to be the most potent HPOB analog with a similar cytotoxicity profile as vorinostat and submicromolar IC_{50} values against all three cell lines. These results are in excellent agreement with the comparable HDAC inhibition profiles of **1g** and vorinostat, both regarding potency and selectivity, as detailed in Table 1. Interestingly, both HPOB and **2b** displayed an identical SI for HDAC1/HDAC6 of 25. However, against the three leukemia cell lines, **2b** showed a significant increase in antiproliferative activity, which was more than five times higher than HPOB. It should be noted that compound **2b** exhibits approximately a 9-fold greater inhibitory activity against HDAC1 than HPOB (see Table 1), suggesting that this enhancement is likely due to increased inhibition of class I isoforms. In addition, differences in cell permeability and physicochemical properties may account for the enhanced antiproliferative activity of compound **2b** compared to HPOB. Consequently, we have calculated drug-likeness properties of all compounds under investigation using SwissADME (Table S1, Supporting Information). The results confirmed that none of the compounds violated any of Lipinski's Rule of Five. Compounds **1a-i** and **2a-d** exhibited calculated Log *P* values ranging from 2.10 to 3.88, suggesting sufficient cell permeability. Notably, compound **2b** (cLog *P* = 3.14) was more lipophilic than HPOB (cLog *P* = 1.41), which could partially explain **2b**'s enhanced antiproliferative effects due to a better cell permeability.

2.5. Selection of hit compounds and extended HDAC isoform profiling

Based on their antiproliferative activities and HDAC6 inhibition profiles we selected compounds **1g** (highest antileukemia activity) and **2b** (highest and most selective HDAC6 inhibition) for additional experiments. First, we performed an extended HDAC isoform profiling and

Table 2

Cytotoxicities of nonfluorinated HDACi **1a-i** and fluorinated analogues **2a-d** in comparison to those of HPOB and vorinostat against selected leukemia cell lines.



1: X = H
2: X = F

	IC_{50} [μ M]				
	R ¹	R ²	HAL01	HL60	Jurkat
1a	H	c-Hex	2.02 ± 0.44	1.83 ± 0.60	2.54 ± 0.43
1b	H	Bn	2.15 ± 0.77	2.31 ± 0.26	2.29 ± 0.71
1c	H	n-Bu	3.12 ± 1.32	2.68 ± 0.81	4.03 ± 1.50
1d	H	t-Bu	4.91 ± 1.49	5.99 ± 1.31	8.31 ± 2.85
1e	2-Me	c-Hex	1.51 ± 0.31	2.31 ± 0.90	2.52 ± 0.74
1f	3-Me	c-Hex	1.89 ± 0.35	1.75 ± 0.99	2.59 ± 0.45
1g	4-Me	c-Hex	0.23 ± 0.10	0.36 ± 0.06	0.41 ± 0.06
1h	2-Et	c-Hex	1.43 ± 0.12	1.34 ± 0.32	2.28 ± 0.15
1i	2-CF ₃	c-Hex	3.03 ± 1.17	2.09 ± 1.74	4.58 ± 0.78
2a	H	c-Hex	2.08 ± 1.04	1.34 ± 0.56	2.42 ± 0.55
2b	2-Me	c-Hex	2.32 ± 0.77	2.04 ± 0.62	3.08 ± 0.59
2c	2-Et	c-Hex	1.44 ± 0.29	1.51 ± 0.89	3.21 ± 0.60
2d	2-CF ₃	c-Hex	1.79 ± 0.26	1.61 ± 0.69	2.28 ± 0.48
HPOB			13.86 ± 3.89	11.33 ± 7.22	16.07 ± 2.52
vorinostat			0.30 ± 0.05	0.22 ± 0.05	0.47 ± 0.02

screened both compounds for their inhibition of HDAC2 and HDAC3 using vorinostat as control; the results are summarized in Table 3. As expected, **1g** emerged as an unselective inhibitor with IC_{50} values of 0.112 and 0.042 μ M against HDAC2 and HDAC3, respectively. In contrast, compound **2b** showed even higher selectivity for HDAC6 against HDAC2 (IC_{50} = 0.787 μ M; $SI^{HDAC2/6}$ = 87) and HDAC3 (IC_{50} = 0.520 μ M; $SI^{HDAC3/6}$ = 58) compared to HDAC1 (IC_{50} = 0.228 μ M; $SI^{HDAC1/6}$ = 25).

2.6. **1g** and **2b** induce cytotoxicity against leukemia cells via activating apoptosis

To comprehensively evaluate the antileukemic properties of **1g** and **2b**, we used a high-throughput drug screening approach by employing a diverse panel of leukemia cell lines. Additionally, we included two commercially available inhibitors, CI994 (tacedinaline) and ricolinostat, as positive controls (Fig. 4A). Notably, **1g** exhibited superior cytotoxic activity against leukemia cell lines compared to the reference inhibitors, as evidenced by its IC_{50} profile (mean IC_{50} fold change = 6.8 compared to CI994 and 9.7 for ricolinostat). In contrast, **2b** demonstrated comparable IC_{50} values, occasionally surpassing the reference inhibitors in specific cases (mean IC_{50} fold change = 0.95 for CI994 and 1.35 for ricolinostat). Notably, the AML cell lines exhibited comparatively higher susceptibility to both **1g** and **2b**. To assess whether **1g** and **2b** operate within a therapeutic window, avoiding general cytotoxicity against healthy cells, we included healthy fibroblast controls in our screening. Importantly, neither **1g** nor **2b** exhibited any cytotoxic activity against healthy fibroblasts.

Subsequent investigation into markers of HDAC inhibition, such as acetylation levels of H3 (HDAC class I target) and α -tubulin (HDAC6 target) through immunoblotting at various inhibitor concentrations, revealed distinct patterns (Fig. 4B). In the case of **1g**, α -tubulin hyperacetylation was relatively weak at lower concentrations, where H3 acetylation levels were significantly increased. When compared, **1g** (24 nM) elicited analogous levels of H3 hyperacetylation at a concentration as low as 2.67 % of that required for ricolinostat (0.9 μ M), while causing minimal changes to α -tubulin acetylation. In contrast, as expected, **2b** efficiently increased α -tubulin acetylation while affecting H3 acetylation to a lesser extent, mirroring the effects of ricolinostat at similar compound concentrations.

Subsequent to this, we examined the potential of **1g** and **2b** to induce apoptosis through annexin-PI staining (Fig. 4C). Relative to the DMSO control, **1g** demonstrated a substantial reduction in the population of viable cells by half, accompanied by an elevation in the percentages of early, late apoptotic, and dead cells. Conversely, **2b** exhibited less pronounced shifts, yet still exhibited a significant increase in the percentage of late apoptotic cells.

2.7. **1g** and **2b** synergize effectively with the standard chemotherapeutic decitabine against AML cells

A cornerstone of every modern leukemia treatment protocol is the combination a several anticancer chemotherapeutics. It is crucial that these agents exhibit synergistic interactions to maximize leukemia cell eradication while minimizing cytotoxic side effects. A widely recognized synergistic drug combination includes the use of DNA methyltransferase inhibitors (DNMTis), such as decitabine, in combination with HDAC

Table 3

Extended HDAC isoform profiles of **1g** and **2b**.

	IC_{50} [μ M]			
	HDAC1	HDAC2	HDAC3	HDAC6
1g	0.083 ± 0.001	0.112 ± 0.004	0.042 ± 0.003	0.042 ± 0.002
2b	0.228 ± 0.035	0.787 ± 0.003	0.520 ± 0.014	0.009 ± 0.003
vorinostat	0.088 ± 0.008	0.140 ± 0.019	0.096 ± 0.012	0.048 ± 0.004

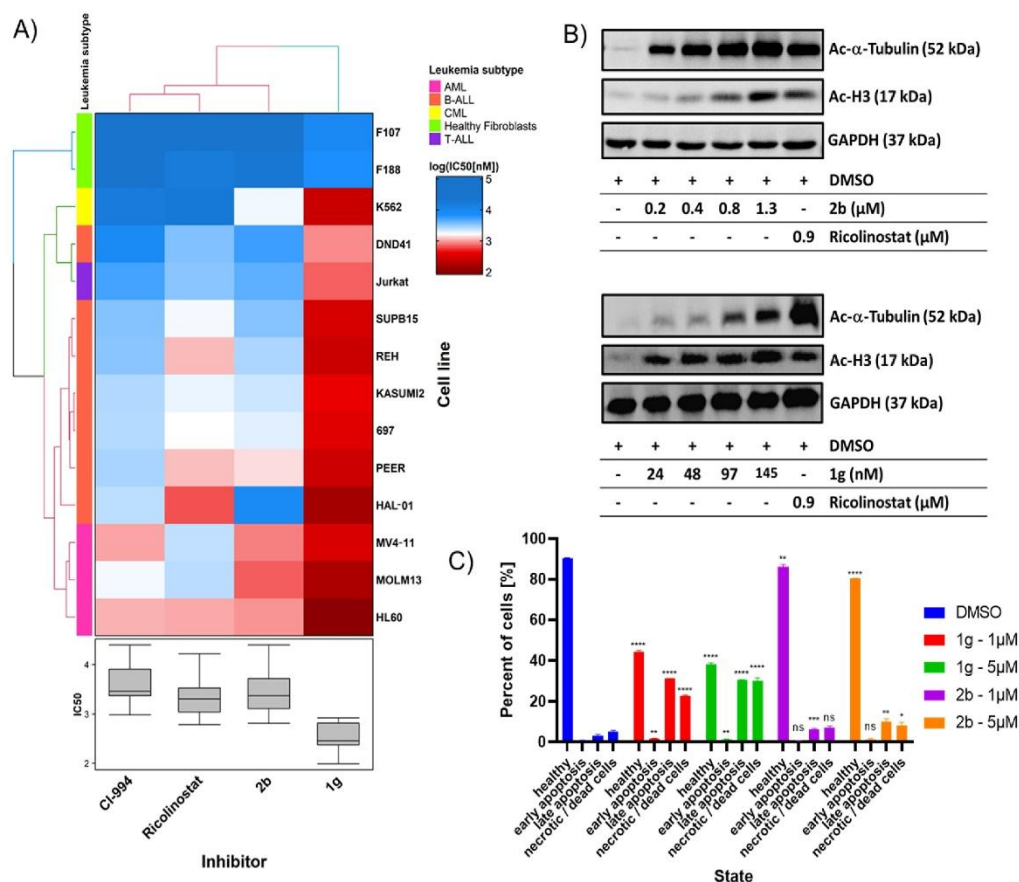


Fig. 4. A) Comparative cellular viability (log IC₅₀ nM) of different sub-groups of leukemic cell lines (K562, DND41, Jurkat, SUPB15, REH, Kasumi 2, 697, PEER, HAL01, MV4-11, MOLM13 and HL60) and healthy fibroblasts (F107 and F188), after treatment with **1g** and **2b** in comparison to the commercially available selective HDAC6 inhibitors ricolinostat and HDAC class I inhibitor CI-994 (n = 3). The log IC₅₀ nM data was visualized through a clustered heat map, accompanied by unsupervised hierarchical clustering. The dendrogram represents the similarity between inhibitors, while the color of each tile indicates its position along a gradient of log IC₅₀ (nM). B) Western Blot analysis was performed to evaluate the impact on HDAC inhibition markers, specifically acetylated H3 (ac-H3) and acetylated Tubulin (ac-Tubulin), after a 24-h treatment with compounds **1g**, **2d**, and the control, ricolinostat, in HL60 leukemia cells (n = 3). The presented blot includes a representative sample, with GAPDH employed as the loading control. C) Annexin-PI staining to evaluate the induction of apoptosis in HL60 leukemia cells exposed to **1g** and **2b** at concentrations of 1 μM and 5 μM over a 48-h period (n = 3). The bar graphs displays statistical significance (unpaired *t*-test), * = *p* < 0.05, ** = *p* < 0.01, *** = *p* < 0.001, **** = *p* < 0.0001.

inhibitors [16,35,36]. To explore the potential synergistic effects of combining **1g** or **2b** with decitabine, we utilized a matrix drug screening approach with the zero interaction potency (ZIP) algorithm (Fig. 5). In the case of **2b**, we observed particularly high ZIP synergy scores of around 60 across a broad range of concentrations in both AML cell lines (HL60 and MOLM13; Fig. 5A and B). Interestingly, in HAL-01 (B-ALL) or JURKAT (T-ALL), no substantial synergy was observed (Fig. 5C and D). In fact, the drug synergy matrices for HAL-01 and JURKAT displayed a trend towards antagonism, highlighting the potential selectivity of this synergistic response in the context of AML. When combined with decitabine, **1g** also showed synergistic effects against HL60 and MOLM13 cells, albeit with notably lower ZIP scores (~10–15) than those observed with **2b** (Fig. 5E and F). This indicates that although **1g** is more potent as an individual cytotoxic inhibitor, **2b** excels significantly in inducing specific drug synergy with decitabine against AML cells.

Consistent with this, a prior study revealed that the combination of a DNMTi and HDAC6 inhibitors can augment anti-tumor immune signaling and reduce tumor burden in ovarian cancer [38]. Taken together, the selective synergistic effects of compounds **1g** and **2b** with decitabine were particularly evident in myeloid-originated leukemia cell lines (HL60 and MOLM13), in contrast to lymphoid-originated leukemia

cell lines (HAL01 and JURKAT). The observed selectivity of the epigenetic combination, which includes HDAC inhibitors and DNMTis, towards AML cell lines is in line with our previous findings and has been corroborated by other studies [16,39,40]. Furthermore, additional studies have underscored the safety, tolerability, and anti-leukemia efficacy of this combined treatment approach, prompting the initiation of clinical trials in AML [41,42]. In regards to potential mechanisms driving this synergistic interaction, it is known that crosstalk exists between DNA methylation and HDAC pathways. Specifically, this process entails the binding of a 5-methylcytosine-binding protein to the target gene promoter, facilitating subsequent recruitment of HDACs [43]. The significance of this interplay in AML cells is underscored by the synergistic activation of the tumor suppressor genes such as CDKN2B [43]. However, further studies will be required to determine the specific mechanisms contributing to the heightened synergistic interaction observed against AML cells upon combining preferential HDAC6 inhibitors with DNMTis.

3. Conclusions

In summary, we have synthesized a series of HPOB analogues via a

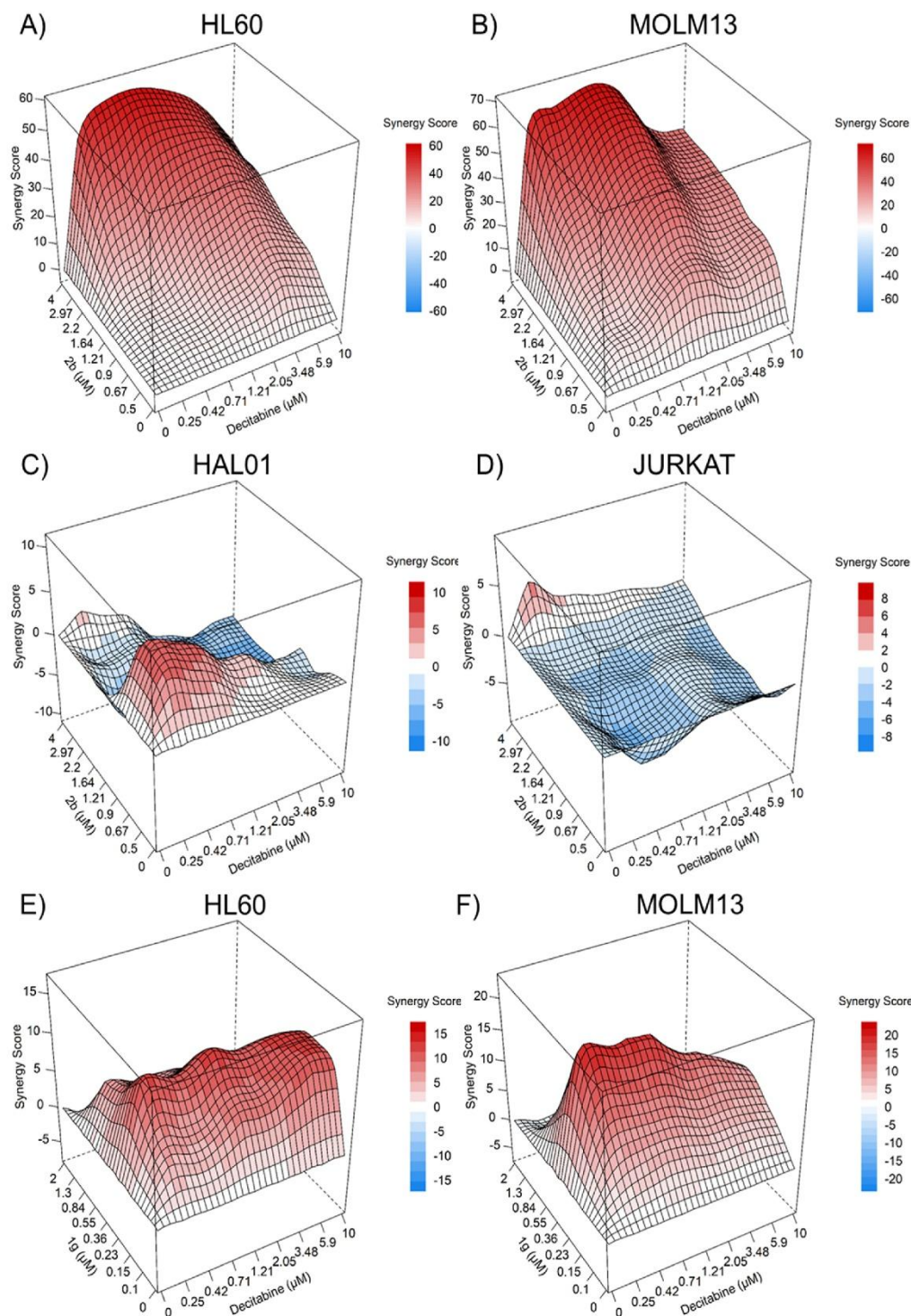


Fig. 5. Illustrative synergy plot representing the effects of **2b** after co-treatment of (A) HL60, (B) MOLM13, (C) HAL01 and (D) Jurkat leukemic cell lines with decitabine for 72 h. Illustrative synergy plot representing the effects **1g** after co-treatment of (E) HL60 and (F) MOLM13 cell lines with decitabine for 72 h. Synergy scores were calculated using the ZIP model, and visualizations were conducted using the SynergyFinder webtool [37].

simple and efficient two-step protocol relying on the U-4CR as the key step. The subsequent isoform profiling against HDAC1 and 6 provided important structure-activity relationships. All synthesized analogues of HPOB (0.085 μM) demonstrated superior HDAC6 inhibitory capacities compared to HPOB, exhibiting IC_{50} values ranging from 0.009 to 0.060 μM . However, they displayed different HDAC1 vs. HDAC6 selectivity profiles, ranging from vorinostat-like to HPOB-like. In this series, compound **2b** stood out as the most potent and selective HDAC6 inhibitor, with an HDAC6 IC_{50} value of 0.009 μM and a selectivity index ($\text{SI}^{\text{HDAC1/6}}$) of 25. The subsequent viability assays in three different leukemia cell lines (HAL01, HL60, and Jurkat) led to the identification of **1g** as the compound with the highest antiproliferative activity. Notably, it demonstrated submicromolar activity against all three cell lines. Considering their antiproliferative activities and HDAC6 inhibition profiles, we chose compounds **1g** (due to its highest antileukemia activity) and **2b** (highest and most selective HDAC6 inhibition) for further experiments.

To investigate the antileukemic properties of **1g** and **2b** in more detail, we used a high throughput drug screening approach using a diverse panel of leukemia cell lines. In particular, the AML subcluster was susceptible to HDAC inhibition. Immunoblot analysis conducted on HL60 cells, focusing on histone H3 vs α -tubulin hyperacetylation, corroborated the selectivity profiles observed in the biochemical HDAC inhibition assays for compounds **1g** (demonstrating unselective inhibition) and **2b** (exhibiting preferential HDAC6 inhibition). In subsequent combination screenings, both **1g** and, in particular, **2b** exhibited synergy with the DNMTi decitabine in AML cell lines. These findings emphasize the therapeutic promise of combining preferential HDAC6 inhibitors with DNMTis, such as decitabine.

4. Experimental section

4.1. Synthesis

4.1.1. General

All reagents and solvents were purchased from commercial sources and used without further purification. The carboxylic acids **5** and **6** were prepared according to a known procedure [44]. Dry solvents, e.g. MeOH and DCM, were obtained from the MBraun MB SPS-800 solvent purification system. All microwave-assisted reactions were carried out with a CEM Focused Microwave System, Model Discover. Flash column chromatography was performed on silica gel (0.040–0.063 mm, 230–400 mesh, pore size 60 Å) with the solvent mixtures specified in the corresponding experiment. Thin layer chromatography (TLC) was carried out using Macherey-Nagel pre-coated aluminium foil sheets, which were visualized using UV light (254 nm). Hydroxamic acids were stained using a 1 % solution of iron(III) chloride in MeOH. ^1H NMR and ^{13}C NMR spectra were recorded at room temperature on a Bruker Avance III HD or Varian/Agilent Mercury-plus (300, 400, 500 or 600 MHz) spectrometer using DMSO- d_6 or CDCl_3 as solvents. Chemical shifts are given in parts per million (ppm), relative to residual solvent peak for ^1H and ^{13}C . Due to the well-known phenomenon of *cis/trans*-amide bond rotamers in peptoids, ^1H and ^{13}C NMR-signals can occur as two distinct sets of signals. Coupling constants (*J*) are reported in Hertz (Hz). Mass-spectra were measured by the Leipzig University Mass Spectrometry Service using electrospray ionisation (ESI) on Bruker Daltonics Impact II and Bruker Daltonics micrOTOF spectrometers. The uncorrected melting points were determined using a Barnstead Electrothermal 9100 apparatus. Analytical HPLC analysis were carried out using a Gynkotek GINA 50 apparatus equipped with a Dionex P680A LPG pump, a Dionex UVD 340 U detector, and a Gina 50 autosampler and a Macherey-Nagel NUCLEODUR 100-5 C_{18} ec columns (250 mm $\times$ 4.6 mm). UV absorption was detected at 254 nm with a linear gradient of 10 % B to 100 % B within 30 min. HPLC-grade water +0.1 % TFA (solvent A) and HPLC-grade acetonitrile + 0.1 % TFA (solvent B) were used for elution at a flow rate of 1 mL/min. The purity of the final compounds was at least

95.0 %.

4.1.2. Synthesis and compound characterization

General procedure A for the preparation of UGI compounds 8a-i and 9a-d. In a 10 mL microwave reaction tube, 1.2 equivalents of amine (freshly distilled) and 1.2 equivalents of paraformaldehyde were dissolved in methanol (4 mL). The reaction mixture was irradiated at $T = 45^\circ\text{C}$ and 150 W for 30 min. Subsequently, 1.0 equivalent of the carboxylic acid component and 1.0 equivalents of the isocyanide component were added to the turbid white suspension. The reaction mixture was subjected to microwave irradiation at $T = 45^\circ\text{C}$ and 100 W for 1–2 h. The reaction was monitored by thin-layer chromatography and HPLC. The solvent was removed under reduced pressure, washed with aqueous Na_2CO_3 ($\text{pH} > 7$), and extracted with dichloromethane. The desired product was purified by column chromatography (cyclohexane/ethyl acetate as a gradient [4:1] to [1:1]).

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(phenyl)amino)-2-oxoethyl)benzoate (8a). Synthesized from **5**, cyclohexyl isocyanide (**7a**), paraformaldehyde (**4**), aniline (**3**) according to the general procedure A in 72 % yield as a colorless oil: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = 7.92 (d, $J = 8.3$ Hz, 2H), 7.46–7.30 (m, 3H), 7.20–7.14 (m, 2H), 7.12 (d, $J = 8.0$ Hz, 2H), 6.14 (d, $J = 7.9$ Hz, 1H), 4.24 (s, 2H), 3.90 (s, 3H), 3.82–3.68 (m, 1H), 3.57 (s, 2H), 1.93–1.78 (m, 2H), 1.72–1.60 (m, 2H), 1.40–1.28 (m, 2H), 1.23–1.00 (m, 4H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 171.3, 167.8, 167.0, 142.7, 140.3, 130.0, 129.8, 129.1, 128.8, 128.1, 55.4, 52.2, 48.2, 41.1, 33.0 (2C), 25.6 (2C), 24.8. HRMS calculated for $\text{C}_{24}\text{H}_{29}\text{N}_2\text{O}_4$: 409.2127, found: 409.2129 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 1/1): $R_f = 0.30$.

Methyl 4-(2-((2-(benzylamino)-2-oxoethyl)(phenyl)amino)-2-oxoethyl)benzoate (8b). Synthesized from **5**, benzyl isocyanide (**7b**), paraformaldehyde (**4**), aniline (**3**) according to the general procedure A in 64 % yield as a colorless oil: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = 7.88 (d, $J = 8.2$ Hz, 2H), 7.45–7.05 (m, 12H), 6.60 (br. s., 1H), 4.47 (d, $J = 4.5$ Hz, 2H), 4.34 (s, 2H), 3.90 (s, 3H), 3.57 (s, 2H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 171.5, 168.5, 167.0, 142.5, 140.1, 138.1, 130.0, 129.8, 129.2, 128.8, 128.1, 127.8, 127.6, 54.4, 52.2, 43.6, 41.0. HRMS calculated for $\text{C}_{25}\text{H}_{25}\text{N}_2\text{O}_4$: 417.1814, found: 417.1808 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 1/1): $R_f = 0.30$.

Methyl 4-(2-((2-(butylamino)-2-oxoethyl)(phenyl)amino)-2-oxoethyl)benzoate (8c). Synthesized from **5**, *n*-butyl isocyanide (**7c**), paraformaldehyde (**4**), aniline (**3**) according to the general procedure A in 37 % yield as a yellowish oil: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = 7.94 (d, $J = 8.2$ Hz, 2H), 7.41 (m, 3H), 7.20 (m, 2H), 7.14 (d, $J = 8.1$ Hz, 2H), 6.27 (br. s., 1H), 4.27 (s, 2H), 3.92 (s, 3H), 3.59 (s, 2H), 3.26 (dd, $J = 6.9$, 13.0 Hz, 2H), 1.46 (dt, $J = 7.3$, 14.8 Hz, 2H), 1.32 (dt, $J = 6.1$, 19.9 Hz, 2H), 0.91 (t, $J = 7.3$ Hz, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 171.4, 168.4, 166.9, 142.5, 140.1, 123.0, 129.7, 129.08, 128.8, 128.72, 127.9, 54.5, 52.1, 40.9, 39.3, 31.6, 20.0, 13.7. HRMS calculated for $\text{C}_{22}\text{H}_{27}\text{N}_2\text{O}_4$: 383.1971, found: 383.1967 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 1/1): $R_f = 0.24$.

Methyl 4-(2-((2-(tert-butylamino)-2-oxoethyl)(phenyl)amino)-2-oxoethyl)benzoate (8d). Synthesized from **5**, *tert*-butyl isocyanide (**7d**), paraformaldehyde (**4**), aniline (**3**) according to the general procedure A in 57 % yield as a colorless oil: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = 7.91 (d, $J = 8.3$ Hz, 2H), 7.39–7.37 (m, 3H), 7.17–7.15 (m, 2H), 7.11 (d, $J = 8.3$ Hz, 2H), 6.10 (br. s., 1H), 4.50 (s, 2H), 3.90 (s, 3H), 3.50 (s, 2H), 3.31 (s, 9H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 171.1, 167.6, 166.9, 142.5, 140.2, 129.9, 129.7, 129.0, 128.7, 128.6, 127.9, 55.3, 52.1, 51.3, 40.9, 28.7 (3C). HRMS calculated for $\text{C}_{22}\text{H}_{27}\text{N}_2\text{O}_4$: 383.1971, found: 383.1962 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 1/1): $R_f = 0.42$.

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(2-methylphenyl)amino)-2-oxoethyl)benzoate (8e). Synthesized from **5**, cyclohexyl isocyanide (**7a**), paraformaldehyde (**4**), 2-methylaniline (**3b**) according to the general procedure A in 61 % yield as a colorless oil: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = 7.93 (d, $J = 8.2$ Hz, 2H), 7.39–7.32 (m, 3H), 7.23 (m, 1H), 7.10 (d, $J = 8.1$ Hz, 2H), 6.14 (d, $J = 7.9$ Hz, 1H), 4.58 (d, $J = 14.5$ Hz, 1H),

3.93 (s, 3H), 3.76 (d, $J = 14.5$ Hz, 1H), 3.78 (m, 1H), 2.11 (s, 3H), 1.92–1.82 (m, 2H), 1.75–1.60 (m, 2H), 1.45–1.25 (m, 3H), 1.20–1.10 (m, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 171.6, 167.8, 167.0, 140.4, 140.0, 138.9, 130.7, 129.9, 129.2, 128.9, 127.7, 54.8, 52.3, 48.2, 40.9, 33.0, 25.6, 24.8, 21.4. HRMS calculated for $\text{C}_{25}\text{H}_{31}\text{N}_2\text{O}_4$: 423.2284, found: 423.2273 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 1/1): $R_f = 0.30$.

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(3-methylphenyl)amino)-2-oxoethyl)benzoate (8f). Synthesized from 5, cyclohexyl isocyanide (7a), paraformaldehyde (4), 3-methylaniline (3c) according to the general procedure A in 75 % yield as a colorless oil: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = 7.94 (d, $J = 8.2$ Hz, 2H), 7.31 (s, 1H), 7.20 (d, 1H), 7.15 (d, $J = 8.1$ Hz, 2H), 6.97 (m, 2H), 6.18 (d, 1H), 5.32 (s, 1H), 4.25 (s, 2H), 3.93 (s, 3H), 3.59 (s, 2H), 2.34 (s, 3H), 1.90–1.82 (m, 2H), 1.70–1.64 (m, 2H), 1.42–1.25 (m, 3H), 1.23–1.06 (m, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 171.6, 167.8, 167.0, 140.4, 140.0, 138.9, 130.7, 129.9, 129.2, 128.9, 127.7, 54.8, 52.3, 48.2, 40.9, 33.0, 25.6, 24.8, 21.2. HRMS calculated for $\text{C}_{25}\text{H}_{31}\text{N}_2\text{O}_4$: 423.2284, found: 423.2282 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 1/1): $R_f = 0.30$.

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(4-methylphenyl)amino)-2-oxoethyl)benzoate (8g). Synthesized from 5, cyclohexyl isocyanide (7a), paraformaldehyde (4), 4-methylaniline (3d) according to the general procedure A in 58 % yield as a colorless oil: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = 7.92 (d, $J = 8.3$ Hz, 2H), 7.22–7.11 (m, 4H), 7.03 (d, $J = 8.2$ Hz, 2H), 6.16 (d, $J = 8.0$ Hz, 1H), 4.22 (s, 2H), 3.90 (s, 3H), 3.73 (m, 1H), 3.56 (s, 2H), 2.38 (s, 3H), 1.91–1.77 (m, 2H), 1.71–1.60 (m, 2H), 1.33 (m, 2H), 1.22–1.01 (m, 4H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 171.6, 167.8, 167.0, 140.4, 140.0, 138.9, 130.7, 129.9, 129.2, 128.9, 127.7, 54.8, 52.3, 48.2, 40.9, 33.0, 25.6, 24.8, 21.3. HRMS calculated for $\text{C}_{25}\text{H}_{31}\text{N}_2\text{O}_4$: 423.2284, found: 423.226 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 1/1): $R_f = 0.30$.

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(2-ethylphenyl)amino)-2-oxoethyl)benzoate (8h). Synthesized from 5, cyclohexyl isocyanide (7a), paraformaldehyde (4), 2-ethylaniline (3e) according to the general procedure A in 54 % yield as a colorless oil: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = 7.91 (d, $J = 8.3$ Hz, 2H), 7.35–7.32 (m, 2H), 7.20 (m, 1H), 7.10 (m, 1H), 7.07 (d, $J = 8.3$ Hz, 2H), 6.43 (d, $J = 8.3$ Hz, 1H), 4.62 (d, $J = 14.5$ Hz, 1H), 3.90 (s, 3H), 3.68 (d, $J = 14.5$ Hz, 1H), 3.44 (s, 2H), 2.45 (ddt, $J = 7.5$ Hz, 2H), 1.90–1.79 (m, 2H), 1.69–1.61 (m, 2H), 1.40–1.25 (m, 3H), 1.19 (t, $J = 7.6$ Hz, 3H), 1.15–1.06 (m, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 171.8, 167.5, 166.8, 140.8, 140.6, 139.8, 129.7 (2C), 129.6, 129.4, 129.2 (2C), 129.0, 128.8, 127.4, 54.5, 52.0, 48.0, 40.8, 32.9, 32.8, 25.5, 24.6, 23.4, 14.2. HRMS calculated for $\text{C}_{26}\text{H}_{32}\text{N}_2\text{O}_4\text{Na}$: 459.2260 found: 459.2268 ($[\text{M}+\text{Na}]^+$), TLC (CH/EtOAc 1/1): $R_f = 0.29$.

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(3-(trifluoromethyl)phenyl)amino)-2-oxoethyl)benzoate (8i). Synthesized from 5, cyclohexyl isocyanide (7a), paraformaldehyde (4), 2-trifluoro-aniline (3f) according to the general procedure A in 38 % yield as a colorless oil: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = 7.93 (d, $J = 8.3$ Hz, 2H), 7.80 (m, 1H), 7.56 (m, 3H), 7.27 (m, 1H), 7.15 (d, $J = 8.2$ Hz, 2H), 6.05 (d, $J = 7.5$ Hz, 1H), 4.85 (d, $J = 15.1$ Hz, 1H), 3.89 (s, 3H), 3.73 (m, 1H), 3.52 (d, $J = 15.1$ Hz, 1H), 3.46 (d, $J = 15.4$ Hz, 1H), 3.35 (d, $J = 15.4$ Hz, 1H), 1.85 (m, 2H), 1.65 (m, 2H), 1.58 (m, 1H), 1.30 (m, 2H), 1.16 (m, 3H). $^{19}\text{F-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = –66.99. $^{13}\text{C-NMR}$ (CDCl_3 , 75 MHz): δ [ppm] = 171.1, 167.2, 166.9, 140.1, 139.7, 133.7, 132.2, 129.7, 129.5, 129.2, 128.8, 127.6 (q, $J = 5.2$ Hz), 123.4 (q, $J = 273.3$ Hz), 54.2, 52.0, 48.3, 40.8, 32.9, 26.9, 25.4, 24.7. HRMS calculated for $\text{C}_{25}\text{H}_{28}\text{F}_3\text{N}_2\text{O}_4$: 477.2001, found: 477.1999 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 2/1): $R_f = 0.30$.

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(phenyl)amino)-2-oxoethyl)-3-fluorobenzoate (9a). Synthesized from 6, cyclohexyl isocyanide (7a), paraformaldehyde (4), aniline (3) according to the general procedure A in 87 % yield as a yellowish solid: $^1\text{H-NMR}$ (CDCl_3 , 300 MHz): δ [ppm] = 7.76 (dd, $J = 7.9$, 1.6 Hz, 1H), 7.64 (dd, $J = 10.3$, 1.6 Hz, 1H), 7.42 (m, 2H), 7.37 (m, 1H), 7.28 (m, 2H), 7.25 (s, 1H), 6.20 (d, $J = 8.6$ Hz, 1H), 4.27 (s, 2H), 3.90 (s, 3H), 3.77 (m, 1H), 3.56 (s, 2H), 1.92–1.85 (m, 2H), 1.72–1.65 (m, 2H), 1.62–1.56 (m, 1H), 1.38–1.30

(m, 2H), 1.20–1.10 (m, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 170.4, 167.5, 165.8, 160.5 (d, $J = 246.7$ Hz), 142.4, 131.5 (d, $J = 4.2$ Hz), 131.0 (d, $J = 7.8$ Hz), 130.1 (2C), 128.7, 127.9 (d, $J = 16.1$ Hz), 127.7 (2C), 125.3 (d, $J = 3.4$ Hz), 116.2 (d, $J = 24.0$ Hz), 54.5, 52.3, 48.2, 34.8, 32.9, 25.5, 24.7. HRMS calculated for $\text{C}_{24}\text{H}_{28}\text{FN}_2\text{O}_4$: 427.2033, found: 427.2060 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 2/1): $R_f = 0.30$.

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(2-methylphenyl)amino)-2-oxoethyl)-3-fluoro-benzoate (9b). Synthesized from 6, cyclohexyl isocyanide (7a), paraformaldehyde (4), 2-methylaniline (3b) according to the general procedure A in 61 % yield as a yellowish solid: $^1\text{H-NMR}$ (CDCl_3 , 300 MHz): δ [ppm] = 7.75 (dd, $J = 7.9$, 1.7 Hz, 1H), 7.64 (dd, $J = 10.3$, 1.6 Hz, 1H), 7.30 (dd, $J = 8.3$ Hz, 2H), 7.26 (m, 1H), 7.23 (m, 2H), 6.49 (d, $J = 8.3$ Hz, 1H), 4.56 (d, $J = 14.6$ Hz, 1H), 3.90 (s, 3H), 3.78 (d, $J = 14.6$ Hz, 1H), 3.77 (m, 1H), 3.42 (q, $J = 16.1$ Hz, 2H), 2.24 (s, 3H), 1.96–1.81 (m, 2H), 1.75–1.64 (m, 2H), 1.60–1.54 (m, 1H), 1.45–1.30 (m, 2H), 1.21–1.12 (m, 3H). $^{19}\text{F-NMR}$ (CDCl_3 , 400 MHz): δ [ppm] = –116.30. $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 170.7, 167.3, 165.9, 160.6 (d, $J = 246.7$ Hz), 141.0, 135.4, 131.8, 131.6 (d, $J = 3.9$ Hz), 131.1 (d, $J = 7.6$ Hz), 129.2, 128.6, 127.7, 127.6, 125.3 (d, $J = 3.1$ Hz), 116.3 (d, $J = 23.9$ Hz), 54.1, 52.2, 48.2, 34.6, 32.9 (2C), 25.5 (2C), 24.7, 17.4. HRMS calculated for $\text{C}_{25}\text{H}_{30}\text{FN}_2\text{NaO}_4$: 441.2190, found: 441.2203 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 2/1): $R_f = 0.23$.

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(2-ethylphenyl)amino)-2-oxoethyl)-3-fluoro-benzoate (9c). Synthesized from 6, cyclohexyl isocyanide (7a), paraformaldehyde (4), 2-ethylaniline (3e) according to the general procedure A in 43 % yield as a yellowish solid: $^1\text{H-NMR}$ (CDCl_3 , 300 MHz): δ [ppm] = 7.76 (d, $J = 7.8$ Hz, 1H), 7.65 (d, $J = 10.2$ Hz, 1H), 7.37 (d, $J = 3.4$ Hz, 2H), 7.27 (m, 1H), 7.23 (m, 2H), 6.48 (d, $J = 7.8$ Hz, 1H), 4.61 (d, $J = 14.6$ Hz, 1H), 3.91 (s, 3H), 3.78 (t, $J = 14.7$ Hz, 1H), 3.76 (d, $J = 14.6$ Hz, 1H), 3.42 (dd, $J_{\text{H-F}} = 50.2$ Hz, $J_{\text{H-H}} = 16.1$ Hz, 2H), 2.58 (q, 2H, $J = 7.4$ Hz), 1.95–1.81 (m, 2H), 1.74–1.63 (m, 2H), 1.62–1.58 (m, 1H), 1.36 (m, 1H), 1.29–1.22 (m, 3H), 1.25 (t, $J = 7.6$ Hz, 3H), 1.16 (m, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 170.9, 165.8, 160.6 (d, $J = 246.9$ Hz), 140.9, 140.5, 131.6 (d, $J = 4.2$ Hz), 131.1 (d, $J = 7.8$ Hz), 129.7, 129.4, 128.6, 127.7, 127.6, 125.3 (d, $J = 3.4$ Hz), 116.2 (d, $J = 23.9$ Hz), 54.6, 52.3, 48.1, 34.7, 32.9 (2C), 26.9, 25.5 (2C), 24.7, 23.3, 14.3. HRMS calculated for $\text{C}_{26}\text{H}_{32}\text{FN}_2\text{O}_4$: 455.2346, found: 455.2375 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 2/1): $R_f = 0.19$.

Methyl 4-(2-((2-(cyclohexylamino)-2-oxoethyl)(2-trifluoromethylphenyl)amino)-2-oxoethyl)-3-fluorobenzoate (9d). Synthesized from 6, cyclohexyl isocyanide (7a), paraformaldehyde (4), 2-trifluoromethylaniline (3f) according to the general procedure A in 28 % yield as a yellowish oil: $^1\text{H-NMR}$ (CDCl_3 , 300 MHz): δ [ppm] = 7.78 (m, 2H), 7.65 (m, 2H), 7.56 (m, 1H), 7.34 (m, 2H), 6.40 (s, 1H), 4.82 (d, $J = 15.0$ Hz, 1H), 3.92 (s, 3H), 3.76 (m, 1H), 3.56 (d, $J_{\text{H-H}} = 15.8$ Hz, 1H), 3.42 (dd, $J_{\text{H-F}} = 49.2$ Hz, $J_{\text{H-H}} = 16.3$ Hz, 2H), 1.85 (m, 2H), 1.68 (m, 3H), 1.34 (m, 2H), 1.13 (m, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz): δ [ppm] = 170.5, 167.3, 167.0 (d, $J = 2.8$ Hz), 160.6 (d, $J = 247.0$ Hz), 140.2 (d, $J = 1.8$ Hz), 134.0, 132.1, 131.8 (d, $J = 4.0$ Hz), 131.2 (d, $J = 7.8$ Hz), 129.7, 127.9 (q, $J = 5.1$ Hz), 127.5 (d, $J = 16.1$ Hz), 125.5 (d, $J = 3.5$ Hz), 123.4 (d, $J = 273.1$ Hz), 116.7 (q, $J = 23.9$ Hz), 116.3 (d, $J = 24.0$ Hz), 54.5, 52.5, 48.5, 33.0 (d, $J = 5.3$ Hz), 27.0, 25.6, 24.9 (d, $J = 2.3$ Hz). HRMS calculated for $\text{C}_{25}\text{H}_{27}\text{F}_4\text{N}_2\text{O}_4$: 495.1907, found: 495.1897 ($[\text{M}+\text{H}]^+$), TLC (CH/EtOAc 2/1): $R_f = 0.08$.

General procedure B for the preparation of target compounds 1a-i and 2a-d. The appropriate ester (1 equivalent) were dissolved 10 mL one-neck flask in 5 mL dichloromethane/methanol (1:2), cooled down to 0 °C and treated with 20 equivalent hydroxylamine (50 wt% in water) dropwise and 10 equivalent powdered potassium hydroxide were added. The reaction mixture was stirred 15 min at 0 °C and additionally 2–4 h at room temperature and monitored via TLC. The solvent was removed under reduced pressure, the crude residue dissolved in 5 mL water and acidified with 1 N hydrochloric acid (pH: 8). The precipitated white solid was filtered, washed with water and dried at high vacuum. Additionally, the product was recrystallized or purified with prep-HPLC (ACN/H₂O+0.05 % TFA-gradient).

4-(2-((2-(Cyclohexylamino)-2-oxoethyl)(phenyl)amino)-2-oxoethyl)-

N-hydroxybenzamide (**1a**). Synthesized from **8a** according to the general procedure B in 45 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ [ppm] = 11.15 (br. s, 1H), 9.16 (br. s, 1H), 7.74 (d, *J* = 7.5 Hz), 7.62 (d, *J* = 7.8 Hz, 2H), 7.48–7.30 (m, 5H), 7.14 (d, *J* = 7.8 Hz, 2H), 4.18 (s, 2H), 3.57–3.39 (m, 3H), 1.75–1.58 (m, 4H), 1.58–1.46 (m, 1H), 1.31–1.16 (m, 2H), 1.13–1.01 (m, 3H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 169.6, 166.6, 164.0, 143.0, 139.0, 130.8, 129.4 (2C), 129.2 (2C), 128.3 (2C), 127.9, 126.6 (2C), 52.0, 47.7, 40.0 (overlap DMSO), 32.4 (2C), 25.2, 24.5 (2C). HRMS calculated for C₂₃H₂₆N₃O₄: 408.1929, found: 408.1896 ([M + H]⁺). HPLC purity: 97.1 %.

4-((2-(Benzyl)-2-oxoethyl)(phenyl)amino)-2-oxoethyl-N-hydroxybenzamide (**1b**). Synthesized from **8b** according to the general procedure B in 70 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ [ppm] = 11.15 (br. s, 1H), 9.00 (br. s, 1H), 8.46 (t, *J* = 5.5 Hz, 1H), 7.63 (d, *J* = 8.0 Hz, 2H), 7.51–7.42 (m, 4H), 7.40–7.33 (m, 1H), 7.30–7.25 (m, 2H), 7.23–7.18 (m, 3H), 7.15 (d, *J* = 8.0 Hz, 2H), 4.30 (s, 2H), 4.28 (s, 2H), 3.50 (s, 2H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 169.7, 167.8, 164.0, 142.9, 139.2, 139.0, 130.8, 129.5, 129.2 (2C), 128.3, 128.2 (4C), 128.0, 127.1 (2C), 126.7, 126.6 (2C), 52.3, 42.1, 40.2 (overlap DMSO). HRMS calculated for C₂₄H₂₉N₃O₄: 440.1586, found: 440.1580 ([M + Na]⁺). HPLC purity: 95.3 %.

4-((2-(tert-Butylamino)-2-oxoethyl)(phenyl)amino)-2-oxoethyl-N-hydroxybenzamide (**1c**). Synthesized from **8c** according to the general procedure B in 45 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ [ppm] = 11.11 (br. s, 1H), 8.97 (br. s, 1H), 7.87 (t, *J* = 5.7 Hz, 1H), 7.62 (d, *J* = 8.0 Hz, 2H), 7.53–7.32 (m, 5H), 7.12 (d, *J* = 8.0 Hz, 2H), 4.20 (s, 2H), 3.47 (s, 2H), 3.04 (q, *J* = 6.5 Hz, 2H), 1.41–1.27 (m, 2H), 1.26–1.13 (m, 2H), 0.83 (t, *J* = 7.2 Hz, 3H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 169.8, 167.5, 164.1, 143.0, 138.9, 131.0, 129.5 (2C), 129.2 (2C), 128.9, 128.7, 128.3 (2C), 128.0, 126.6, 52.2, 40.0 (overlap DMSO) 31.2, 19.5, 13.7. HRMS calculated for C₂₁H₂₅N₃O₄: 406.1743, found: 432.1735 ([M + Na]⁺). HPLC purity: 95.8 %.

4-((2-(tert-Butylamino)-2-oxoethyl)(phenyl)amino)-2-oxoethyl-N-hydroxybenzamide (**1d**). Synthesized from **8d** according to the general procedure B in 41 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ [ppm] = 10.97 (br. s, 1H), 9.06 (br. s, 1H), 7.63 (d, *J* = 7.9 Hz, 2H), 7.50–7.31 (m, 6H), 7.14 (d, *J* = 7.9 Hz, 2H), 4.15 (s, 2H), 3.46 (s, 2H), 1.24 (s, 9H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 169.5, 166.9, 164.0, 143.0, 139.0, 130.9, 129.4 (2C), 129.2 (2C), 128.3 (2C), 127.9, 126.6 (2C), 52.3, 50.2, 40.0 (overlap with DMSO), 28.5 (3C). HRMS calculated for C₂₁H₂₅N₃O₄: 406.1743, found: 406.1736 ([M + Na]⁺). HPLC purity: 98.1 %.

4-((2-(2-(Cyclohexylamino)-2-oxoethyl)(2-methylphenyl)amino)-2-oxoethyl)-N-hydroxybenzamide (**1e**). Synthesized from **8e** according to the general procedure B in 80 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ [ppm] = 11.06 (br. s, 1H), 9.00 (br. s, 1H), 7.69 (d, *J* = 7.7 Hz, 1H), 7.61 (d, *J* = 8.2 Hz, 2H), 7.45–7.39 (m, 1H), 7.37–7.30 (m, 2H), 7.29–7.22 (m, 1H), 7.08 (d, *J* = 8.2 Hz, 2H), 4.62 (d, *J* = 15.7 Hz, 1H), 3.54 (d, *J* = 15.7 Hz, 1H), 3.54–3.44 (m, 1H), 3.37 (d, *J* = 15.3 Hz, 1H), 3.30 (d, *J* = 15.3 Hz, 1H, overlap with water), 2.11 (s, 3H), 1.77–1.56 (m, 4H), 1.56–1.46 (m, 1H), 1.31–1.19 (m, 2H), 1.18–0.98 (m, 3H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 169.7, 166.4, 164.0, 141.4, 138.7, 135.5, 131.2, 130.9, 129.6, 129.1 (2C), 128.5, 127.0, 126.6 (2C), 50.8, 47.6, 40.0 (overlap with DMSO), 32.4, 32.3, 25.2, 24.5 (2C), 17.2. HRMS calculated for C₂₄H₂₉N₃O₄: 446.2056, found: 446.2049 ([M + Na]⁺). HPLC purity: 95.5 %.

4-((2-(2-(Cyclohexylamino)-2-oxoethyl)(3-methylphenyl)amino)-2-oxoethyl)-N-hydroxybenzamide (**1f**). Synthesized from **8f** according to the general procedure B in 59 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ [ppm] = 11.12 (br. s, 1H), 9.21 (br. s, 1H), 7.74 (d, *J* = 7.3 Hz, 1H), 7.63 (d, *J* = 7.9 Hz, 2H), 7.31 (t, *J* = 7.6 Hz, 1H), 7.18 (m, 3H), 7.12 (d, *J* = 7.9 Hz, 2H), 4.61 (s, 2H), 3.48 (m, 1H), 3.46 (s, 2H), 2.29 (s, 3H), 1.72–1.57 (m, 4H), 1.50–1.48 (m, 1H), 1.28–1.17 (m, 2H), 1.16–1.02 (m, 3H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 169.6, 166.6, 164.0, 142.9, 138.9, 138.7, 131.3, 129.2, 129.1 (2C), 128.8, 128.5, 126.5 (2C), 125.2, 52.0, 47.7, 39.9 (overlap with DMSO),

32.4 (2C), 25.2, 24.5 (2C), 20.8. HRMS calculated for C₂₄H₂₉N₃O₄: 446.2056, found: 446.2055 ([M + Na]⁺). HPLC purity: 95.5 %.

4-((2-(2-(Cyclohexylamino)-2-oxoethyl)(3-methylphenyl)amino)-2-oxoethyl)-N-hydroxybenzamide (**1g**). Synthesized from **8g** according to the general procedure B in 39 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ [ppm] = 11.13 (br. s, 1H), 8.97 (br. s, 1H), 7.72 (d, *J* = 7.2 Hz, 1H), 7.62 (d, *J* = 7.7 Hz, 2H), 7.27–7.24 (d, *J* = 7.9 Hz, 2H), 7.15–7.13 (d, *J* = 7.7 Hz, 2H), 4.15 (s, 2H), 3.39 (m, 1H), 2.32 (s, 3H), 1.70–1.62 (m, 3H), 1.52–1.48 (m, 2H), 1.28–1.18 (m, 3H), 1.16–1.04 (m, 2H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 169.6, 166.5, 140.4, 139.1, 137.3, 136.6, 130.8, 129.9 (2C), 129.2 (2C), 128.0 (2C), 126.6 (2C), 52.0, 47.6, 39.9 (overlapped with DMSO), 32.4 (2C), 25.2, 24.5 (2C), 20.6. HRMS calculated for C₂₄H₂₉N₃O₄: 446.2056, found: 446.2050 ([M + Na]⁺). HPLC purity: 95.4 %.

4-((2-(2-(Cyclohexylamino)-2-oxoethyl)(2-ethylphenyl)amino)-2-oxoethyl)-N-hydroxybenzamide (**1h**). Synthesized from **8h** according to the general procedure B in 49 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ [ppm] = 11.34 (br. s, 1H), 8.98 (br. s, 1H), 7.90 (d, *J* = 7.9 Hz, 1H), 7.61 (d, *J* = 8.2 Hz, 1H), 7.44 (m, 1H), 7.36 (m, 2H), 7.25 (m, 1H), 7.07 (d, *J* = 8.2 Hz, 2H), 4.64 (d, *J* = 15.8 Hz, 1H), 3.51 (d, *J* = 15.8 Hz, 1H), 3.48 (m, 1H), 3.33 (q, *J* = 7.7 Hz, 2H), 3.30 (s, 2H), 1.73–1.57 (m, 4H), 1.56–1.42 (m, 1H), 1.31–1.18 (m, 2H), 1.14 (t, *J* = 7.7 Hz, 3H), 1.10–1.01 (m, 2H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 169.8, 166.3, 163.9, 140.9, 140.8, 138.3, 131.4, 129.5, 129.3, 129.2, 129.0 (2C), 128.7, 126.8, 126.5 (2C), 51.5, 47.6, 32.4, 32.3, 25.1, 24.5, 24.4, 22.9, 14.1. HRMS calculated for C₂₅H₃₁N₃O₄: 460.2212, found: 460.2209 ([M + Na]⁺). HPLC purity: 95.4 %.

4-((2-(2-(Cyclohexylamino)-2-oxoethyl)(2-trifluoromethylphenyl)amino)-2-oxoethyl)-N-hydroxybenzamide (**1i**). Synthesized from **8i** according to the general procedure B in 34 % yield as a white solid: ¹H-NMR (methanol-*d*₄, 400 MHz): δ [ppm] = 10.0 (br. s, 1H), 7.88 (d, *J* = 7.3 Hz, 1H), 7.79 (d, *J* = 7.6 Hz, 1H), 7.72–7.68 (m, 1H), 7.65 (d, *J* = 8.1 Hz, 3H), 7.18 (d, *J* = 8.1 Hz, 2H), 4.85 (d, *J* = 16.3 Hz, 1H), 3.68–3.59 (m, 1H), 3.60 (d, *J* = 16.1 Hz, 1H), 3.52–3.37 (m, 2H), 1.93–1.56 (m, 5H), 1.38–1.30 (m, 2H), 1.23–1.12 (m, 3H). ¹³C-NMR (methanol-*d*₄, 101 MHz): δ [ppm] = 173.1, 168.9, 167.6, 141.6, 139.5 (d, *J* = 4.6 Hz), 135.1, 133.4, 132.7, 131.0, 130.6 (2C), 128.7 (q, *J* = 5.1 Hz), 128.4 (q, *J* = 29.7 Hz), 127.9 (2C), 124.9 (q, *J* = 272.5 Hz), 54.0, 49.9, 41.6, 33.7, 33.7, 26.6, 26.0 (2C). HRMS calculated for C₂₄H₂₆F₃N₃O₄: 500.1750, found: 500.1750 ([M + Na]⁺). HPLC purity: 98.5 %.

4-((2-(2-(Cyclohexylamino)-2-oxoethyl)(phenyl)amino)-2-oxoethyl)-3-fluoro-N-hydroxybenzamide (**2a**). Synthesized from **9a** according to the general procedure B in 71 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ [ppm] = 10.01 (br. s, 1H), 7.74 (d, *J* = 7.8 Hz, 1H), 7.50 (d, *J* = 7.9 Hz, 1H), 7.44 (d, *J* = 4.5 Hz, 4H), 7.33 (t, *J* = 7.8 Hz, 2H), 4.19 (s, 1H), 3.58–3.23 (m, 4H), 1.74–1.59 (m, 4H), 1.52 (dt, *J* = 13.1, 3.8 Hz, 1H), 1.21 (tt, *J* = 10.7, 3.0 Hz, 2H), 1.16–1.04 (m, 3H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 168.7, 166.5, 162.7, 160.1 (d, *J* = 244.6 Hz), 142.8, 133.8 (d, *J* = 6.8 Hz), 131.9, 129.6 (2C), 128.2 (2C), 128.1, 125.9 (d, *J* = 16.1 Hz), 122.4, 113.1 (d, *J* = 23.6 Hz), 64.9, 52.1, 47.7, 33.7, 32.4, 25.2, 24.5, 15.2. HRMS calculated for C₂₃H₂₆FN₃O₄: 450.1805, found: 450.1795 ([M + Na]⁺). HPLC purity: 95.4 %.

4-((2-(2-(Cyclohexylamino)-2-oxoethyl)(2-methylphenyl)amino)-2-oxoethyl)-3-fluoro-N-hydroxybenzamide (**2b**). Synthesized from **9b** according to the general procedure B in 44 % yield as a white solid: ¹H-NMR (DMSO-*d*₆, 400 MHz): δ = 7.70 (d, *J* = 7.8 Hz, 1H), 7.48 (ddd, *J* = 10.1, 7.9, 1.7 Hz, 2H), 7.42 (dd, *J* = 10.7, 1.6 Hz, 1H), 7.38–7.26 (m, 3H), 4.61 (d, *J* = 15.7 Hz, 1H), 3.57 (d, *J* = 15.7 Hz, 1H), 3.50 (dq, *J* = 7.1, 3.4 Hz, 1H), 3.41–3.34 (m, 2H), 2.22 (s, 3H), 1.77–1.57 (m, 5H), 1.21 (dddd, *J* = 19.9, 14.3, 6.7, 2.7 Hz, 3H), 1.14–1.03 (m, 2H). ¹³C-NMR (DMSO-*d*₆, 101 MHz): δ [ppm] = 168.7, 166.3, 162.6, 160.1 (d, *J* = 244.8 Hz), 141.2, 135.5, 133.9 (d, *J* = 7.3 Hz), 132.0, 131.2, 129.5, 128.5, 127.0, 125.6 (d, *J* = 16.2 Hz), 122.3, 113.0 (d, *J* = 23.4 Hz), 50.9, 47.6, 33.6, 32.4, 32.3, 25.2, 24.5, 24.4, 17.0. HRMS calculated for C₂₄H₂₈FN₃O₄: 464.1962, found: 464.1964 ([M + Na]⁺). HPLC purity

(250 nm): 95.6 %.

4-(2-((2-(Cyclohexylamino)-2-oxoethyl)(2-ethylphenyl)amino)-2-oxoethyl)-3-fluoro-N-hydroxy-benzamide (**2c**). Synthesized from **9c** according to the general procedure B in 61 % yield as a white solid: ¹H-NMR (methanol-d₄, 400 MHz): δ [ppm] = 7.81 (weak d, 1H), 7.57 (d, *J* = 7.6 Hz, 1H), 7.34 (d, *J* = 7.8 Hz, 2H), 7.29–7.20 (m, 2H), 7.23–7.06 (m, 2H), 4.50 (d, *J* = 15.7 Hz, 1H), 3.80–2.90 (m, 4H, H₂O peak), 2.49–2.37 (m, 2H), 1.70–1.43 (m, 4H), 1.37 (d, *J* = 11.4 Hz, 1H), 1.08 (s, 2H), 1.04 (t, *J* = 7.5 Hz, 3H), 0.93 (dq, *J* = 22.6, 10.6, 9.3 Hz, 3H). ¹³C-NMR (methanol-d₄, 101 MHz): δ [ppm] = 178.6, 175.9, 172.2, 169.7 (d, *J* = 244.4 Hz), 150.5, 150.26, 144.4 (d, *J* = 5.6 Hz), 139.1, 138.9, 138.4, 136.5, 134.4 (d, *J* = 15.4 Hz), 131.7, 122.4 (d, *J* = 23.4 Hz), 61.2, 57.2, 43.2, 41.9 (2C), 34.7, 34.0 (2C), 32.3, 23.7. HRMS calculated for C₂₅H₃₀FN₃NaO₄: 478,2118, found: 478.2122 ([M+Na]⁺). HPLC purity: 97.0 %.

4-(2-((2-(Cyclohexylamino)-2-oxoethyl)(2-trifluoromethylphenyl)amino)-2-oxoethyl)-3-fluoro-N-hydroxy-benzamide (**2d**). Synthesized from **9d** according to the general procedure B in 34 % yield as a white solid: ¹H-NMR (methanol-d₄, 400 MHz): δ [ppm] = 7.88 (t, *J* = 6.9 Hz, 2H), 7.74 (t, *J* = 7.3 Hz, 1H), 7.66 (t, *J* = 7.6 Hz, 1H), 7.51 (dd, *J* = 7.9, 1.5 Hz, 1H), 7.43–7.35 (m, 2H), 4.84 (d, *J* = 16.3 Hz, 1H), 3.62 (d, *J* = 15.9 Hz, 2H), 3.55–3.33 (m, 2H), 1.92–1.55 (m, 5H), 1.35 (qdd, *J* = 13.2, 5.8, 3.1 Hz, 2H), 1.26–1.07 (m, 3H). ¹³C-NMR (methanol-d₄, 101 MHz): δ [ppm] = 172.0, 168.9, 166.4, 162.1 (d, *J* = 246.4 Hz), 141.5 (d, *J* = 10.4 Hz), 135.2, 134.9 (d, *J* = 7.3 Hz), 133.4, 133.1 (d, *J* = 3.9 Hz), 131.0, 129.2–128.6 (2C), 126.9 (d, *J* = 16.1 Hz), 124.9 (q, *J* = 265.3 Hz), 123.6 (d, *J* = 3.2 Hz), 114.7 (d, *J* = 24.2 Hz), 54.1, 49.9, 35.1, 33.7, 33.6, 26.6, 26.0 (2C). HRMS calculated for C₂₄H₂₅F₄N₃NaO₄: 518.1679, found: 518.1663 ([M+Na]⁺). HPLC purity: 99.9 %.

4.2. Molecular docking

For the molecular docking the available protein structure of human HDAC6 was downloaded from the Protein Data Bank (PDB; PDB ID: 5EDU) [29]. Only chain A was used for the docking. The protein was prepared using the software Molecular Operating Environment (MOE, Chemical Computing Group, version 2022.02) [45] by adding hydrogen atoms and building missing loops. Additionally, water molecules and ions (except the catalytic zinc ion) were removed from the crystal structure. The active water molecule (H₂O 921) from the crystal structure of *Danio rerio* HDAC6 (PDB ID: 5EF7) [29] was placed to the active site by structural alignment of both protein structures. Compound **2b** was drawn with ChemDraw (version 21.0.0.28) in its deprotonated form and prepared in MOE by generating the 3D structure and energy minimization using AMBER:EHT force field. The docking experiment was performed with GOLD [46] in combination with the HERMES visualizer (Cambridge Crystallographic Data Centre, version 2022.3.0). The active site was defined by the co-crystallized ligand within a radius of 15 Å. All poses were optimized within 50 GA runs and scored with ChemPLP. The early termination option of the docking runs was not allowed. The search efficacy was set to 200 %. The docking was carried out with a scaffold match constraint to place the ligand onto a given scaffold location within the active site. The hydroxamate substructure (CONO) of the co-crystallized ligand in *Danio rerio* HDAC6 (PDB ID: 5EF7) was used as a template. The docking solutions were visual inspected according to a proper coordination of the zinc ion and a reasonable orientation of the cap groups. The pose depicted in Fig. 3 represents the second top ranked docking solution out of 50.

4.3. Biological evaluation

4.3.1. Inhibition assay for HDAC1, 2, 3, and 6

The *in vitro* inhibitory activities against HDAC1–3 and HDAC6 were measured using a previously published protocol [47]. For test compounds and controls, serial dilutions of the respective DMSO-stock solution in assay buffer (50 mM Tris–HCl, pH 8.0, 137 mM NaCl, 2.7 mM

KCl, 1.0 mM MgCl₂•6H₂O, 0.1 mg/mL BSA), were prepared and 5.0 μL of this serial dilution were transferred into OptiPlate-96 black micro-plates (PerkinElmer). 35 μL of the fluorogenic substrate ZMAL (Z-Lys(Ac)-AMC) [48] (21.43 μM in assay buffer) and 10 μL enzyme solution (human recombinant HDAC1 (BPS Bioscience, Catalog# 50051); HDAC2 (BPS Bioscience, Catalog# 50052); HDAC3/NcoR2 (BPS Bioscience, Catalog# 50003); HDAC6 (BPS Bioscience, Catalog# 50006). The total assay volume of 50 μL (max. 1 % DMSO) was incubated at 37 °C for 90 min. Subsequently, 50 μL trypsin solution (0.4 mg/mL trypsin in buffer: 50 mM Tris–HCl, pH 8.0, 100 mM NaCl) was added, followed by additional 30 min of incubation at 37 °C. Fluorescence (excitation: 355 nm, emission: 460 nm) was measured using an Ascent Fluoroskan microplate reader (Thermo Scientific). All compounds were evaluated in duplicates in at least two independent experiments.

4.3.2. Cell culture

Leukemia cell lines were provided from the German collection of microorganisms and cell culture (DSMZ, Braunschweig, Germany) and two healthy fibroblast control cells (ATCC, Manassas, VA) were cultured according to the supplier's recommendations. All cell lines were cultured in RPMI1640 with 10–20 % fetal bovine serum at 37 °C with 5 % CO₂. In general cells were only used for experiments if the viability exceeded 90 %.

4.3.3. High throughput drug screening

Compounds were dissolved in DMSO as 10 mM stock solutions [16]. Plates for drug screening were preprinted via the Tecan D300e in a 384 well set up and stored afterward at –80 °C. All plates were individually randomized to avoid plate effects, and at least one row/column of the outer wells was kept empty to prevent evaporation at the plate edges. Wells with lower DMSO content were normalized to the well with the highest DMSO volume. The cell viability and density were determined prior to the seeding via the Vi-CELL BLU cell counter. Cell lines were seeded in fresh media at 0.04 × 10⁶ cell/mL density with the help of a Multidrop reagent dispenser. After 72 h CellTiter-Glo reagent was added and the luminescence was determined by a Tecan Spark microplate reader. The initial determination of the experimental inhibitors used seven different compound doses ranging from 0.005 μM to 25 μM. All three cell lines were measured in biological triplicates. The focused drug screening was performed in technical triplicates with 12 different concentrations, ranging from 0.005 μM to 25 μM. The viability [%] was determined by using the mean of the DMSO wells as a reference. Drug response curves & IC₅₀ values were determined via prism software (GraphPad Prism Inc., San Diego, CA) using (log(inhibitor) vs. normalized response – Variable slope) function. Heatmaps were generated with the help of the R package complex heatmap.

4.3.4. Combinatorial drug screening

A matrix drug screening approach was utilized to investigate drug synergy [16]. Both **1g** and **2b** were combined with decitabine with an increased concentration in an 8 × 8 dose-response matrices (1g: 0.05–4 μM, 2b: 0.05–4 μM, Decitabine 0.025–10 μM). Viability was assessed after a 72-h period employing the CellTiter-Glo luminescent assay, as detailed earlier (Spark, Tecan). The ZIP scores and graphical analysis were calculated with the help of the R SynergyFinder package.

4.3.5. Immunoblotting

Cells were treated with the indicated concentration of compound over the course of 24 h in cell culture dishes. 0.5 × 10⁶ cells/mL were seeded in 10 mL of media. Cells were washed three times with cold PBS and snap-frozen in liquid nitrogen. Lysis was accomplished via Pierce RIPA buffer (with cComplete Protease Inhibitor cocktail, PhosSTOP & Dithiothreitol). After the removal of DNA the protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). All samples were mixed with 5x laemmli sample

buffer and heated at 95 °C for 5 min to ensure protein denaturation. 20 µg of protein were loaded on an SDS polyacrylamide gel. Afterward, the proteins were transferred from the gel to a nitrocellulose membrane in 1x tris-glycine transfer buffer with 10 % methanol at 100 mV for 90 min.

3 % bovine serum albumin (BSA) in tris buffered saline with Tween (TBST) was used for 1 h to block the membrane and prevent unspecific antibody binding. Membranes were incubated with BSA diluted antibody solutions (1:1000) overnight at 4 °C. Antibodies used were anti-acetyl- α -tubulin (catalog no. 5335), anti-acetyl-histone H3 (catalog no. 9677S), and anti- β -actin (catalog no. 5125S) or anti-GAPDH (catalog no. 97166), following the supplier's guidelines (Cell Signaling Technology, Danvers, MA). The next day the membranes were washed three times with TBST. Horseredish peroxidase (HRP) coupled secondary antibodies (Cell Signaling Technology) were diluted 1:1000 in a BSA solution and the membranes were incubated for 2 h. Blots were visualized using Simple Western Jess machine (Biotechnie).

4.3.6. Annexin PI staining

Leukemia cells (0.1×10^6 cells/mL) were seeded in 1 mL in 24 well plates and treated for 48 h at the indicated concentrations. The treated cells were washed once with PBS and afterward treated with Annexin-PI-staining solution for 15 min at 4 °C, following the supplier's guidelines (Invitrogen, Carlsbad, CA). FACS analysis was done via the Beckman Coulter CytoFLEX. Significance was calculated via an unpaired t-test.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.2024.116447>.

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3.3 - Deciphering the therapeutic potential of novel pentyloxy-amide-based class I, IIb HDAC inhibitors against therapy-resistant leukemia

Starting from an already improved vorinostat analog, in this paper, highly potent and selective class I/IIb inhibitors were designed and characterized. After a HBTU-mediated coupling and purification, the HDAC inhibition profile of the compounds was determined. Molecular docking studies, binding pose prediction, and the crystal structure of HDAC6 determined the binding method of the novel compounds. The inhibitory potential against three leukemic cell lines was investigated, followed by a western blot validation of HDAC inhibition of the three most promising structures **4m**, **4d** & **4l**. An extensive screening with 12 reference inhibitors against healthy controls, leukemia cell lines, and patient derived xenografts (PDX) revealed that **4d** and **4m** had a preferable safety profile. Through a cell cycle assay and a caspase-based assay, the cell death mechanism via apoptosis was identified. A matrix synergy approach determined the synergistic potential of the HDACi with decitabine. Subsequently, a western blot analysis highlighted that decitabine enhances the HDAC1-HDAC3 inhibition of **4d** & **4m**. An additional synergistic interaction partner was found in clofarabine. The metabolic stability was determined *in vitro* in liver microsomes and *in vivo* in mice. Following these results, **4d** was able, in an *in vivo* experiment, to decrease the leukemia growth in mice at a similar level of vorinostat. These findings highlight the preclinical potential of these novel class I/IIb inhibitors.

Contributions: In general, writing of the manuscript and experimental design, execution, evaluation, and visualization of the following biological experiments:

- Initial screening of the compound library (see Table 3)
- Western blot analysis of downstream HDAC deacetylation targets after HDACi treatment (see Figure 6)
- Extended screening with reference inhibitors in leukemia cell lines and PDX (see Figure 7A-7B)
- Cell cycle analysis upon experimental HDACi treatment (see Figure 8)
- Apoptosis induction and reversal via caspase 3/7 activity (see Figure 9A-9B)
- Matrix synergy screening of **4d** & **4m** with decitabine (see Figure 10A-10B)
- Western blot analysis and quantification of enhancement of H3 acetylation and PARP cleavage through the drug combination of HDACi and decitabine (see Figure 10C-10F)
- Matrix synergy screening of **4d** & **4m** with clofarabine (see Figure 11A-11B)

Deciphering the Therapeutic Potential of Novel Pentyloxyamide-Based Class I, IIb HDAC Inhibitors against Therapy-Resistant Leukemia

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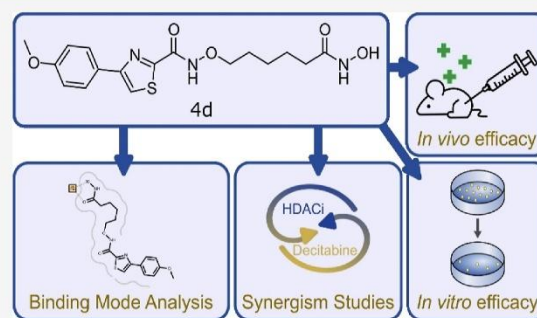
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ABSTRACT: Histone deacetylase inhibitors (HDACi) are established anticancer drugs, especially in hematological cancers. This study aimed to design, synthesize, and evaluate a set of HDACi featuring a pentyloxyamide connecting unit linker region and substituted phenylthiazole cap groups. A structural optimization program yielded HDACi with nanomolar inhibitory activity against histone deacetylase class I/IIb enzymes. The novel inhibitors (**4d** and **4m**) showed superior antileukemic activity compared to several approved HDACi. Furthermore, **4d** and **4m** displayed synergistic activity when combined with chemotherapeutics, decitabine, and clofarabine. In vitro pharmacokinetic studies showed the most promising profile for **4d** with intermediate microsomal stability, excellent plasma stability, and concentration-independent plasma protein binding. Additionally, **4d** demonstrated comparable in vivo pharmacokinetics to vorinostat. When administered in vivo, **4d** effectively inhibited the proliferation of leukemia cells without causing toxicity. Furthermore, the binding modes of **4d** and **4m** to the catalytic domain 2 of HDAC6 from *Danio rerio* were determined by X-ray crystallography.



1. INTRODUCTION

Leukemia is characterized by genetic and epigenetic heterogeneity due to the presence of diverse molecular alterations, contributing to the formation of distinct subgroups and influencing the prognosis and therapeutic outcome of patients.^{1–3} Leukemia is the most common cancer in children, with a 5 year-overall survival rate exceeding 85%.⁴ However, approximately 20–25% of childhood leukemia patients experience relapsed or refractory leukemia; among those cases only around 15–50% of patients are able to achieve remission.⁵ Another ongoing challenge in leukemia treatment is the therapy-related toxicity of routinely used drugs,^{6,7} along with certain leukemia subtypes that continue to be linked to unfavorable outcomes.⁸ In order to prevent long-term therapy-related health damage and to successfully treat therapy-refractory subtypes, new antileukemic drugs are urgently needed.

A possible target for new therapies is the characteristic perturbations of cancer cells in their epigenetic regulation.⁹ Epigenetic dysregulation is common for all kinds of human cancer irrespective of solid tumors or hematological

malignancies.¹⁰ It has been argued that epigenetic disruption is the causative mechanism behind all hallmarks of cancer steering proliferation, growth suppressor evasion, or apoptosis of cancer cells.¹¹ Previous studies revealed that hematological malignant cells are especially sensitive to treatment with HDAC inhibitors (HDACi),¹² although the exact mechanism behind this observation remains elusive as it is not allocated to a single cause.¹³ Today, all approved HDACi are approved for the therapy of hematological malignancies.¹⁴ The epigenetic regulation of transcription is significantly governed by the acetylation status of histones.¹⁵ This status is tightly regulated by the interplay of two families of enzymes that control the acetylation or deacetylation of ϵ -amino-groups of

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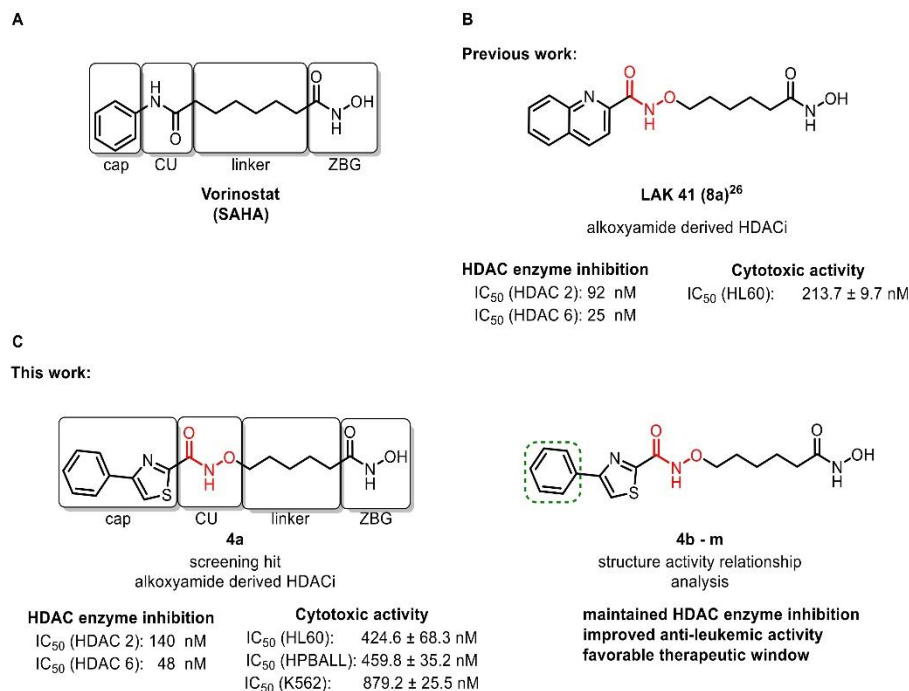


Figure 1. Development of novel pentyloxyamide-based HDACi (A) pharmacophore model of HDAC-inhibitors exemplified on vorinostat. CU = connecting unit and ZBG = zinc-binding-group. (B) Previously published pentyloxyamide-derived HDAC inhibitor.²⁶ (C) Initial screening hit **4a** showed nanomolar HDAC-isoenzyme inhibition and high nanomolar cytotoxic activity on three different leukemic cell lines. Derivatisation of the terminal phenyl moiety improved the antileukemic activity while retaining HDAC isoenzyme inhibition.

lysine residues: histone acetyltransferases acetylate the ϵ -amino group of lysine residues while HDAC remove acetyl groups from lysines.¹⁶ HDAC enzymes are classified into four classes related to their homology toward deacetylases.¹⁷ Three of the four classes, namely class I, II, and IV HDAC enzymes, are zinc-dependent metalloenzymes. Class I consists of HDAC enzymes 1, 2, 3, and 8. Especially the nucleic HDAC enzymes 1, 2, and 3 are directly involved in the regulation of the acetylation status of histones.¹⁸ Class IIb HDACs are formed by the cytosolic enzymes HDAC6 and 10. Substrates of HDAC6 are, among others, proteins that are found to be dysregulated in cancer like HSP90¹⁹ or Ku70.²⁰

In addition to their potent antileukemic effects used as mono treatment, HDACi are displaying strong synergistic effects in combination with a broad variety of anticancer drugs.²¹ For instance, HDACi act synergistically with alkylating^{22,23} and hypomethylating agents,²⁴ proteasome inhibitors,^{25–27} and immunomodulating drugs.^{28,29} In light of the promising results seen in preclinical studies, clinical trials are currently underway to investigate the potential of HDACi (vorinostat) in combination with the hypomethylating agent (azacitidine) for the treatment of pediatric or adult acute myeloid leukemia (AML) patients (NCT03843528 and NCT00392353). This provides the rationale for a systematic investigation of HDACi as synergistic combination partners for the therapy of resistant leukemic cells.

Our newly developed HDAC inhibitors, targeting class I and IIb, have demonstrated remarkable efficacy against different types of leukemic cells, including those resistant to standard therapies. Notably, these inhibitors exhibit a higher selectivity toward leukemic cells while minimizing damage to

healthy cells, distinguishing them from several approved HDACi. Moreover, our most promising candidates (**4d** and **4m**) have displayed significant synergistic effects when combined with the approved chemotherapeutic agents, decitabine and clofarabine. Promising in vitro pharmacokinetic characteristics and in vivo activity encourage their further investigation.

2. RESULTS AND DISCUSSION

We screened our internal library for novel and easily accessible lead structures against three leukemic cell lines K562—(chronic myeloid leukemia or CML); HL60—(AML or AML); and HPBALL—(T-cell acute lymphoblastic leukemia or T-ALL) and identified HDACi **4a** as a hit compound. Strikingly, **4a** outperformed the approved HDACi, vorinostat, both in terms of the HDAC enzyme inhibitory activity and on cytotoxic activity on various leukemic cell lines. **4a** combines a phenylthiazole cap group that has been successfully deployed in the development of HDACi before^{30–33} and an oxyamide group as a bioisosteric replacement for the common amide moiety^{26,34} (Figure 1). Currently, the two clinically approved Mitogen-activated protein kinase (MEK) 1/2 inhibitors, binimetinib and selumetinib, are establishing the oxyamide group as a valuable structural motif in the field of anticancer drugs.^{35,36} We have shown previously that HDACi with an oxyamide linker region are equally or even more potent than some approved HDACi with an amide connecting unit.^{26,34} The oxyamide connecting unit extends the chemical space of amide bioisosters and may contribute to the recently emerging

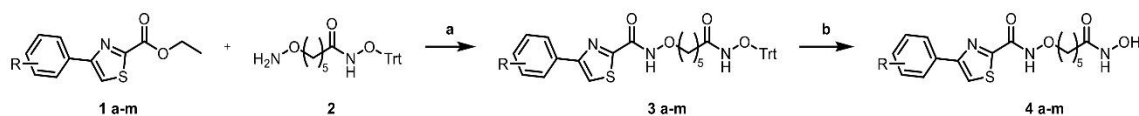


Figure 2. Reagents and conditions: (a) (1) 1M NaOH, THF, RT o.n., (2) HBTU, DIPEA, DMF, RT, o.n., and (b) TFA, Et₃SiH, DCM, RT, 30 min.

Table 1. Inhibition of Human HDAC2, 4, 6, and 8 by Compounds 4a–m and Reference Compounds Vorinostat, Panobinostat, and Tubastatin A

compound	R	IC ₅₀ (HDAC2) [μM]	IC ₅₀ (HDAC4) [μM]	IC ₅₀ (HDAC6) [μM]	IC ₅₀ (HDAC8) [μM]
4a		0.059 ± 0.012	> 100	0.075 ± 0.03	4.01 ± 0.9
4b		0.046 ± 0.009	> 100	0.026 ± 0.006	2.16 ± 0.3
4c		0.056 ± 0.013	> 100	0.026 ± 0.008	5.20 ± 1.0
4d		0.015 ± 0.004	> 100	0.038 ± 0.01	5.75 ± 0.9
4e		0.065 ± 0.008	> 100	0.051 ± 0.05	1.29 ± 0.3
4f		0.045 ± 0.011	> 100	0.047 ± 0.015	2.32 ± 0.3
4g		0.048 ± 0.012	> 100	0.045 ± 0.012	6.38 ± 1.6
4h		0.047 ± 0.008	> 100	0.081 ± 0.03	2.51 ± 0.3
4i		0.105 ± 0.016	> 100	0.049 ± 0.017	5.41 ± 0.3
4j		0.060 ± 0.03	> 100	0.064 ± 0.03	7.01 ± 1.9
4k		0.065 ± 0.03	> 100	0.108 ± 0.004	4.86 ± 0.5
4l		0.010 ± 0.003	> 100	0.030 ± 0.01	3.68 ± 0.5
4m		0.0045 ± 0.0007	> 100	0.064 ± 0.03	4.80 ± 1.6
Vorinostat	-	0.165 ± 0.006	47.8 ± 6.0	0.047 ± 0.003	5.85 ± 0.7
Panobinostat	-	N.D.	0.725 ± 0.06	N.D.	0.299 ± 0.07
Tubastatin	-	N.D.	N.D.	0.033 ± 0.017	N.D.

^aAll compounds were evaluated in duplicate in two independent experiments. Values represent the mean ± SEM. N.D. = not determined.

Table 2. Inhibition of Human HDAC1, 2, 3, 4, 6, 8, and 11 by Compounds 4d and 4m and Reference Compounds Vorinostat, Panobinostat, and Tubastatin A

compound	R	IC ₅₀ (HDAC1) [μM]	IC ₅₀ (HDAC2) [μM]	IC ₅₀ (HDAC3) [μM]	IC ₅₀ (HDAC4) [μM]	IC ₅₀ (HDAC6) [μM]	IC ₅₀ (HDAC8) [μM]	IC ₅₀ (HDAC11) [μM]
4d		0.0045 ± 0.0010	0.015 ± 0.004	0.013 ± 0.002	> 100	0.038 ± 0.012	5.8 ± 0.8	26 ± 4
4m		0.012 ± 0.003	0.0045 ± 0.0007	0.048 ± 0.012	> 100	0.060 ± 0.03	4.8 ± 1.6	36 ± 7
Vorinostat	-	0.082 ± 0.018	0.16 ± 0.01	0.073 ± 0.010	47 ± 6	0.047 ± 0.003	5.9 ± 0.7	> 100
Panobinostat	-	0.0082 ± 0.0015	N.D.	0.014 ± 0.003	0.72 ± 0.06	N.D.	0.30 ± 0.07	2.9 ± 0.2
Tubastatin	-	N.D.	N.D.	N.D.	N.D.	0.033 ± 0.017	N.D.	N.D.

^aAll compounds were evaluated in duplicate in two independent experiments. Values represent the mean ± SEM. N.D. = not determined.

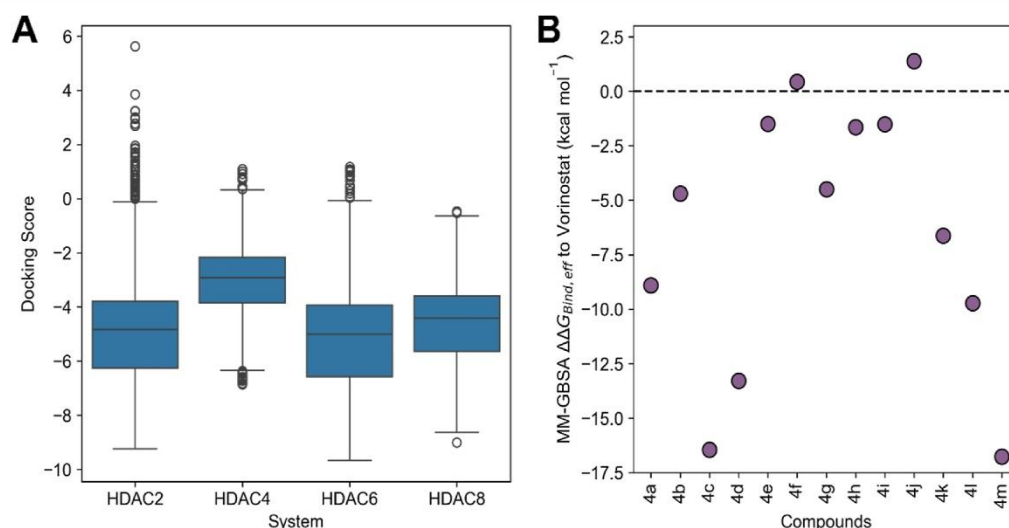


Figure 3. (A) Comparison of the docking results between the isoenzymes HDAC2, 4, 6, and 8 for compounds 4a–4m. The results mirror the enzyme assay data in that the compounds on average show better inhibition of HDAC2, HDAC6, and HDAC8 than HDAC4. (B) MM-GBSA results of the best-docked pose of a compound in HDAC2. In line with the results of the enzyme assay, 4m is predicted to have the best effective binding energy.

topic of hydroxylamine analogs to established amine moieties.^{37,38}

As starting materials for a diverse set of phenylthiazole analogs were readily available, we aimed in this study to further develop 4a into a new antileukemic lead structure by elucidating the structure–activity relationship of its terminal phenyl moiety.

2.1. Synthesis. The synthesis of the novel HDACi (4a–m) started with the saponification of phenylthiazole carboxylic esters (1a–m) followed by HBTU-mediated coupling reactions of the generated corresponding sodium salts with the hydroxylamine linker 2 containing the *O*-trityl-protected hydroxamic acid zinc binding group using a modified protocol of Goodreid et al.³⁹ (Figure 2). The coupling reactions for the construction of the pentoxoyamide moiety were performed utilizing the in situ generated sodium salts of the respective carboxylic acids as the corresponding acids of 1a–m were prone to decarboxylation. The synthesis of the hydroxylamine linker 2 was previously published by our group.²⁶ Subsequent TFA and triethylsilane-mediated

deprotection of the *O*-trityl-protected hydroxamates yielded the hydroxamic acids 4a–m.

2.2. HDAC Enzyme Inhibition. All synthesized HDACi are nanomolar inhibitors of HDAC2, a representative of HDAC class I, and of HDAC6, a representative of HDAC class IIb. The HDAC enzyme inhibition profile did not differ remarkably among the improved analogs (Table 1). Derivate 4m was the most potent HDAC2 inhibitor improving the IC₅₀ of the parent compound 4a (IC₅₀ 0.140 μM) significantly by a factor of 13 (IC₅₀ 0.0045 μM). Also, compounds 4d (IC₅₀ 0.015 μM) and 4l (IC₅₀ 0.010 μM) improved HDAC2 inhibition in comparison to 4a by a factor of 4 or 6, respectively. Interestingly, HDAC6 inhibition was not improved to the same extent as HDAC2 inhibition. 4c is the most active HDAC6 inhibitor in this series and exceeded the activity of the parent compound by a factor of about three (IC₅₀ 0.026 μM). However, each newly synthesized HDAC inhibitor surpassed vorinostat in regards to HDAC2 inhibition and was similarly potent as tubastatin A in HDAC6 enzyme inhibition.

Encouraged by the promising results of **4m** and **4d**, these compounds were subjected to an expanded HDAC isozyme analysis (Table 2), which demonstrated nanomolar inhibition of HDAC 1 and 3 but only weak HDAC11 inhibition.

2.3. Docking Studies. Molecular docking was performed to assess the binding affinities of the compounds **4a–4m** to human HDAC2, 4, 6, and 8 from a structural perspective. Binding affinities predicted with the Glide SP scoring function of the Schrödinger Suite⁴⁰ generally align with the enzyme assay results when considered on a per-isoform basis (Table 1, Figure 3): On average, **4a–4m** yield more favorable docking scores in HDAC2, HDAC6, and HDAC8 (-5.1 ± 1.8 kcal mol⁻¹, -5.3 ± 1.5 kcal mol⁻¹, and -4.7 ± 1.2 kcal mol⁻¹, respectively) than in HDAC4 (-3.0 ± 1.4 kcal mol⁻¹). Although predicting binding affinities with scoring functions requires caution when the differences are small,⁴¹ this agreement mirrors that IC₅₀ values for HDAC4 are one to two orders of magnitude larger than for the other isoforms. To support the results, Molecular Mechanics - Generalized Born Surface Area (MM-GBSA) computations were used to predict effective binding energies for the best-docked pose of each compound in HDAC2. The results identify **4m** as the compound with the highest binding affinity, which agrees with the experimental data. The other compounds cannot be expected to be ranked well due to the small value range (a factor of ~ 2 except for **4d**, **4l**, which are predicted second and third best, qualitatively in line with the experimental data; **4c** is overpredicted; Table 1) and SEM values of up to $\sim 50\%$ of the mean value.

The docking poses of **4m** and the unsubstituted compound **4a** (Figure 4) show an interaction of the hydroxamate moiety with the zinc ion, hydrogen bonds with D100 and Y304, and stacking interactions of the phenyl

moiety with H29 close to the rim of the binding tunnel. The *p*-substitution of the phenyl ring with pyrrolidine in **4m** increases the interaction surface of the complex, allowing for additional apolar interactions. The entropic penalty for extending the ligand with the pyrrolidine moiety is likely small since free rotation about the C–N axis is already restricted in solution due to conjugation such that only a few degrees of freedom are lost upon binding.

2.4. Crystal Structures of **4d and **4m** in Complex with HDAC6.** To analyze the binding mode of **4d** and **4m** to HDAC6, both inhibitors were cocrystallized with the catalytic domain 2 (CD2) of HDAC6 from *Danio rerio* (zebrafish). This enzyme can be used as a model for human HDAC6 due to high structural similarity and high conservation of the active site.⁴³

The crystal structures of CD2 from DrHDAC6 bound to **4d** and **4m** were solved at 1.90 and 2.14 Å resolutions, respectively (Figure 5 and Supporting Information Table S1),

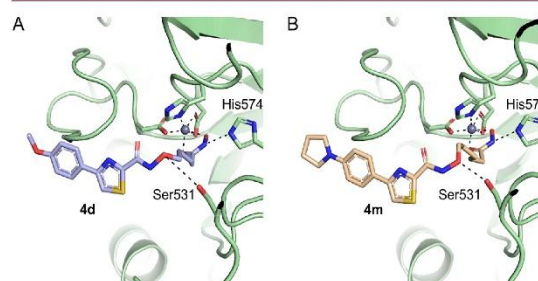


Figure 5. Crystal structures of (A) HDAC6–**4d** complex and (B) HDAC6–**4m** complex. The HDAC6 protein is shown as a cartoon and colored pale green. Close-up view of the active site with the ligands and the side chains of the amino acids coordinated to the zinc ion shown as a stick model and colored light blue (**4d**) and wheat (**4m**). The ligands and amino acid side chains are colored according to the element-specific color code. The zinc ion is shown as a sphere and colored gray. A figure displaying the simulated annealing omit map of the ligands is shown in the Supporting Information (Figure S1) (PDB ID: 9GGH and 9GGK).

providing, for the first time, insights into the binding mode of pentoxoyamide-based HDACi. The structures do not reveal pronounced conformational changes relative to the HDAC6 structure in the absence of any ligands (PDB ID: SEEM).⁴⁴ The root-mean-square deviation (rmsd) between unliganded and **4d**-bound structures is 0.4 Å for 353 Cα atoms and between unliganded and **4m**-bound structures is 0.5 Å for 353 Cα atoms.

The inhibitors **4d** and **4m** bind in a similar conformation to DrHDAC6 with the hydroxamate group coordinated to the zinc ion in the active site. The flexible pentoxoyamide connecting unit linker region is able to enter the narrow channel to the active site and enable a tight coordination to the zinc with O–Zn distances of 2.0 Å. The higher electron density observed in the simulated annealing omit map of the inhibitor shows that the sulfur atom of the thiazole group has the same orientation in both inhibitors, suggesting that only one conformation is adopted (Supporting Information Figure S1). Interestingly, the oxygen and nitrogen atoms of the oxyamide connecting unit of **4d** and **4m** are in a similar position despite not forming a specific interaction with the protein surface. In addition to the coordination of the hydroxamate group to the zinc ion, we observe two

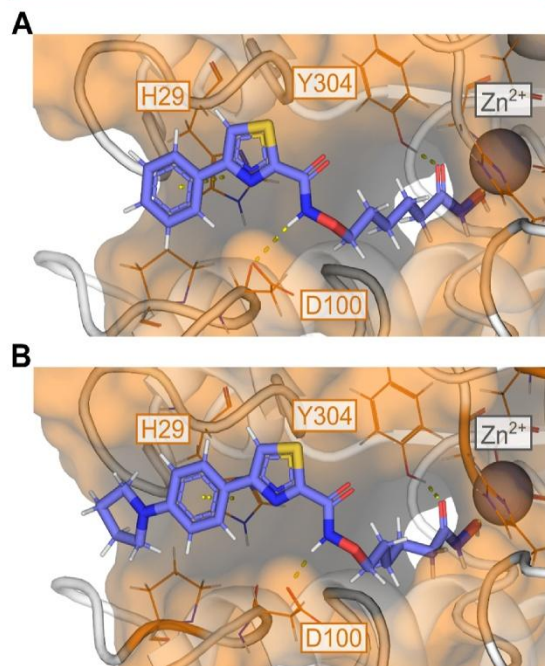
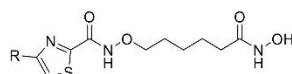


Figure 4. Predicted binding poses of compounds **4a** (A) and **4m** (B) to human HDAC2 (PDB-ID: 7KBG).⁴²

Table 3. Comparison of the IC₅₀ of the Experimental Compounds in Three Different Leukemia Entities

compound	R	IC ₅₀ (HL60) [nM]	IC ₅₀ (HPBALL) [nM]	IC ₅₀ (K562) [nM]
4a		424.6 ± 68.3	459.8 ± 35.2	879.2 ± 25.5
4b		332.1 ± 56.2	523.1 ± 58.2	993 ± 128.2
4c		670.7 ± 74.8	714.6 ± 55.2	1478.7 ± 53
4d		76.8 ± 8.2	110.6 ± 15.2	180.8 ± 55.7
4e		352.4 ± 25.5	359.9 ± 20	873.9 ± 238.4
4f		249.8 ± 84.5	421.2 ± 44.7	656.1 ± 264.7
4g		380.3 ± 36.8	465.3 ± 73.1	786.4 ± 230.9
4h		168.1 ± 104	355.9 ± 19.6	526.5 ± 28
4i		313.7 ± 96.5	403.8 ± 46.2	722 ± 164
4j		192.4 ± 22.4	279 ± 20.7	414.6 ± 38.8
4k		263.7 ± 70.7	446.4 ± 65.7	629.6 ± 69.6
4l		86 ± 10.4	119.4 ± 6.2	242.5 ± 52
4m		49.1 ± 5.4	100.1 ± 6	141.9 ± 13.2
Vorinostat	-	318.03 ± 36.7	823.53 ± 27	577.4 ± 119.8
Ricolinostat	-	2190.3 ± 24.9	2583.7 ± 71.16	2933 ± 79.5
CI994	-	1578.7 ± 92.5	4703.3 ± 117.2	5331.3 ± 948.17
Belinostat	-	157.7 ± 13.9	269.1 ± 5	217.9 ± 51.8

interactions with the HDAC6 protein. The imidazole ring of HS74 interacts with the nitrogen atom in the hydroxamate group of the inhibitor, and the oxygen atom in the hydroxy group of SS31 interacts with the bivalent oxygen atom of the oxyamide connecting unit. Notably, the predicted binding mode of **4m** to human HDAC2 differs from the conformation observed in the crystal structure of DrHDAC6, which may be due to the differences in the sequence between human HDAC2 and zebrafish HDAC6. Taken together, the

crystal structures of **4d** and **4m** in CD2 of DrHDAC6 provide important insights into the binding mode of the novel pentyloxamide-based HDACi.

2.5. High Throughput Drug Screening on Cytotoxicity of Novel HDACi. A HTDS⁴⁵ was conducted to evaluate the activity of 13 new synthesized HDACi across three leukemia cell lines: K562, HPBALL, and HL60 (Table 3). By representing the IC₅₀ values of each HDACi through heatmaps and performing unsupervised clustering, three

distinct clusters were identified (Supporting Information Figure S2). Compared to the lead inhibitor **4a**, the first cluster had similar activity against the three entities, while the second cluster consisted of HDACi with slightly lower IC_{50} and the third one showed significantly lower IC_{50} (~ 0.7 log₁₀ fold change). In the HL60 cell line, which is the most sensitive, HDACi **4d**, **4m**, and **4l** showed IC_{50} values between 0.049 and 0.086 μ M, whereas in the K562 cell line, the range was 0.141 to 0.242 nM. Throughout all three cell lines the inhibitor with the most potent anticancer activity is **4m**, followed by **4d** and **4l**.

2.6. Structure–Activity–Relationship Analysis. First, we modulated the electronic properties of the terminal phenyl ring by introducing a methoxy or fluorine substituent at the 1', 2', or 3'-position. Though the introduction of fluorine did not influence the antileukemic effect of the HDAC inhibitors significantly regardless of the ring position (**4e–g**), the introduction of a methoxy substituent at the 4'-position (**4d**) enhanced the cytotoxic activity against leukemia cells in comparison to the 2' or the 3' derivatives (**4b**, **4c**).

Second, the replacement of the phenyl ring with thiophene was tolerated (**4h** and **4i**). Interestingly, the sulfur in the 2'-position (**4h**) proved to be beneficial for the antileukemic activity. However, the overall influence on the cytotoxic activity against leukemic cells remained rather small.

Third, we focused on the optimization of the substituent in 4'-position. The molecular docking studies suggested that the introduction of a larger substituent would favor additional apolar interactions with the HDAC2 enzyme. Correlating with the molecular docking studies, the pyrrolidine analog **4m** is a compound with strong antileukemic activity and low nanomolar inhibition of the HDAC2 enzyme. Further agreeing with that, extending the size of the cap group with a 2'-benzofuran moiety (**4l**) also enhanced the cytotoxic activity remarkably in comparison to **4a**. Overall, the introduction of larger substituents in 4'-position in combination with electron donating heteroatoms (**4d**, **4m**) and the 2'-substituted benzofuran (**4l**) improved the antileukemic activity in comparison to the unsubstituted compound **4a** remarkably (Figure 5). In addition, there is also a good correlation between the antileukemic activity and the inhibition of HDAC2 and 6.

2.7. Western Blot Analysis. The HDAC inhibitory effects of the three most promising derivatives (**4d**, **4l**, and **4m**) were next evaluated through cell-based assays using the HL60 cell line. Treatment of HL60 cells with compounds **4d**, **4l**, and **4m** resulted in notable α -tubulin acetylation (indicating effective inhibition of HDAC6), as determined by Western blot analysis. Vorinostat was utilized as a control for comparison (Figure 6). When evaluating HDAC class I inhibition, the optimized inhibitors displayed higher histone H3 acetylation levels compared to vorinostat. Furthermore, correlating with the H3 acetylation, we observed higher PARP cleavage (indicator of apoptosis induction) with **4d**, **4l** and **4m**, suggesting their enhanced effectiveness in comparison to vorinostat.

2.8. Selective Cytotoxicity of 4d and 4m against Leukemia Cells Surpasses Various Commercial HDAC Inhibitors. Following the confirmation of cellular HDAC inhibition by the promising new HDACi series, we selected two most potent HDACi (**4d** and **4m**) for an extended drug screening analysis. This analysis involved evaluating their

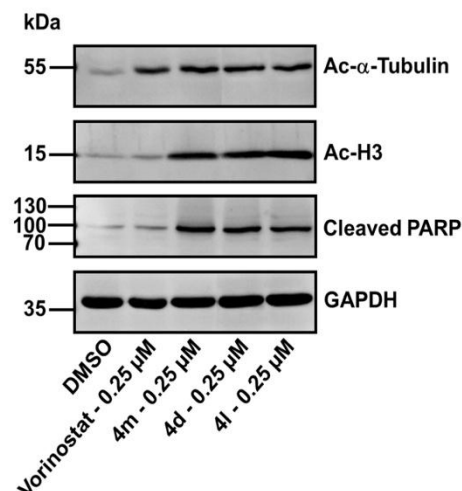


Figure 6. Western Blot analysis was conducted to assess the effects on HDAC inhibition markers (ac-H3 and ac-Tubulin) and the induction of apoptosis marker (cleaved PARP) following a 24 h treatment with **4d**, **4m**, and vorinostat (control) in HL60 leukemia cells ($n = 3$). A representative blot is shown here with GAPDH serving as the loading control.

effectiveness against 12 commercially available HDAC inhibitors using 7 leukemia cell lines (Figure 7A and Supporting Information Tables S2–S4) and 27 leukemia patient-derived xenograft (PDX) cells (Figure 7B and Supporting Information Tables S2–S4) across distinct therapy response subgroups. We incorporated healthy controls into our screens to determine whether **4d** and **4m** operate within a safe therapeutic window. These controls comprised CD34⁺ hematopoietic stem and progenitor cells derived from cord blood and T-cells from healthy donors. By determining drug sensitivity scores (DSS) and generating selective or differential DSS (dDSS),⁴⁶ we obtained comparative data to evaluate their selectivity on the leukemia cells compared to the healthy controls. In comparative studies, both **4d** and **4m** demonstrated superior efficacy compared to clinically advanced HDAC inhibitors including tubastatin A, ricolinostat, CI994, romidepsin, and vorinostat. This was evidenced by their significantly elevated sDSS values when assayed against a panel of leukemia cell lines and PDX cells. Moreover, **4d** and **4m** consistently displayed selective antileukemic effects in the majority of tested leukemia cell types, suggesting their superiority over other examined HDACi. Additionally, standard chemotherapeutics used in leukemia treatment protocols, including cytarabine, idarubicin, daunorubicin, and azacitidine, were incorporated into the PDX drug screening analysis. Both **4d** and **4m** demonstrated significantly higher sDSS values, indicative of an improved therapeutic window and relatively similar cytotoxic profiles toward both chemotherapy-sensitive and chemotherapy-resistant subgroups (Figure 7B).

2.9. 4d and 4m Induce Cell Cycle Arrest and Apoptosis in Leukemia Cells. HDAC inhibition commonly leads to cell cycle arrest as an initial response, followed by the induction of apoptosis.^{47,48} Analysis of the cell cycle demonstrated that treatment with **4d** in a concentration of 0.15 μ M and **4m** at both concentrations significantly increased the proportion of HL60 cells in the G1 phase.

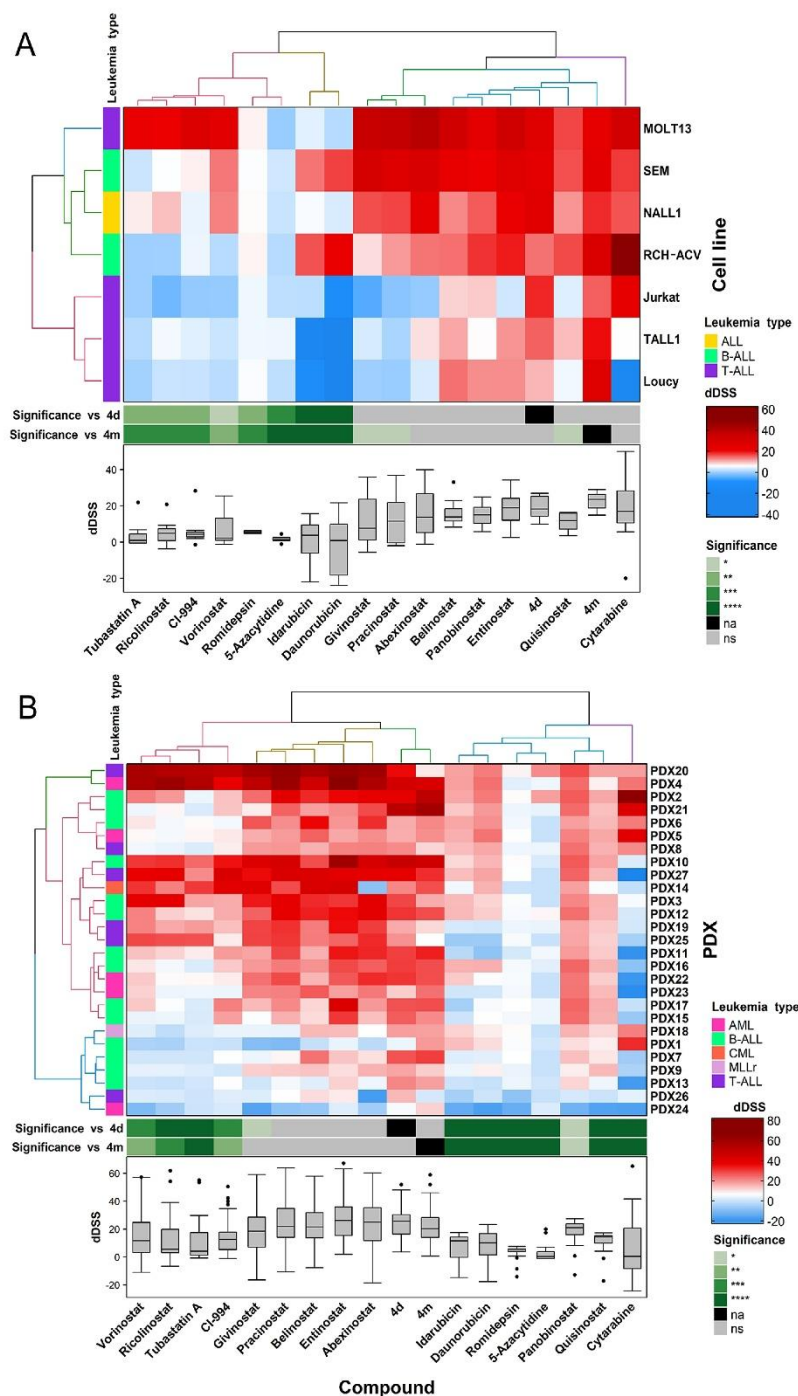


Figure 7. Unsupervised clustered heatmap displaying the differential drug sensitivity score (dDSS) of (A) leukemia cell lines and (B) PDX-grown leukemia cells. The statistical analysis was conducted using one-way ANOVA, $n = 1$. ALL = acute lymphoblastic leukemia, AML = acute myeloid leukemia, B-ALL = B-cell acute lymphoblastic leukemia, CML = chronic myeloid leukemia, MLLr = MLL rearranged leukemia, T-ALL = T-cell acute lymphoblastic leukemia, na = not applicable, ns = not significant, * = $p(\text{adj}) > 0.05$, ** = $p(\text{adj}) > 0.01$, *** = $p(\text{adj}) > 0.001$, and **** = $p(\text{adj}) > 0.0001$.

4d and **4m** reduced the number of cells in the S and G2/M phases significantly (Figure 8). Furthermore, an increased population of cells exhibited sub G1 labeling, indicating DNA fragmentation and confirming the induction of apoptosis. In

comparison, **4m** exhibited greater potency than **4d** in inducing cell cycle arrest at the same concentration, which can be attributed to the lower IC_{50} value reported for **4m** as compared to **4d** (Table 3).

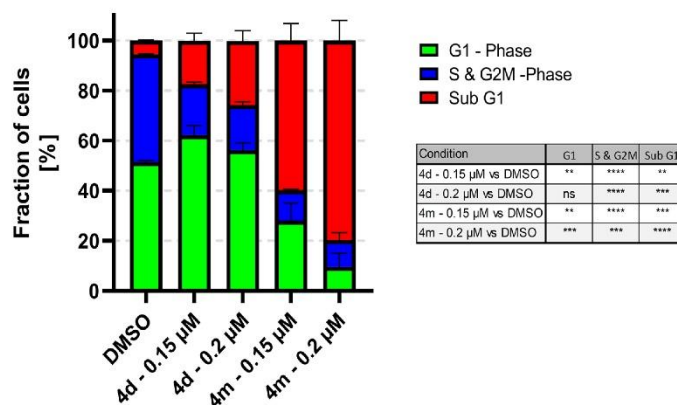


Figure 8. Bar graph depicting a cell cycle analysis via propidium iodide staining of HL60 leukemia cells treated with **4m** or **4d**, while the bar graphs displays statistical significance (unpaired student *t*-test, *n* = 3). ns = not significant, ** = *p*(adj) > 0.01, *** = *p*(adj) > 0.001, and **** = *p*(adj) > 0.0001.

To assess apoptosis induction by **4d** and **4m** further, we employed a trypan blue staining in conjunction with a caspase 3/7 assay on HL60 leukemia cells. As a control, we used the pan-caspase inhibitor Q-VD.⁴⁹ Treatment with **4d** or **4m** resulted in a significant reduction of viability ~20% after 24 h and 70% after 48 h (Figure 9A). Q-VD reduced the loss of viability by 50%, suggesting that the cytotoxic effects triggered by both compounds are associated with apoptosis and are largely caspase-dependent. The caspase 3/7 assay further validated these findings, showing a marked increase in caspase activity after 48 h treatments with **4d** or

4m (Figure 9B). Notably, Q-VD effectively negated this caspase 3/7 activity. In sum, the findings robustly indicate that both **4d** and **4m** induce apoptosis, leading to cytotoxic impacts on leukemia cells.

2.10. 4d and 4m Synergize Effectively with the Standard Chemotherapy Drugs Decitabine and Clofarabine. Current clinical therapeutic strategies acknowledge the shortcomings of singular drug treatments, advocating for the utilization of synergistic drug combinations. These combinations aim to reduce cytotoxicity, exploit the unique susceptibilities of cancer cells, and avert drug resistance development. Therefore, we investigated potential synergistic drug combinations with the novel HDACi **4d** and **4m**. Decitabine, a first line chemotherapeutic, functions as a DNA methyltransferase inhibitor resulting in DNA hypomethylation. Synergistic drug effects between decitabine and HDACi have been well characterized, particularly in the context of AML.^{50,51} Utilizing a matrix synergy screening method with the zero-interaction potency (ZIP) algorithm, we identified high ZIP synergy scores ranging from 60 to 80 across various concentration for both **4d** and **4m** when combined with decitabine (Figure 10A,B). Subsequently, we looked into the underlying mechanism of this drug synergy by evaluating the acetylation levels of histone H3 (Figure 10C). Although decitabine alone did not augment H3 acetylation, its combination with **4d** or **4m** showcased more pronounced bands compared to the individual HDACi treatment.

The combination ratio of decitabine to **4d** (5:1) was determined through synergy analysis, as this concentration ratio exhibited the highest synergistic interaction between them against AML cells. We have conducted additional combination experiments using a broad range of **4d** concentrations in conjunction with decitabine (Figure 10D). We included vorinostat as a positive control, maintaining the same concentrations as for **4d**. Notably, at equivalent concentrations, **4d** significantly increased acetylated-H3 levels and cleaved PARP levels (a marker of apoptosis) compared to vorinostat in combination with decitabine (Figure 10E,F). Therefore, this enhanced effect on protein acetylation (Ac-H3) likely contributes to the observed synergistic cytotoxicity outcomes.

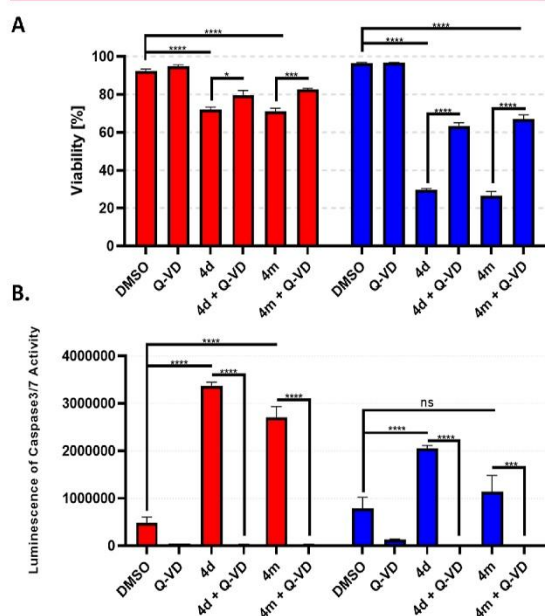


Figure 9. Bar diagram comparing the changes in (A) viability and (B) apoptosis induction via caspase 3/7 activity of HL60 leukemia cells treated with **4m** or **4d** and Q-VD (pan-Caspase inhibitor) via trypan blue or caspase3/7 assay, respectively. The statistical analysis was conducted using unpaired student *t*-test, *n* = 3. ns = not significant, * = *p*(adj) > 0.05, ** = *p*(adj) > 0.01, *** = *p*(adj) > 0.001, and **** = *p*(adj) > 0.0001.

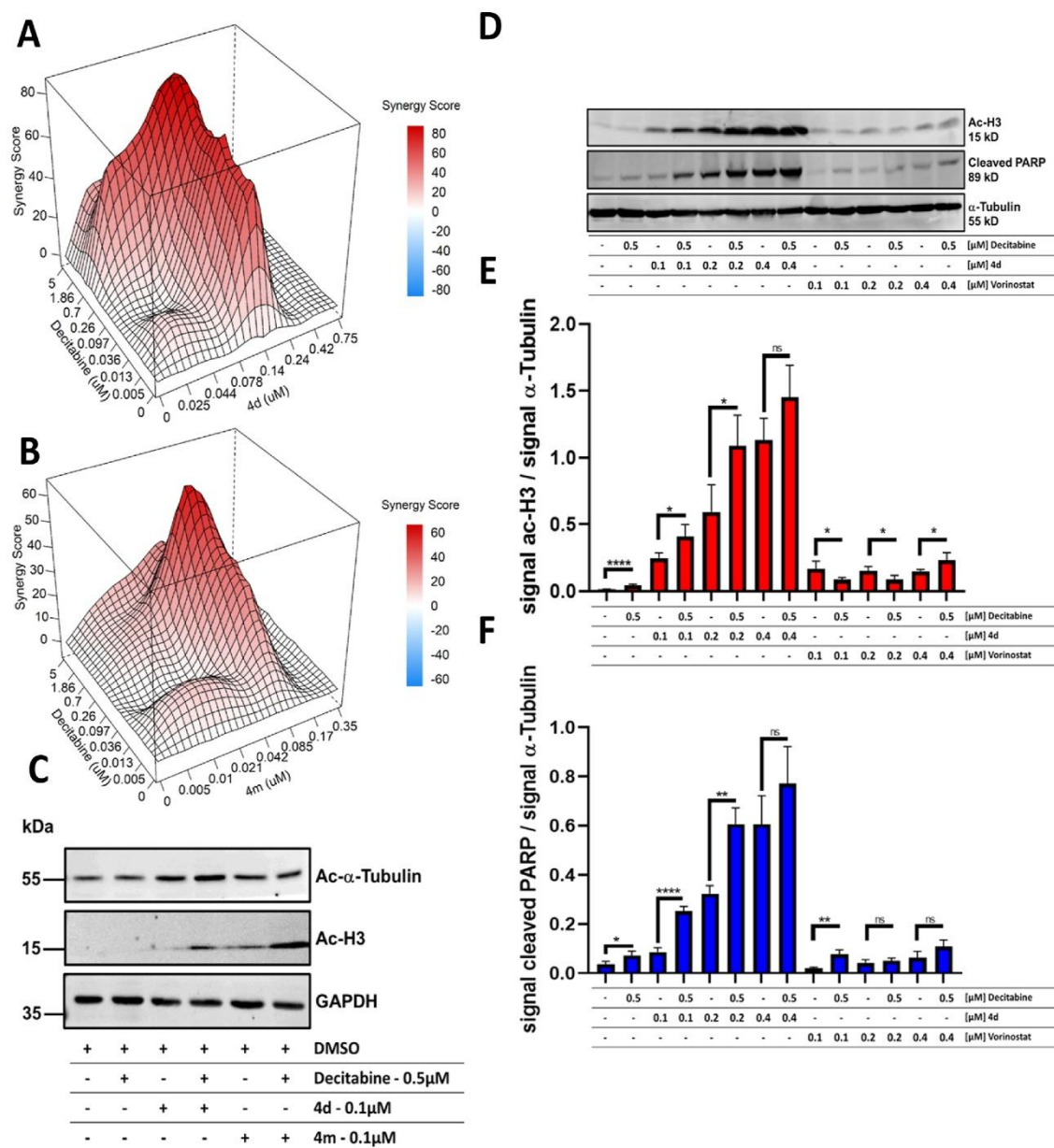


Figure 10. 3D synergy plots of HL60 cells treated with drug combinations for 72 h. The plots represent synergy scores from a combination matrix analyzed by the ZIP algorithm: (A) **4d** + decitabine and (B) **4m** + decitabine ($n = 2$). (C) Western blot analysis illustrating the differences in HDAC inhibition markers, ac-H3 and ac-Tubulin, in HL60 cells. The comparison is between solo treatment and combination treatments of either **4d** or **4m** with decitabine ($n = 3$) for 24 h. A representative blot is displayed with GAPDH as the loading control. (D) Western blot with increasing concentrations of **4d** and vorinostat combined with a fixed concentration of decitabine ($n = 3$). (E/F) Quantification of the signal of ac-H3 or cleaved PARP of D (unpaired student t -test, $n = 3$). ns = not significant, ** = $p(\text{adj}) > 0.01$, *** = $p(\text{adj}) > 0.001$, and **** = $p(\text{adj}) > 0.0001$.

The observed synergy of the epigenetic combination, which includes HDACi (4d) and DNMTi (Decitabine), has been corroborated by our studies and others, demonstrating significant synergistic activity against AML cells.^{24,50–52} In fact, the safety, tolerability, and antileukemia activity of this combination have led to clinical trials involving AML patients.^{53,54} Mechanistic insights suggest that the observed synergistic interaction is due to the crosstalk between DNA

methylation and HDAC pathways. This mechanism involves a 5-methylcytosine-binding protein binding to gene promoters and recruiting HDACs.⁵⁵ In AML cells, this interaction is significant for the activation of tumor suppressor genes, including CDKN2B.⁵⁶ However, we acknowledge that the detailed mechanism underlying enhanced cytotoxicity upon combining HDAC and DNMT inhibitors is still unclear.

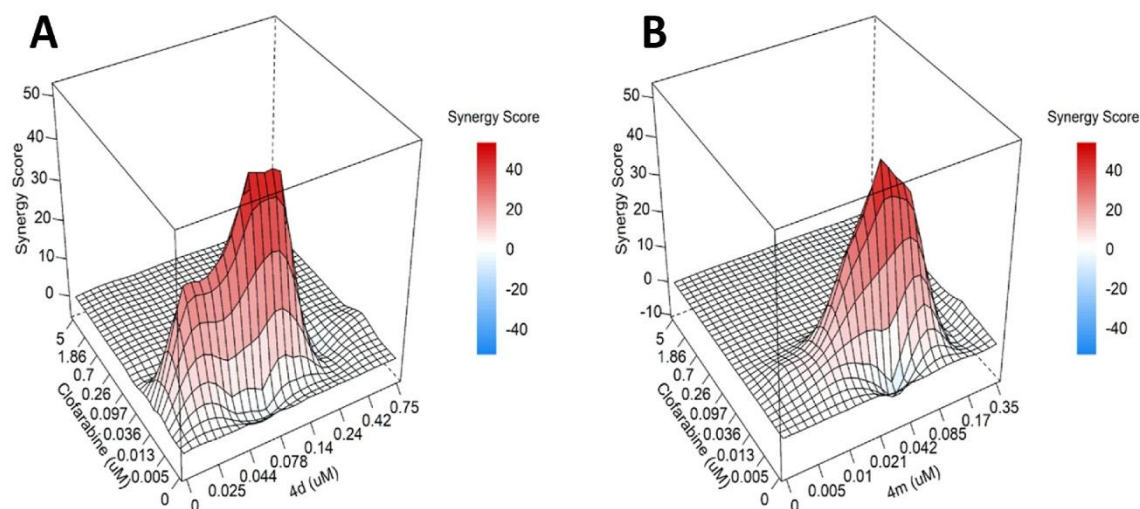


Figure 11. 3D synergy plots of HL60 cells treated with drug combinations for 72 h. The plots represent synergy scores from a combination matrix analyzed by the ZIP algorithm: (A) **4d** + clofarabine and (B) **4m** + clofarabine ($n = 2$).

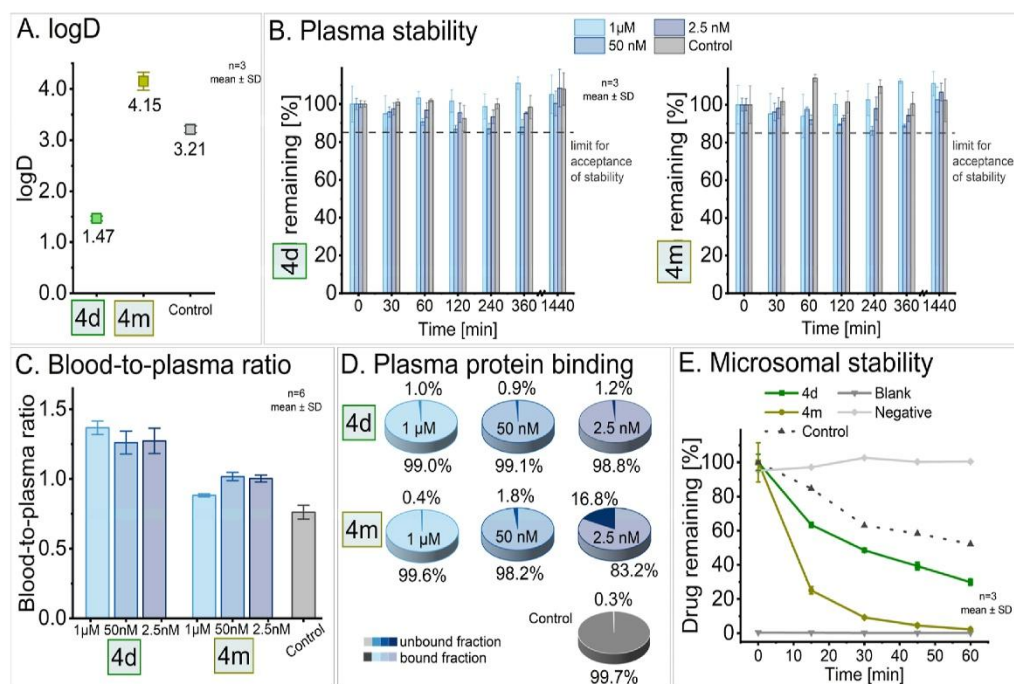


Figure 12. Overview of in vitro pharmacokinetics of **4d** and **4m**. (A) Interval plot of the determination of the coefficient of distribution ($\log D$). Carvedilol was used as a control. (B) Stability of **4d** and **4m** in human plasma over 24 h at three different concentrations. A limit of -15% was set as acceptance criterium for stability according to bioanalytical guidelines. 4% bovine serum albumin (BSA) (absence of plasma enzymes) was used as a control spiked with a concentration of 50 nM of the drug. (C) Blood-to-plasma ratio of **4d** and **4m** for the three assessed concentration levels applying carvedilol as a control. (D) Plasma protein binding for **4d** and **4m** at three different concentration levels. The mean value of $n = 3$ is displayed. Itraconazol was used as a control. (E) Microsomal stability of **4d** and **4m** in HLMs. Propranolol was used as a control. The blank contains no drug, while the negative is free of HLMs. BSA: bovine serum albumin, HLM: HLMs, and SD: standard deviation.

HDAC inhibition contributes to increased DNA damage.⁵⁷ We examined the synergistic impact of combining **4d** and **4m** with clofarabine, a purine nucleoside analog and DNA synthesis inhibitor. In line with our previous observations, we found in a similar matrix synergy approach, ZIP synergy

scores around 50 when **4d** and **4m** were administered in combination with clofarabine (Figure 11A,B).

These findings indicate that HDAC inhibitors **4d** and **4m** enhance the effectiveness of first line chemotherapeutics

Table 4. Comparison of In Vitro Pharmacokinetic Data of 4d and 4m to Vorinostat^a

	vorinostat	4d	4m
log <i>D</i>	1.46 ⁶³	1.47 ± 0.05	4.15 ± 0.18
plasma stability/ <i>t</i> _{1/2}	75 min ⁶³	>24 h	>24 h
<i>K</i> _{B/P}	2.0 ⁶⁴	1.26 ± 0.16 (50 nM)	1.02 ± 0.06 (50 nM)
plasma protein binding	71% ⁶⁵ (1.89 μM)	99.0% (1 μM)	99.6% (1 μM)
microsomal stability/ <i>t</i> _{1/2} (clearance category)	60 min ⁶⁶ (intermediate)	35.9 min ⁶⁶ (intermediate)	11.2 min (high)

^a*K*_{B/P}: blood-to-plasma ratio, log *D*: coefficient of distribution at pH 7.4, *t*_{1/2}: half-life.

Table 5. Pharmacokinetic Parameters (AUC_{last}, *C*_{max}, *T*_{max}, and *t*_{1/2}) Were Calculated According to a Noncompartmental Analysis (NCA) from the Blood Concentration of 4d Based on Actual Blood Sampling Time Pre-Dose and Post-Dose^a

variable	mouse 1	mouse 2	mouse 3	mean [±SD]	vorinostat	
	intraperitoneal 10 mg/kg				per oral 50 mg/kg	
<i>C</i> _{max} (ng/mL)	1490	1030	1230	1250 [±232]	501 ⁶⁷	580 ⁶⁸
<i>T</i> _{max} (h)	0.25	0.25	0.25	0.25 [±0]	na ⁶⁷	0.08 ⁶⁸
AUC _{last} (h·ng/mL)	523	396	480	466 [±65]	619 ⁶⁷	347 ⁶⁸
<i>t</i> _{1/2} (h)	0.275	0.554	0.227	0.352 [±0.177]	0.75 ⁶⁷	0.8 ⁶⁸

^aNa: not applicable.

decitabine and clofarabine potentially allowing for dose reduction and decreasing the likelihood of drug resistance.

2.11. 4d Shows Promising Human In Vitro Pharmacokinetics Superior to 4m. Determination of in vitro pharmacokinetic properties of new chemical entities is a key step in drug discovery and aids in nonclinical evaluation. To assess the initial ADME characteristics (absorption, distribution, metabolism, and elimination) of 4d and 4m, coefficient of distribution, plasma stability, blood-to-plasma ratio, microsomal stability, and plasma protein binding were investigated.

Good oral absorption can be assumed for the coefficient of distribution (log *D*) between 0 and 3.⁵⁸ The log *D* value was determined as 1.47 ± 0.05 for 4d and 4.15 ± 0.18 for 4m (Figure 12A), favoring 4d. Carvedilol acted as a control and confirmed the assay validity (obtained: 3.21 vs in literature: 3.2).⁵⁹

Further, in vitro plasma stability was determined, as instability is an indicator for rapid clearance or a short half-life (*t*_{1/2}), resulting in poor in vivo performance. 4d and 4m demonstrated excellent stability over the 24 h period monitored in human plasma at 37 °C (Figure 12B).

4m showed equal affinity to plasma and red blood cells, while 4d tended toward higher distribution into red blood cells (Figure 12C), as determined by the blood-to-plasma ratio (*K*_{B/P}). One-way ANOVA confirmed no concentration dependency over the range of 2.5 nM to 1 μM for 4d (*K*_{B/P}: 1.27 at 2.5 nM, 1.26 at 50 nM, and 1.37 at 1 μM), while for 4m the *K*_{B/P} at the highest concentration level investigated (1 μM) was significantly lower if compared to the lower concentration levels (*K*_{B/P}: 1.00 at 2.5 nM, 1.02 at 50 nM, and 0.88 at 1 μM). The control compound carvedilol validated the assay performance (*K*_{B/P} obtained: 0.76 vs *K*_{B/P} in literature: 0.76).⁶⁰

Plasma protein binding was high for 4d with a mean of 99.0% over the observed concentration range with no concentration-dependency. 4m exhibited an atypical concentration-dependent plasma protein binding with lower binding for lower concentrations (Figure 12D). If proceeding in drug development, this phenomenon needs to be further investigated.⁶¹ Itraconazole as control showed high plasma

protein binding throughout all cavities confirming no leakage and being in line with values reported in the literature.⁶²

While 4m was extensively metabolized by hepatic enzymes, such as Cytochrome P450 and Uridine 5'-diphosphoglucuronosyltransferases, in Human liver microsomes (HLMs), 4d showed moderate metabolism (Figure 12E). The half-life of 4d was 35.9 min, with an intrinsic clearance of 38.6 μL/min/mg. When corrected for nonspecific protein binding in the incubation mixture, the unbound intrinsic clearance was determined as 54.7 μL/min/mg. The half-life of 4m was substantially shorter with 11.2 min. While the corresponding intrinsic clearance resulted in 123.6 μL/min/mg, its unbound intrinsic clearance was 466.4 μL/min/mg.

Using the collected data, the in vivo hepatic clearance was estimated to be 0.39 L/min for 4d and 8.18 L/min for 4m. Assuming a hepatic blood flow of 1500 mL/min, 4d can therefore be classified as a low hepatic extraction drug (calculated hepatic extraction ratio (*E*_h) < 0.3) and 4m as high hepatic extraction drug (*E*_h > 0.7).

Finally, the collected in vitro pharmacokinetic data of 4d and 4m were compared to that of the approved drug vorinostat (Table 4). In comparison to vorinostat, 4d appeared most promising for further pharmacokinetic investigation.

2.12. Preclinical Pharmacokinetic Study of 4d in Mice Showed Comparability to Vorinostat. Building on our human in vitro pharmacokinetic data, an in vivo pharmacokinetic study using 4d was performed in three C57BL/6 mice. After intraperitoneal application of 10 mg/kg, a rapid onset of the compound was observed with a *t*_{max} of 0.25 h (mean) and *C*_{max} of 1250 ± 232 ng/mL (mean ± SD). Remarkably, dose adjusted overall exposure (AUC_{inf}/dose) of 4d in mice was higher if compared to vorinostat (47 vs 12 h·kg·ng/mL/mg). Similar to vorinostat (*t*_{1/2} = 0.75), 4d was characterized by a short elimination half-life of 0.35 h (mean), indicating a substantial metabolism/elimination of 4d in mice. A summary of pharmacokinetic data and a comparison with vorinostat is shown in Table 5.

2.13. 4d Suppresses In Vivo Growth of Myeloid Leukemia (MV4-11 and C1498) Cells. The pronounced cytotoxic effect of 4d on leukemia cells, combined with the comparatively promising in vitro pharmacokinetic character-

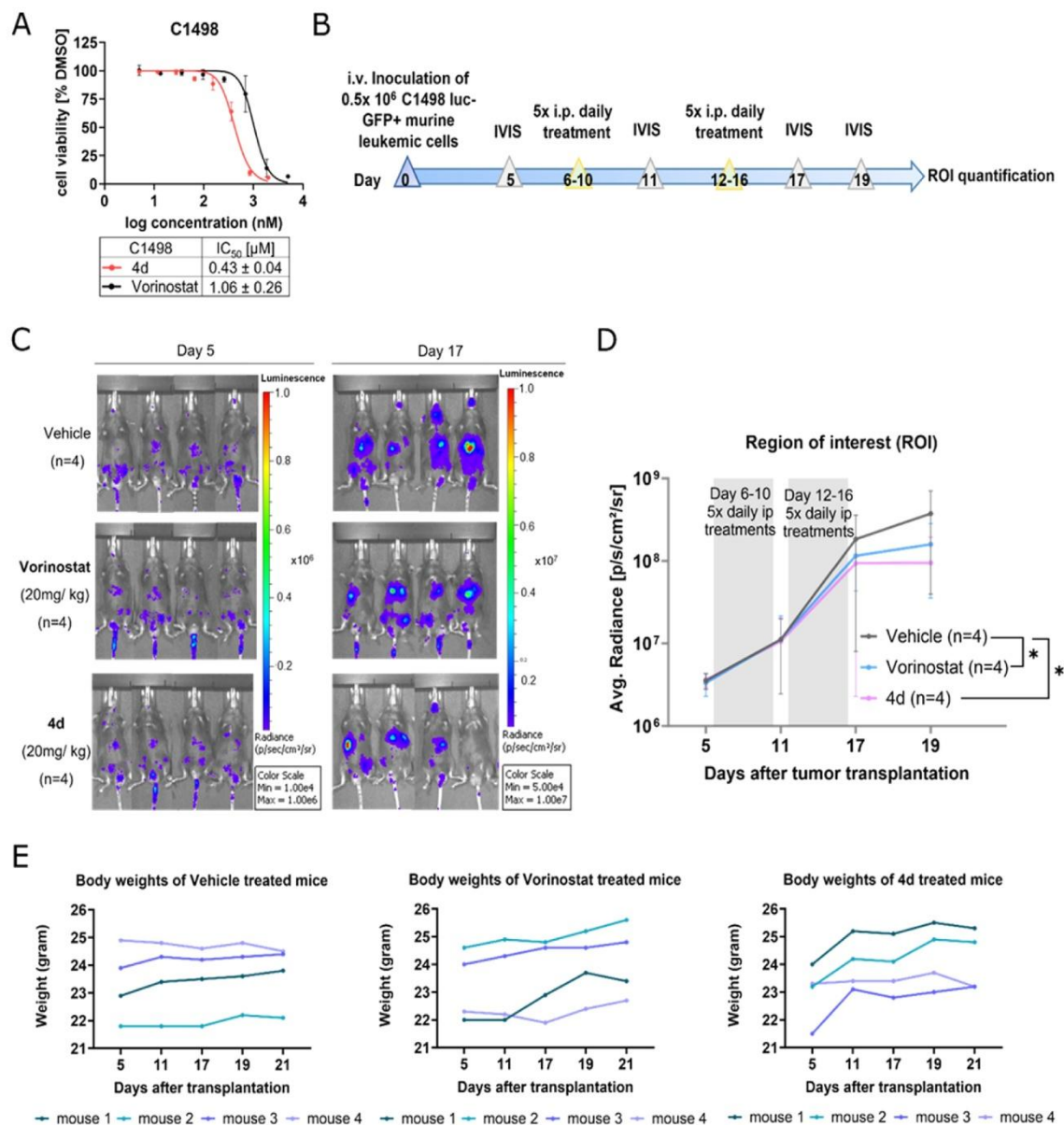


Figure 13. (A) Dose–response curve with IC₅₀ value showing the inhibitory effect of vorinostat and compound **4d** on the proliferation of the C1498 cell line. The curve and IC₅₀ values were generated with GraphPad Prism software by measuring the percentage of viable cells after 72 h incubation of cells with vorinostat and compound **4d** via CellTiter-Glo assay. Cell viability was normalized to DMSO (solvent)-treated controls. This curve and each point represent the results of the mean ± SD of biological and technical triplicates. (B) Schematic timeline of the experimental design. Each mouse received 0.5 × 10⁶ C1498 luc-GFP⁺ murine leukemic cells. After confirmation of tumor engraftment via monitoring the bioluminescence-based in vivo imaging system (IVIS) on day5, mice were grouped into three treatment groups, Vehicle (*n* = 4), Vorinostat (*n* = 4), and **4d** (*n* = 4). All mice received daily treatments accordingly via intraperitoneal injection. Tumor progression was examined by measuring bioluminescent signals with IVIS and ROI (p/s/cm²/sr) quantification. (C) IVIS images of treated mice were taken on days 5 and 17. (D) ROI values at various time points, representing the measurement of leukemia cell growth. Statistical analysis was performed using a two-way mixed ANOVA with Greenhouse-Geisser correction. (E) Changes in body weight of each mouse from the three treatment groups before and after the treatment course.

istics of **4d**, prompted us to evaluate its effectiveness in a preclinical leukemic xenograft mouse (NSG) model using human AML cell line MV4-11. Initially, we determined the IC₅₀ of **4d** in the MV4-11 cell line using the CellTiter-Glo assay, with vorinostat serving as a positive control, as described in prior methods. The dose–response curve and

corresponding IC₅₀ values were generated by applying a nonlinear regression algorithm to plot the log of inhibitor concentrations against normalized cell viability (Supporting Information Figure S3A). **4d** demonstrated an IC₅₀ of 0.036 μM in MV4-11 cells, exhibiting over a 7-fold increase in potency compared to vorinostat. The in vivo experimental

design is illustrated in Supporting Information Figure S3B. Briefly, mice were first intravenously (i.v.) injected with (0.5×10^6) human AML (MV4-11) cells, stably expressing luciferase-GFP for tracking in vivo transplantation. The in vivo growth of the leukemia cells was examined after 14 days of injection via bioluminescence measurement using in vivo imaging system (IVIS). Next, the leukemia bearing mice were distributed randomly to two groups. From day 16 onward, the mice were treated for 8 consecutive days with intraperitoneal injections of either with a vehicle control or **4d** (at a dose of 10 mg/kg). The IVIS images (Supporting Information Figure S3C) and the quantified region of interest (ROI) measurements during the treatment period indicated that the treatment with **4d** significantly ($p = 0.002$) suppresses the in vivo growth of MV4-11 leukemia cells as compared to the vehicle control group (Supporting Information Figure S3D). At day 42, which is 3 weeks after discontinuing the **4d** treatment, no differences in the growth of leukemia cells was observed between **4d** treated and vehicle group. Importantly, for measuring the potential signs of toxicity, such as body weight alterations, the **4d** treatment group exhibited a minor (not significant) reduction during and after the treatment course when compared to the vehicle control group (Supporting Information Figure S3E).

Next, given the growing evidence of immunomodulatory effects of HDAC inhibitors in cancer therapy,⁶⁹ we utilized an allograft leukemia model. In this model, leukemia is established by injecting (C57BL/6) derived murine AML (C1498) cells by intravenous injection in immunocompetent wildtype (C57BL/6) mice.⁷⁰ Similarly, we initially determined the IC_{50} of **4d** in C1498 cells, with vorinostat serving as a positive control. Both **4d** and vorinostat affected C1498 cell growth in a concentration-dependent manner with IC_{50} of 0.425 and 1.06 μM , respectively (Figure 13A). Similar to human leukemia cells, exposure to **4d** or vorinostat increased acetylated-H3 levels in murine C1498 leukemia cells (Supporting Information Figure S4). The in vivo experimental design is illustrated in Figure 13B. Briefly, leukemia was established by injecting (0.5×10^6) murine AML (C1498) cells, stably expressing luciferase-GFP by intravenous injection in C57BL/6 mice.⁷⁰ Confirmation of tumor engraftment was assessed on day 5 after injection of leukemia cells by measuring the luminescence signals in the mice following D-Luciferin intraperitoneal injection (Figure 13C). Starting on day 6, the mice were divided into three treatment groups and administered intraperitoneally with either vehicle, positive control vorinostat (20 mg/kg), or **4d** (20 mg/kg) for 2 weeks. Compared to the vehicle control group, both the vorinostat- ($p = 0.0249$) and **4d** ($p = 0.0326$)-treated groups exhibited significantly lower leukemia burden after the second treatment cycle, with this difference becoming more pronounced by day 19 (Figure 13D). Additionally, no significant differences in body weight were observed during the treatment course (Figure 13E). Additionally, for the target engagement studies, we isolated liver cells from mice 24 h post-treatment with **4d**. Consistent with our in vitro observations, the intracellular staining intensity of acetyl-histone H3 in liver cells was markedly increased in the **4d**-treated mice, pointing toward target engagement inside the organ tissues (Supporting Information Figure S5).

3. CONCLUSIONS

We developed a novel pentyloxyamide-based HDACi with substituted phenylthiazole cap groups and evaluated their potential as novel antileukemic lead structures. The leading candidates from this series, **4d** and **4m**, exhibited nanomolar inhibition toward HDAC2 and HDAC6 and demonstrated cytotoxic effects against a wide range of therapy-sensitive and therapy-resistant leukemia cell lines as well as against patient-derived leukemia cells. In addition, the binding modes of both inhibitors were determined by X-ray crystallography, revealing specific interactions with the CD2 of HDAC6 from *D. rerio* (zebrafish).

Importantly, **4d** and **4m** exhibited comparable or even higher DSS than well-established and clinically used HDAC inhibitors, along with greater selectivity toward malignant cells relative to healthy controls. Considering the current reliance on combination therapies in modern cancer treatment, drugs that demonstrate synergistic interactions can significantly influence the therapeutic outcome, delay or prevent the development of resistance, and increase the treatable population of patients. In this regard, the combination of **4d** and **4m** with the routinely used chemotherapeutic agents clofarabine and decitabine exhibited a notable synergistic cytotoxic effect. The promising in vitro pharmacokinetic characteristics and first in vivo pharmacokinetic data support comprehensive in vivo investigation of **4d**. Notably, in vivo administration of **4d** effectively suppressed the growth of leukemia cells without inducing any observable signs of toxicity in mice. Overall, these findings position **4d** and **4m** as novel lead structures with potential for further preclinical development, as they not only demonstrate a therapeutic window but also promising antileukemic effects when used in combination with established antileukemic drugs. Current challenges, such as the improvable in vivo pharmacokinetics of HDAC inhibitors **4d** and **4m** will be addressed along with further structure–activity relationship (SAR) studies in a follow-up lead optimization program.

4. EXPERIMENTAL SECTION

4.1. Chemistry. Chemicals and solvents were purchased from commercial suppliers (Acros Organics, TCI, abcr, Alfa Aesar, Ambeed, BLDpharm, and Merck) and used without further purification. Dry solvents were purchased from Acros Organics. The reactions were monitored by thin-layer chromatography (TLC) using Macherey-Nagel precoated ALUGRAM Xtra SIL G/UV₂₅₄ plates. Spots were visualized either by irradiation with ultraviolet light (254 nm) or staining in potassium permanganate solution. Hydroxamic acids were further stained using a 1% solution of iron(III) chloride in ethanol. Flash chromatography was performed on a CombiFlash RF 200 or a Büchi Pure C-810 Flash using RediSep Rf-columns using the solvent mixtures of *n*-hexane/ethyl acetate or dichloromethane/methanol according to the separation. Melting points (mp) analyses were performed using a Büchi M-565 melting point apparatus and are uncorrected. Proton (¹H) and carbon (¹³C) NMR spectra were recorded on a Bruker Avance III 300 or 600 MHz using DMSO-*d*₆ as the solvent. Chemical shifts are given in parts per million (ppm) relative to the residual solvent peak for ¹H and ¹³C NMR. Coupling constants, *J*, were reported in Hertz (Hz). High-resolution mass spectra (HRMS) analysis was performed on a UHR-QTOF maXis 4G, Bruker Daltonics, by electrospray ionization (ESI). Analytical HPLC analyses were carried out on an Knauer AZURA system equipped with AZURA 6.1L (pumps), AZURA column thermostat CT 2.1 and AZURA UVD 2.1L (UV-detector), and a Spark Holland B.V. OPTIMAS model 820 autosampler, or on a Agilent LC 1260 Infinity II system equipped

with an G7116A Infinity II Multicolumn Thermostat, a G7104C Infinity II Flexible Pump a G7129C Infinity II Vialsampler and a G7114A Infinity II Variable Wavelength Detector. The separation took place on a Eurospher II 100 S C18 (150 × 4 mm) column. UV absorption was detected at 254 nm. HPLC-grade water +0.1% TFA (solvent A) and HPLC-grade acetonitrile +0.1% TFA (solvent B) were used for elution at a flow rate of 1 mL/min. A linear gradient of 10% B to 100% B within 30 min was used. In the case of the *O*-trityl protected compounds **3a–m** HPLC-grade water (solvent A) and HPLC-grade acetonitrile (solvent B) were used for elution at a flow rate of 1 mL/min. A linear gradient of 10% B to 100% B within 30 min was used. All compounds are >95.0% pure by HPLC.

4.1.1. General Procedure for the Preparation of Phenylthiazoles 1a–1m. The respective 2-bromoketone (1.0 equiv) and ethyl thiooxamate (1.0 equiv) were dissolved in ethanol (7 mL/mmol) and heated at 70 °C. If the TLC (eluent: ethyl acetate/*n*-hexane) showed incomplete conversion after 7 h of heating, the reaction was stirred at room temperature overnight. After evaporation of the solvent, the resulting residue was diluted with ethyl acetate and washed three times with 50 mL of saturated sodium bicarbonate solution and once with 50 mL of brine. The organic phase was dried over anhydrous sodium sulfate and filtrated, and the solvent was evaporated under reduced pressure. The crude product was purified using flash chromatography using ethyl acetate and *n*-hexane as the eluent (0–30% ethyl acetate) to yield phenylthiazoles **1a–m**.

4.1.1.1. 1a Ethyl 4-Phenylthiazole-2-carboxylate. Synthesis according to general procedure A using phenacyl bromide (4.1 g, 20.0 mmol) afforded **1a** as a light yellow solid (3.3 g, 14.1 mmol, 71%). mp 36.9 °C, *t*_r 14.47 min, purity: 99.3%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.36 (t, *J* = 7.1 Hz, 3H), 4.42 (q, *J* = 7.1 Hz, 2H), 7.36–7.44 (m, 1H), 7.45–7.53 (m, 2H), 7.98–8.00 (m, 1H), 8.02 (d, *J* = 1.5 Hz, 1H), 8.53 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 14.1, 62.2, 121.0, 126.2, 128.7, 129.0, 133.2, 156.3, 157.5, 159.4.

HRMS (*m/z*): MH⁺ calcd for C₁₂H₁₁NO₂S 234.0583; found, 234.0584.

4.1.1.2. 1b Ethyl 4-(2-Methoxyphenyl)thiazole-2-carboxylate. Synthesis according to general procedure A using 2-bromo-1-(2-methoxyphenyl)ethanone (2.3 g, 10.0 mmol) afforded **1b** as a white solid (2.0 g, 7.6 mmol, 76%). mp 70.3 °C, *t*_r 15.21 min purity: 99.1%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.36 (t, *J* = 7.1 Hz, 3H), 3.94 (s, 3H), 4.41 (q, *J* = 7.1 Hz, 2H), 7.09 (ddd, *J* = 7.8, 7.3, 1.1 Hz, 1H), 7.18 (dd, *J* = 8.4, 1.1 Hz, 1H), 7.40 (ddd, *J* = 8.3, 7.3, 1.8 Hz, 1H), 8.13 (dd, *J* = 7.7, 1.8 Hz, 1H), 8.45 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 14.1, 55.6, 62.1, 111.9, 120.7, 121.6, 124.2, 129.4, 129.9, 152.3, 155.8, 156.6, 159.5.

HRMS (*m/z*): MH⁺ calcd for C₁₃H₁₃NO₂S 264.0689; found, 264.0696.

4.1.1.3. 1c Ethyl 4-(3-Methoxyphenyl)thiazole-2-carboxylate. Synthesis according to general procedure A using 3-methoxyphenacyl bromide (2.3 g, 10.0 mmol) afforded **1c** as a white solid (1.9 g, 7.4 mmol, 74%). mp 76.6 °C, *t*_r 14.36 min purity: 98.9%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.36 (t, *J* = 7.1 Hz, 3H), 3.83 (s, 3H), 4.42 (q, *J* = 7.1 Hz, 2H), 6.98 (ddd, *J* = 8.2, 2.6, 1.0 Hz, 1H), 7.40 (ddd, *J* = 8.2, 7.7, 0.4 Hz, 1H), 7.51–7.62 (m, 2H), 8.56 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 14.1, 55.2, 62.2, 111.6, 114.4, 118.6, 121.3, 130.1, 134.6, 156.1, 157.3, 159.3, 159.7.

HRMS (*m/z*): MH⁺ calcd for C₁₃H₁₃NO₂S 264.0689; found, 264.0693.

4.1.1.4. 1d Ethyl 4-(4-Methoxyphenyl)thiazole-2-carboxylate. Synthesis according to general procedure A using 4-methoxyphenacyl bromide (2.3 g, 10.0 mmol) afforded **1d** as a white solid (1.9 g, 7.3 mmol, 73%). mp 150.5 °C, *t*_r 14.40 min purity: 97.7%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.36 (t, *J* = 7.1 Hz, 3H), 3.81 (s, 3H), 4.41 (q, *J* = 7.1 Hz, 2H), 6.99–7.09 (m, 2H), 7.90–7.98 (m, 2H), 8.37 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 14.1, 55.2, 62.1, 114.3, 119.0, 126.0, 127.7, 156.3, 157.2, 159.4, 159.7.

HRMS (*m/z*): MH⁺ calcd for C₁₃H₁₃NO₂S 264.0689; found, 264.0693.

4.1.1.5. 1e Ethyl 4-(2-Fluorophenyl)thiazole-2-carboxylate. Synthesis according to general procedure A using 2-bromo-2'-fluoroacetophenone (2.2 g, 10.0 mmol) afforded **1e** as a white solid (1.3 g, 5.2 mmol, 52%). mp 65.7 °C, *t*_r 15.25 min purity: 99.8%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.36 (t, *J* = 7.1 Hz, 3H), 4.42 (q, *J* = 7.1 Hz, 2H), 7.31–7.43 (m, 2H), 7.48 (tdd, *J* = 7.2, 5.3, 2.7 Hz, 1H), 8.11 (td, *J* = 7.8, 1.7 Hz, 1H), 8.37 (d, *J* = 2.4 Hz, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 14.0, 62.3, 116.3 (d, *J* = 22.0 Hz), 121.0 (d, *J* = 11.5 Hz), 124.8–125.2 (m), 129.8 (d, *J* = 2.8 Hz), 130.6 (d, *J* = 8.7 Hz), 149.8 (d, *J* = 2.3 Hz), 157.1, 157.8, 159.3, 161.1.

HRMS (*m/z*): MH⁺ calcd for C₁₂H₁₀FNO₂S 252.0489; found, 252.0490.

4.1.1.6. 1f Ethyl 4-(3-Fluorophenyl)thiazole-2-carboxylate. Synthesis according to general procedure A using 2-bromo-1-(3-fluorophenyl)ethan-1-one (2.2 g, 10.0 mmol) afforded **1f** as a white solid (1.6 g, 6.5 mmol, 65%). mp 66.0 °C, *t*_r 14.81 min purity: 99.7%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.36 (t, *J* = 7.1 Hz, 3H), 4.42 (q, *J* = 7.1 Hz, 2H), 7.24 (dddd, *J* = 9.1, 8.3, 2.6, 1.0 Hz, 1H), 7.41–7.66 (m, 1H), 7.73–7.92 (m, 2H), 8.64 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 14.1, 62.3, 112.8 (d, *J* = 23.2 Hz), 115.5 (d, *J* = 21.2 Hz), 121.1–123.0 (m), 131.1 (d, *J* = 8.4 Hz), 135.5 (d, *J* = 8.3 Hz), 154.8 (d, *J* = 2.9 Hz), 157.7, 159.3, 161.0, 164.2.

HRMS (*m/z*): MH⁺ calcd for C₁₂H₁₀FNO₂S 252.0489; found, 252.0489.

4.1.1.7. 1g Ethyl 4-(4-Fluorophenyl)thiazole-2-carboxylate. Synthesis according to general procedure A using 2-bromo-1-(4-fluorophenyl)ethan-1-one (1.1 g, 5.0 mmol) afforded **1g** as a white solid (0.8 g, 3.3 mmol, 66%). mp 74.0 °C, *t*_r 14.79 min purity: 99.9%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.36 (t, *J* = 7.1 Hz, 3H), 4.41 (q, *J* = 7.1 Hz, 2H), 7.25–7.39 (m, 2H), 7.96–8.13 (m, 2H), 8.51 (s, 1H).

¹³C NMR (126 MHz, DMSO-*d*₆): δ 13.9, 62.1, 115.7 (d, *J* = 21.7 Hz), 120.6, 128.3 (d, *J* = 8.3 Hz), 129.8 (d, *J* = 3.1 Hz), 155.2, 157.5, 159.2, 161.3, 163.2.

HRMS (*m/z*): MH⁺ calcd for C₁₂H₁₀FNO₂S 252.0489; found, 252.0494.

4.1.1.8. 1h Ethyl 4-(Thiophen-2-yl)thiazole-2-carboxylate. Synthesis according to general procedure A using 2-bromo-1-(thiophen-2-yl)ethanone (2.1 g, 10.0 mmol) afforded **1h** as a black solid (1.3 g, 10.0 mmol, 54%). mp 53.4 °C, *t*_r 13.81 min purity: 96.5%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.35 (t, *J* = 7.1 Hz, 3H), 4.41 (q, *J* = 7.1 Hz, 2H), 7.16 (dd, *J* = 5.1, 3.6 Hz, 1H), 7.61 (dd, *J* = 5.1, 1.2 Hz, 1H), 7.68 (dd, *J* = 3.6, 1.2 Hz, 1H), 8.37 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 14.1, 62.3, 119.3, 125.5, 126.9, 128.2, 136.8, 151.1, 157.4, 159.2.

HRMS (*m/z*): MH⁺ calcd for C₁₀H₉NO₂S₂ 240.0147; found, 240.0149.

4.1.1.9. 1i Ethyl 4-(Thiophen-3-yl)thiazole-2-carboxylate. Synthesis according to general procedure A using 3-(bromoacetyl)-thiophene (1.0 g, 4.7 mmol) afforded **1i** as a brown oil (0.7 g, 3.0 mmol, 63%). *T*_b 13.43 min purity: 99.0%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.35 (t, *J* = 7.1 Hz, 3H), 4.41 (q, *J* = 7.1 Hz, 2H), 7.63–7.69 (m, 2H), 8.03 (dd, *J* = 2.6, 1.6 Hz, 1H), 8.36 (s, 1H).

¹³C NMR (151 MHz, DMSO-*d*₆): δ 14.1, 62.2, 120.3, 123.3, 126.2, 127.4, 135.4, 152.6, 157.3, 159.4.

HRMS (*m/z*): MH⁺ calcd for C₁₀H₉NO₂S₂ 240.0147; found, 240.0151.

4.1.1.10. 1j Ethyl 4-(*p*-Tolyl)thiazole-2-carboxylate. Synthesis according to general procedure A using 2-bromo-1-(*p*-tolyl) (2.2 g, 10.0 mmol) afforded **1j** as a white solid (1.7 g, 6.9 mmol, 69%). mp 60.0 °C, *t*_r 15.59 min purity: 99.3%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.36 (t, *J* = 7.1 Hz, 3H), 2.35 (s, 3H), 4.41 (q, *J* = 7.1 Hz, 2H), 7.29 (dd, *J* = 7.9, 0.7 Hz, 2H), 7.84–7.94 (m, 2H), 8.45 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 14.1, 20.8, 62.2, 120.1, 126.2, 129.5, 130.6, 138.2, 156.4, 157.3, 159.4.

HRMS (*m/z*): MH⁺ calcd for C₁₃H₁₃NO₂S 248.0740; found, 248.0744.

4.1.1.11. 1k Ethyl 4-(4-(Trifluoromethyl)phenyl)thiazole-2-carboxylate. Synthesis according to general procedure A using 2-bromo-4'-(trifluoromethyl)acetophenone (2.8 g, 10.0 mmol) afforded 1k as a white solid (2.3 g, 7.7 mmol, 77%). mp 134.3 °C, *t*_r 16.36 min purity: 96.4%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.36 (t, *J* = 7.1 Hz, 3H), 4.43 (q, *J* = 7.1 Hz, 2H), 7.85 (d, *J* = 8.1 Hz, 2H), 8.23 (d, *J* = 8.0 Hz, 2H), 8.74 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 14.1, 62.3, 123.3, 125.9 (d, *J* = 4.1 Hz), 126.9, 128.5, 128.9, 136.9, 154.5, 158.0, 159.3.

HRMS (*m/z*): MH⁺ calcd for C₁₃H₁₀F₃NO₂S 302.0457; found, 302.0465.

4.1.1.12. 1l Ethyl 4-(Benzofuran-2-yl)thiazole-2-carboxylate. Synthesis according to general procedure A using 2-(bromoacetyl)-benzofuran (1.0 g, 4.1 mmol) afforded 1l as a brown solid (0.5 g, 1.7 mmol, 42%). mp 128.6 °C, *t*_r 16.02 min purity: 99.4%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.37 (t, *J* = 7.1 Hz, 3H), 4.43 (q, *J* = 7.1 Hz, 2H), 7.25–7.41 (m, 2H), 7.42 (d, *J* = 0.9 Hz, 1H), 7.69 (ddq, *J* = 17.4, 8.1, 0.8 Hz, 2H), 8.49 (s, 1H).

¹³C NMR (151 MHz, DMSO-*d*₆): δ 14.1, 62.4, 104.2, 111.3, 121.8, 122.5, 123.5, 125.3, 128.2, 147.3, 150.3, 154.3, 158.7, 159.1.

HRMS (*m/z*): MH⁺ calcd for C₁₄H₁₁NO₃S 274.0532; found, 274.0534.

4.1.1.13. 1m Ethyl 4-(4-(Pyrrolidine-1-yl)phenyl)thiazole-2-carboxylate. Synthesis according to general procedure A using 2-bromo-4'-(1-pyrrolidinyl)acetophenone (2.8 g, 10.0 mmol) afforded 1m as a dark yellow solid (0.9 g, 2.9 mmol, 29%). mp 180.5 °C, *t*_r 15.15 min purity: 99.3%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.35 (t, *J* = 7.1 Hz, 3H), 1.88–2.04 (m, 4H), 3.21–3.32 (m, 4H), 4.40 (q, *J* = 7.1 Hz, 2H), 6.54–6.67 (m, 2H), 7.75–7.87 (m, 2H), 8.16 (s, 1H).

¹³C NMR (151 MHz, DMSO-*d*₆): δ 14.1, 25.0, 47.2, 62.0, 111.6, 116.5, 120.5, 127.3, 147.8, 156.7, 157.5, 159.5.

HRMS (*m/z*): MH⁺ calcd for C₁₆H₁₈N₂O₂S 303.1162; found, 303.1167.

4.1.1.14. 6-(Aminoxy)-N-(trityloxy)hexanamide. The synthesis of the linker 2 was performed after an adapted protocol of Avelar et al.⁷¹

4.1.1.15. 6-Bromo-N-(trityloxy)hexanamide. To a stirred solution of 6-bromohexanoic acid (7.4 g, 38 mmol, 1.0 equiv) in 100 mL THF, isobutyl chloroformate (IBCF) (5.8 mL, 45 mmol, 1.2 equiv) and *N*-methylmorpholine (NMM) (5.0 mL, 45 mmol, 1.2 equiv) were added at 10 °C. After stirring for 15 min *O*-tritylhydroxylamine (13 g, 38 mmol, 1.0 equiv) dissolved in 50 mL THF was carefully added, and the reaction was left stirring at room temperature overnight. The precipitate was filtered off, the filtrate was evaporated, and the crude residue was dissolved in ethyl acetate and washed three times with 100 mL of saturated sodium bicarbonate solution and once with 100 mL of brine. The organic phase was dried over anhydrous sodium sulfate, filtrated, and evaporated. The crude product was recrystallized from ethyl acetate/*n* hexanes to yield 1 (14 g, 30 mmol, 80%) as a white solid. mp 128.0 °C, *t*_r 17.58 min purity: 99.6%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.07 (dd, *J* = 15.6, 6.6 Hz, 1H), 1.14–1.28 (m, 1H), 1.64 (p, *J* = 6.9 Hz, 1H), 1.79 (t, *J* = 7.2 Hz, 1H), 3.41 (t, *J* = 6.8 Hz, 2H), 7.33 (s, 8H), 10.18 (s, 1H). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 23.8, 26.9, 31.7, 31.9, 34.9, 91.7, 127.4, 127.5, 128.9, 142.4, 170.1.

HRMS (*m/z*): MNa⁺ calcd for C₂₅H₂₆BrNO₂ 474.1039; found, 474.1041.

4.1.1.16. 6-((1,3-Dioxoisindolin-2-yl)oxy)-N-(trityloxy)-hexanamide. 1 (7.1 g, 16 mmol, 1.0 equiv), *N*-hydroxyphthalimide (NHPI) (2.6 g, 16 mmol, 1.0 equiv), and triethylamine (4.5 mL,

31 mmol, 2.0 equiv) were suspended in 100 mL acetonitrile and refluxed for 12 h. The solvent was removed, the residue dissolved in ethyl acetate and extracted with saturated sodium bicarbonate solution until the aqueous phase did not show any left NHPI. The organic phase was dried over anhydrous sodium sulfate, filtrated, and evaporated. The crude product was recrystallized using ethyl acetate/*n* hexanes to yield 2 (6.2 g, 12 mmol, 74%) as a white solid. mp 142.0 °C, HPLC *t*_r 16.72 min purity: 96.9%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.11 (d, *J* = 16.0 Hz, 2H), 1.18–1.30 (m, 2H), 1.52 (t, *J* = 7.4 Hz, 2H), 1.72–1.85 (m, 2H), 4.03 (t, *J* = 6.7 Hz, 2H), 7.32 (s, 15H), 7.86 (s, 4H), 10.19 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.4, 27.3, 77.5, 91.7, 123.2, 127.4, 127.5, 128.6, 129.0, 134.7, 142.5, 163.3, 170.2.

HRMS (*m/z*): MH⁺ calcd for C₃₃H₃₀N₂O₅ 535.2227; found, 535.2237.

4.1.1.17. Synthesis of 6-(Aminoxy)-N-(trityloxy)hexanamide. 2 (2.1 g, 4.0 mmol, 1.0 equiv) was dissolved in 40 mL dichloromethane; subsequently, hydrazine monohydrate (0.4 mL, 8 mmol, 2.0 equiv) was added, and the reaction was left stirring overnight. The precipitate was filtered off, and the filtrate was washed three times with 50 mL of saturated sodium bicarbonate solution and once with 50 mL of brine. The organic phase was dried over anhydrous sodium sulfate, filtrated, and evaporated to yield the 2 (1.5 g, 3.7 mmol, 94%) as a white wax, which was suitable to use without any further purification. HPLC: *t*_r 13.17 min purity: 96.0%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 0.98 (q, *J* = 8.5 Hz, 2H), 1.18 (t, *J* = 7.6 Hz, 2H), 1.32 (q, *J* = 7.0 Hz, 2H), 1.77 (t, *J* = 7.4 Hz, 2H), 3.40 (t, *J* = 6.7 Hz, 2H), 5.84 (s, 2H), 7.32 (s, 15H), 10.16 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.7, 25.0, 27.7, 31.9, 74.7, 91.7, 127.4, 127.5, 128.9, 142.5, 170.2.

HRMS (*m/z*): MH⁺ calcd for C₂₅H₂₈N₂O₃ 405.2173; found, 405.2172.

4.1.2. General Procedure for the Preparation of the *O*-Trityl-Protected Hydroxamates 3a–m. The synthesis of the *O*-trityl-protected hydroxamates was performed after an adapted protocol of Goodreid et al.³⁹

After solving the corresponding phenylthiazole (1a–m) (1.0 equiv) in tetrahydrofuran (15 mL/mmol), an equimolar amount of 1 M sodium hydroxide solution in water was added. The reaction was monitored via TLC (eluent ethyl acetate/*n*-hexane). After complete consumption of the starting material, the solvent was evaporated under reduced pressure, and the resulting solid was dried in a vacuum. The dried product was suspended in *N,N*-dimethylformamide (2.2 mL/mmol). HBTU (1.1 equiv) and DIPEA (1.1 equiv) were added, and the resulting suspension was left stirring until a clear solution was formed (usually after 1 h). To that solution 2 (1.1 equiv) solved in *N,N*-dimethylformamide (2.2 mL/mmol) was added, and the reaction was left stirring overnight. The solvent was removed under reduced pressure, the resulting product diluted with 50 mL dichloromethane and washed three times with 50 mL of saturated sodium bicarbonate solution and three times with 50 mL of brine. The organic layer was dried over anhydrous sodium sulfate, filtrated, and the solvent was removed under reduced pressure. The obtained crude product was purified via flash chromatography, unless otherwise stated, using *n*-hexane/ethyl acetate (0–100%) as the eluent to yield the *O*-trityl-protected hydroxamates 3a–m.

4.1.2.1. 3a N-((6-Oxo-6-((trityloxy)amino)hexyl)oxy)-4-phenylthiazole-2-carboxamide. Synthesis according to general procedure B using 2a (114 mg, 0.5 mmol) afforded 3a as a white solid (150 mg, 0.25 mmol, 51%). mp 130.0 °C, *t*_r 17.46 min purity: 99.6%.

¹H NMR (600 MHz, DMSO-*d*₆): δ 1.11 (dd, *J* = 14.8, 7.9 Hz, 2H), 1.25 (dq, *J* = 11.3, 5.4 Hz, 2H), 1.45–1.53 (m, 2H), 1.82 (t, *J* = 7.3 Hz, 2H), 3.86 (t, *J* = 6.6 Hz, 2H), 7.26–7.38 (m, 15H), 7.40 (t, *J* = 7.4 Hz, 1H), 7.49 (t, *J* = 7.7 Hz, 2H), 8.07 (d, *J* = 7.3 Hz, 2H), 8.44 (s, 1H), 10.21 (s, 1H), 12.15 (s, 1H).

¹³C NMR (151 MHz, DMSO-*d*₆): δ 25.0, 25.2, 27.8, 32.4, 76.0, 92.2, 119.8, 126.8, 127.9, 128.0, 129.1, 129.3, 129.4, 133.8, 142.9, 155.9, 157.1, 162.0, 170.7.

HRMS (*m/z*): MNa⁺ calcd for C₃₅H₃₃N₃O₄S 614.2084; found, 614.2092.

4.1.2.2. 3b 4-(2-Methoxyphenyl)-N-((6-oxo-6-((trityloxy)amino)hexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure B using **2b** (0.8 g, 3.0 mmol) afforded **3b** as a white solid (1.7 g, 2.2 mmol, 74%). mp 190.6 °C, *t*_r 17.66 min purity: 98.8%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.04–1.17 (m, 2H), 1.18–1.31 (m, 2H), 1.48 (t, *J* = 7.8 Hz, 2H), 1.80 (t, *J* = 7.0 Hz, 2H), 3.84 (t, *J* = 6.7 Hz, 2H), 3.94 (s, 3H), 7.03–7.13 (m, 1H), 7.17 (d, *J* = 8.4 Hz, 1H), 7.33 (s, 16H), 8.29–8.37 (m, 2H), 10.19 (s, 1H), 12.12 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.5, 24.7, 27.3, 31.9, 55.6, 75.5, 91.7, 111.7, 120.4, 121.7, 122.6, 127.4, 127.5, 129.0, 129.7, 129.8, 142.4, 151.4, 156.6, 159.8, 170.2.

HRMS (*m/z*): MH⁺ calcd for C₃₆H₃₅N₃O₅S 622.2370; found, 622.2373.

4.1.2.3. 3c 4-(3-Methoxyphenyl)-N-((6-oxo-6-((trityloxy)amino)hexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure B using **2c** (0.8 g, 3.0 mmol) afforded **3c** as a white solid (1.2 g, 1.9 mmol, 62%). mp 142.8 °C, *t*_r 17.36 min purity: 99.3%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 12.15 (s, 1H), 10.20 (s, 1H), 8.46 (s, 1H), 7.73–7.58 (m, 2H), 7.33 (d, *J* = 4.6 Hz, 16H), 6.96 (dd, *J* = 8.2, 2.6 Hz, 1H), 3.84 (d, *J* = 5.5 Hz, 5H), 1.81 (t, *J* = 6.9 Hz, 2H), 1.59–1.40 (m, 2H), 1.24 (s, 2H), 1.19–1.01 (m, 2H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.5, 24.7, 27.3, 31.9, 55.2, 75.5, 91.7, 111.9, 114.2, 118.6, 119.6, 127.4, 127.5, 129.0, 129.9, 134.7, 142.4, 155.3, 156.5, 159.7, 161.4, 170.2.

HRMS (*m/z*): MH⁺ calcd for C₃₆H₃₅N₃O₅S 622.2370; found, 622.2373.

4.1.2.4. 3d 4-(4-Methoxyphenyl)-N-((6-oxo-6-((trityloxy)amino)hexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure B using **2d** (263 mg, 1.0 mmol) afforded **3d** as a white solid (150 mg, 0.24 mmol, 24%). mp 195.4 °C, *t*_r 17.34 min purity: 98.2%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.11 (q, *J* = 7.7 Hz, 2H), 1.16–1.33 (m, 2H), 1.48 (p, *J* = 7.0 Hz, 2H), 1.81 (t, *J* = 7.3 Hz, 2H), 3.83 (d, *J* = 10.2 Hz, 5H), 6.99–7.08 (m, 2H), 7.33 (d, *J* = 4.7 Hz, 15H), 7.95–8.04 (m, 2H), 8.27 (s, 1H), 10.20 (s, 1H), 12.11 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.5, 24.7, 27.3, 31.9, 55.2, 75.5, 91.7, 114.2, 117.3, 126.2, 127.4, 127.5, 127.7, 129.0, 142.5, 155.4, 156.6, 159.6, 161.3, 170.2.

HRMS (*m/z*): MNa⁺ calcd for C₃₆H₃₅N₃O₅S 644.2189; found, 644.2196.

4.1.2.5. 3e 4-(2-Fluorophenyl)-N-((6-oxo-6-((trityloxy)amino)hexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure B using **2e** (0.8 g, 3.0 mmol) afforded **3e** as a white solid (1.3 g, 2.1 mmol, 70%). mp 159.2 °C, *t*_r 17.87 min purity: 98.6%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.00–1.18 (m, 2H), 1.24 (s, 2H), 1.47 (d, *J* = 8.2 Hz, 2H), 1.80 (t, *J* = 7.1 Hz, 2H), 3.85 (t, *J* = 6.6 Hz, 2H), 7.33 (s, 16H), 7.46 (dt, *J* = 7.9, 5.4 Hz, 1H), 8.21–8.34 (m, 2H), 10.19 (s, 1H), 12.18 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.5, 24.7, 27.3, 31.9, 75.5, 91.7, 116.2 (d, *J* = 22.0 Hz), 121.0 (d, *J* = 11.5 Hz), 123.5 (d, *J* = 13.5 Hz), 124.8 (d, *J* = 3.4 Hz), 127.4, 127.5, 129.0, 130.1, 130.5 (d, *J* = 8.8 Hz), 142.5, 148.9, 156.4, 157.9, 161.2, 170.2.

HRMS (*m/z*): MH⁺ calcd for C₃₅H₃₂FN₃O₄S 610.2170; found, 610.2163.

4.1.2.6. 3f 4-(3-Fluorophenyl)-N-((6-oxo-6-((trityloxy)amino)hexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure B using **2f** (0.8 g, 3.0 mmol) afforded **3f** as a white solid (0.6 g, 1.0 mmol, 34%). mp 142.8 °C, *t*_r 17.85 min purity: 99.1%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.11 (d, *J* = 7.2 Hz, 2H), 1.23 (d, *J* = 7.0 Hz, 2H), 1.48 (t, *J* = 7.3 Hz, 2H), 1.80 (t, *J* = 7.1 Hz, 2H), 3.85 (t, *J* = 6.6 Hz, 2H), 7.17–7.41 (m, 16H), 7.53 (td, *J* = 8.2, 6.2 Hz, 1H), 7.88–7.97 (m, 2H), 8.55 (s, 1H), 10.19 (s, 1H), 12.16 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.7, 27.3, 75.6, 91.8, 113.3, 115.3 (d, *J* = 21.5 Hz), 120.6, 122.3, 127.4, 127.5, 129.0, 130.9 (d, *J* = 9.1 Hz), 135.7 (d, *J* = 8.4 Hz), 142.5, 154.0, 156.4, 161.7.

HRMS (*m/z*): MH⁺ calcd for C₃₅H₃₂FN₃O₄S 610.2170; found, 610.2173.

4.1.2.7. 3g 4-(4-Fluorophenyl)-N-((6-oxo-6-((trityloxy)amino)hexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure B using **2g** (253 mg, 1.0 mmol) afforded **3g** as a white solid (313 mg, 0.5 mmol, 51%). mp 163.0 °C, *t*_r 17.57 min purity: 99.6%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.11 (d, *J* = 7.3 Hz, 2H), 1.24 (s, 2H), 1.40–1.55 (m, 2H), 1.79 (d, *J* = 7.5 Hz, 2H), 3.84 (t, *J* = 6.6 Hz, 2H), 7.24–7.41 (m, 17H), 8.06–8.15 (m, 2H), 8.41 (s, 1H), 10.18 (s, 1H), 12.13 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.5, 24.7, 27.3, 31.9, 75.5, 91.7, 115.7 (d, *J* = 21.7 Hz), 119.1, 127.4, 127.5, 128.4 (d, *J* = 8.3 Hz), 128.9, 130.0 (d, *J* = 2.9 Hz), 142.4, 154.4, 156.5, 160.6, 161.6, 163.9, 170.2.

HRMS (*m/z*): MH⁺ calcd for C₃₅H₃₂FN₃O₄S 610.2170; found, 610.2180.

4.1.2.8. 3h N-((6-Oxo-6-((trityloxy)amino)hexyl)oxy)-4-(thiophen-2-yl)thiazole-2-carboxamide. Synthesis according to general procedure B using **2h** (0.7 g, 3.0 mmol) afforded **3h** as a white solid (1.6 g, 2.6 mmol, 88%). mp 174.6 °C, *t*_r 17.23 min purity: 98.1%.

¹H NMR (600 MHz, DMSO-*d*₆): δ 1.09 (p, *J* = 7.9 Hz, 2H), 1.24 (p, *J* = 7.5 Hz, 2H), 1.47 (p, *J* = 7.0 Hz, 2H), 1.80 (q, *J* = 7.9 Hz, 2H), 3.84 (t, *J* = 6.6 Hz, 2H), 7.15 (dd, *J* = 5.0, 3.6 Hz, 1H), 7.25–7.38 (m, 15H), 7.58–7.62 (m, 1H), 7.64–7.68 (m, 1H), 8.25 (s, 1H), 10.19 (s, 1H), 12.09 (s, 1H).

¹³C NMR (151 MHz, DMSO-*d*₆): δ 24.5, 24.7, 27.3, 31.9, 75.4, 91.7, 118.0, 125.4, 126.8, 127.4, 127.5, 128.1, 129.0, 136.8, 142.5, 150.3, 156.4, 161.8, 170.2.

HRMS (*m/z*): MH⁺ calcd for C₃₃H₃₁N₃O₄S₂ 598.1829; found 598.1832.

4.1.2.9. 3i N-((6-Oxo-6-((trityloxy)amino)hexyl)oxy)-4-(thiophen-3-yl)thiazole-2-carboxamide. Synthesis according to general procedure B using **2i** (665 mg, 2.8 mmol) afforded **3i** after recrystallization in *n*-hexane/ethyl acetate as a white solid (1.5 g, 2.6 mmol, 92%). mp 214.7 °C, *t*_r 16.91 min purity: 96.9%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.10 (d, *J* = 7.2 Hz, 2H), 1.23 (d, *J* = 6.8 Hz, 2H), 1.46 (d, *J* = 7.2 Hz, 2H), 1.79 (d, *J* = 7.5 Hz, 2H), 3.84 (t, *J* = 6.6 Hz, 2H), 7.33 (d, *J* = 4.3 Hz, 15H), 7.62–7.73 (m, 2H), 8.01 (dd, *J* = 2.8, 1.5 Hz, 1H), 8.25 (s, 1H), 10.19 (s, 1H), 12.09 (s, 1H).

¹³C NMR (126 MHz, DMSO-*d*₆): δ 24.9, 25.1, 27.7, 32.3, 75.9, 92.2, 119.0, 123.3, 126.7, 127.6, 127.8, 127.9, 129.3, 136.1, 142.9, 152.1, 157.0, 161.8, 170.6.

HRMS (*m/z*): MH⁺ calcd for C₃₃H₃₁N₃O₄S₂ 598.1829; found, 598.1836.

4.1.2.10. 3j N-((6-Oxo-6-((trityloxy)amino)hexyl)oxy)-4-(*p*-tolyl)thiazole-2-carboxamide. Synthesis according to general procedure B using **2j** (742 mg, 3.0 mmol) afforded **3j** as a white solid (313 mg, 0.5 mmol, 17%). mp 163.2 °C, *t*_r 17.93 min purity: 95.1%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.10 (d, *J* = 6.4 Hz, 2H), 1.23 (s, 2H), 1.46 (d, *J* = 7.7 Hz, 3H), 1.79 (d, *J* = 7.6 Hz, 2H), 2.35 (s, 3H), 3.84 (t, *J* = 6.6 Hz, 2H), 7.24–7.41 (m, 17H), 7.95 (d, *J* = 8.1 Hz, 2H), 8.36 (s, 1H), 10.19 (s, 1H), 12.11 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 20.9, 24.5, 24.7, 27.3, 31.9, 75.5, 91.7, 118.5, 126.2, 127.4, 127.5, 129.0, 129.4, 130.7, 138.0, 142.5, 156.6, 161.4, 170.2.

HRMS (*m/z*): MNa⁺ calcd for C₃₆H₃₅N₃O₄S 628.2240; found, 628.2244.

4.1.2.11. 3k N-((6-Oxo-6-((trityloxy)amino)hexyl)oxy)-4-(4-(trifluoromethyl)phenyl)thiazole-2-carboxamide. Synthesis according to general procedure B using **2k** (904 mg, 3.0 mmol) afforded **3k** as a white solid (499 mg, 0.8 mmol, 25%). mp 182.5 °C, *t*_r 19.44 min purity: 99.8%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.11 (q, *J* = 7.7 Hz, 2H), 1.25 (t, *J* = 7.3 Hz, 2H), 1.48 (t, *J* = 7.4 Hz, 2H), 1.81 (t, *J* = 7.0 Hz, 2H), 3.85 (t, *J* = 6.6 Hz, 2H), 7.33 (s, 15H), 7.86 (d, *J* = 8.2

Hz, 2H), 8.29 (d, $J = 8.1$ Hz, 2H), 8.66 (s, 1H), 10.19 (s, 1H), 12.22 (s, 1H).

^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 24.4, 24.6, 27.2, 31.7, 75.4, 91.6, 121.6, 125.7, 126.8, 127.3, 127.4, 128.8, 137.0, 142.3, 153.6, 156.3, 162.0, 170.1.

HRMS (m/z): MNa^+ calcd for $\text{C}_{36}\text{H}_{32}\text{F}_3\text{N}_3\text{O}_4\text{S}$ 682.1958; found, 682.1966.

4.1.2.12. 3l *N*-((6-Benzofuran-2-yl)-*N*-((6-oxo-6-((trityloxy)amino)hexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure B using **2l** (547 mg, 2.0 mmol) afforded **3l** after recrystallization in dichloromethane/ethyl acetate as a light brown solid (408 mg, 0.7 mmol, 32%). mp 200.7 °C, t_r 18.46 min purity: 97.9%.

^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 1.03–1.19 (m, 2H), 1.14–1.34 (m, 3H), 1.42–1.56 (m, 2H), 1.74–1.87 (m, 2H), 3.85 (t, $J = 6.6$ Hz, 2H), 7.20–7.43 (m, 18H), 7.63–7.69 (m, 1H), 7.74 (dd, $J = 7.3$, 1.4 Hz, 1H), 8.42 (s, 1H), 10.19 (s, 1H), 12.24 (s, 1H).

^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 24.5, 24.7, 27.3, 31.9, 75.5, 91.7, 104.0, 111.2, 121.2, 121.8, 123.5, 125.3, 127.4, 127.5, 128.1, 129.0, 142.5, 146.5, 150.5, 154.3, 156.3, 163.1, 170.2.

HRMS (m/z): MH^+ calcd for $\text{C}_{37}\text{H}_{33}\text{N}_3\text{O}_5\text{S}$ 532.2214; found, 632.2211.

4.1.2.13. 3m *N*-((6-Oxo-6-((trityloxy)amino)hexyl)oxy)-4-(4-(pyrrolidin-1-yl)phenyl)thiazole-2-carboxamide. Synthesis according to general procedure B using **2m** (907 mg, 3.0 mmol) afforded **3m**, after flash chromatography with dichloromethane/30% methanol in dichloromethane as a eluent, as a yellow solid (487 mg, 0.7 mmol, 25%). mp 192.1 °C, t_r 20.03 min purity: 95.3%.

^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 1.05–1.17 (m, 2H), 1.18–1.30 (m, 2H), 1.43–1.53 (m, 2H), 1.79 (d, $J = 7.2$ Hz, 2H), 1.92–2.02 (m, 4H), 3.27 (q, $J = 5.1$ Hz, 4H), 3.84 (t, $J = 6.6$ Hz, 2H), 6.56–6.64 (m, 2H), 7.31 (q, $J = 5.1$ Hz, 15H), 7.81–7.91 (m, 2H), 8.05 (s, 1H), 10.20 (s, 1H), 12.05 (s, 1H).

^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 24.5, 24.7, 25.0, 26.3, 27.3, 31.9, 47.3, 75.5, 91.7, 111.5, 114.7, 120.7, 127.4, 127.5, 129.0, 142.5, 147.8, 156.6, 156.8, 170.2.

HRMS (m/z): MH^+ calcd for $\text{C}_{39}\text{H}_{40}\text{N}_4\text{O}_5\text{S}$ 661.2843; found, 661.2838.

4.1.2.14. General Procedure for the Preparation of the Hydroxamates 4a–4m. *O*-trityl-protected hydroxamate (**4a–m**) was dissolved in DCM (30 mL/mmol). Et_3SiH (10 equiv) and TFA (10 equiv) were added successively, and the reaction was left stirring for 30 min at room temperature. After that time, the reaction mixture was purified via flash chromatography using dichloromethane and 30% methanol in dichloromethane as eluents to yield hydroxamic acids **4a–m**.

4.1.2.15. 4a *N*-((6-(Hydroxyamino)-6-oxohexyl)oxy)-4-phenylthiazole-2-carboxamide. Synthesis according to general procedure C using **3a** (296 mg, 0.5 mmol) afforded **4a** as a colorless wax (240 mg, 0.41 mmol, 81%). t_r 9.32 min purity: 95.1%.

^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ 1.38 (qd, $J = 9.0$, 6.2 Hz, 2H), 1.55 (p, $J = 7.5$ Hz, 2H), 1.62 (p, $J = 6.8$ Hz, 2H), 1.98 (t, $J = 7.4$ Hz, 2H), 3.94 (t, $J = 6.5$ Hz, 2H), 7.40 (t, $J = 7.3$ Hz, 1H), 7.49 (t, $J = 7.7$ Hz, 2H), 8.07 (d, $J = 7.4$ Hz, 2H), 8.43 (s, 1H), 8.68 (s, 1H), 10.37 (s, 1H), 12.18 (s, 1H).

^{13}C NMR (151 MHz, $\text{DMSO}-d_6$): δ 25.3, 25.5, 27.8, 32.7, 76.1, 119.8, 126.8, 129.1, 129.3, 133.8, 155.9, 157.1, 162.0, 169.5.

HRMS (m/z): MH^+ calcd for $\text{C}_{16}\text{H}_{19}\text{N}_3\text{O}_4\text{S}$ 349.1096; found, 350.1170.

4.1.2.16. 4b *N*-((6-(Hydroxyamino)-6-oxohexyl)oxy)-4-(2-methoxyphenyl)thiazole-2-carboxamide. Synthesis according to general procedure C using **3b** (622 mg, 1.0 mmol) afforded **4b** as a white solid (284 mg, 0.75 mmol, 75%). mp 137.4 °C, t_r 9.84 min, purity: 99.6%.

^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 1.26–1.47 (m, 2H), 1.58 (dp, $J = 22.2$, 7.0 Hz, 4H), 1.97 (t, $J = 7.3$ Hz, 2H), 3.93 (d, $J = 4.0$ Hz, 5H), 7.08 (td, $J = 7.5$, 1.1 Hz, 1H), 7.17 (dd, $J = 8.4$, 1.1 Hz, 1H), 7.39 (ddd, $J = 8.4$, 7.3, 1.8 Hz, 1H), 8.31 (dd, $J = 7.8$, 1.8 Hz, 1H), 8.35 (s, 1H), 8.67 (s, 1H), 10.35 (s, 1H), 12.14 (s, 1H).

^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 24.9, 25.0, 27.4, 32.2, 55.6, 75.6, 111.8, 120.5, 121.7, 122.6, 129.8, 129.8, 151.4, 156.6, 156.7, 159.8, 169.0.

HRMS (m/z): MH^+ calcd for $\text{C}_{17}\text{H}_{21}\text{N}_3\text{O}_5\text{S}$ 380.1275; found, 380.1272.

4.1.2.17. 4c *N*-((6-(Hydroxyamino)-6-oxohexyl)oxy)-4-(3-methoxyphenyl)thiazole-2-carboxamide. Synthesis according to general procedure C using **3c** (622 mg, 1.0 mmol) afforded **4c** as a yellow wax (253 mg, 0.67 mmol, 67%). t_r 9.53 min purity 99.9%.

^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 1.38 (ddd, $J = 12.4$, 6.0, 3.2 Hz, 2H), 1.58 (dp, $J = 22.5$, 7.0 Hz, 4H), 1.97 (t, $J = 7.3$ Hz, 2H), 3.84 (s, 3H), 3.93 (t, $J = 6.4$ Hz, 2H), 6.96 (ddd, $J = 8.3$, 2.6, 1.0 Hz, 1H), 7.39 (t, $J = 7.9$ Hz, 1H), 7.57–7.72 (m, 2H), 8.46 (s, 1H), 8.67 (d, $J = 1.8$ Hz, 1H), 10.35 (s, 1H), 12.17 (s, 1H).

^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 24.9, 25.0, 27.4, 32.2, 55.3, 75.6, 111.9, 114.2, 118.6, 119.6, 129.9, 134.7, 155.3, 156.5, 159.7, 161.3, 169.0.

HRMS (m/z): MH^+ calcd for $\text{C}_{17}\text{H}_{21}\text{N}_3\text{O}_5\text{S}$ 380.1275; found, 380.1276.

4.1.2.18. 4d *N*-((6-(Hydroxyamino)-6-oxohexyl)oxy)-4-(4-methoxyphenyl)thiazole-2-carboxamide. Synthesis according to general procedure C using **3d** (1.8 g, 3.0 mmol) afforded **4d** as a white solid (603 mg, 1.59 mmol, 53%). mp 151.1 °C, t_r 9.46 min purity 98.1%.

^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 1.38 (q, $J = 8.1$ Hz, 2H), 1.58 (dp, $J = 22.1$, 7.0 Hz, 4H), 1.97 (t, $J = 7.3$ Hz, 2H), 3.81 (s, 3H), 3.92 (t, $J = 6.4$ Hz, 2H), 6.92–7.18 (m, 2H), 7.85–8.13 (m, 2H), 8.27 (s, 1H), 8.67 (d, $J = 1.8$ Hz, 1H), 10.35 (d, $J = 1.9$ Hz, 1H), 12.13 (s, 1H).

^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 24.9, 25.0, 27.4, 32.2, 55.2, 75.6, 114.2, 117.3, 126.2, 127.7, 155.4, 156.6, 159.6, 161.3, 169.0.

HRMS (m/z): MH^+ calcd for $\text{C}_{17}\text{H}_{21}\text{N}_3\text{O}_5\text{S}$ 380.1275; found, 380.1277.

4.1.2.19. 4e 4-(2-Fluorophenyl)-*N*-((6-(hydroxyamino)-6-oxohexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure C using **3e** (610 mg, 1.0 mmol) afforded **4e** as an orange wax (221 mg, 0.6 mmol, 60%). t_r 9.93 min purity 99.2%.

^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 1.28–1.46 (m, 2H), 1.58 (dp, $J = 22.5$, 7.0 Hz, 4H), 1.97 (t, $J = 7.3$ Hz, 2H), 3.93 (t, $J = 6.4$ Hz, 2H), 7.28–7.41 (m, 2H), 7.41–7.52 (m, 1H), 8.21–8.36 (m, 2H), 8.67 (d, $J = 1.8$ Hz, 1H), 10.35 (d, $J = 1.8$ Hz, 1H), 12.21 (s, 1H).

^{13}C NMR (126 MHz, $\text{DMSO}-d_6$): δ 24.7, 24.9, 27.3, 32.1, 75.5, 116.1 (d, $J = 22.0$ Hz), 121.0 (d, $J = 11.5$ Hz), 123.2 (d, $J = 13.4$ Hz), 124.7 (d, $J = 3.4$ Hz), 130.0 (d, $J = 2.8$ Hz), 130.3 (d, $J = 8.7$ Hz), 148.9, 156.4, 158.4, 160.4, 161.0, 168.9.

HRMS (m/z): MH^+ calcd for $\text{C}_{16}\text{H}_{18}\text{FN}_3\text{O}_4\text{S}$ 368.1075; found, 368.1067.

4.1.2.20. 4f 4-(3-Fluorophenyl)-*N*-((6-(hydroxyamino)-6-oxohexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure C using **3f** (505 mg, 0.8 mmol) afforded **4f** as a colorless wax (258 mg, 0.7 mmol, 85%). t_r 9.89 min purity 95.9%.

^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 1.30–1.48 (m, 2H), 1.46–1.70 (m, 4H), 1.97 (t, $J = 7.3$ Hz, 2H), 3.93 (t, $J = 6.5$ Hz, 2H), 7.23 (td, $J = 8.6$, 2.5 Hz, 1H), 7.53 (td, $J = 8.1$, 6.1 Hz, 1H), 7.87–7.99 (m, 2H), 8.54 (s, 1H), 8.67 (s, 1H), 10.36 (s, 1H), 12.18 (s, 1H).

^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 24.9, 25.0, 27.4, 32.2, 75.6, 113.1 (d, $J = 23.6$ Hz), 115.3 (d, $J = 20.7$ Hz), 120.5, 122.3, 130.9 (d, $J = 8.5$ Hz), 135.8, 154.0, 156.4, 161.0, 161.7, 164.3, 169.0.

HRMS (m/z): MH^+ calcd for $\text{C}_{16}\text{H}_{18}\text{FN}_3\text{O}_4\text{S}$ 368.1075; found, 368.1081.

4.1.2.21. 4g 4-(4-Fluorophenyl)-*N*-((6-(hydroxyamino)-6-oxohexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure C using **3g** (260 mg, 0.4 mmol) afforded **4g** as a white solid (99 mg, 0.3 mmol, 63%). mp 170.7 °C, t_r 9.65 min purity 98.5%.

^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 1.38 (q, $J = 7.8$ Hz, 2H), 1.45–1.67 (m, 4H), 1.97 (t, $J = 7.2$ Hz, 2H), 3.92 (t, $J = 6.4$ Hz, 2H), 7.26–7.39 (m, 2H), 8.03–8.16 (m, 2H), 8.41 (s, 1H), 8.68 (s, 1H), 10.36 (s, 1H), 12.17 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.9, 25.0, 27.4, 32.2, 75.6, 115.7 (d, *J* = 21.6 Hz), 119.1, 128.5 (d, *J* = 8.3 Hz), 130.0 (d, *J* = 3.0 Hz), 154.4, 156.5, 160.6, 161.6, 163.9, 169.0.

HRMS (*m/z*): MH⁺ calcd for C₁₆H₁₈FN₃O₄S 368.1075; found, 368.1074.

4.1.2.22. 4h *N*-((6-(Hydroxyamino)-6-oxohexyl)oxy)-4-(thiophen-2-yl)thiazole-2-carboxamide. Synthesis according to general procedure C using **3h** (598 mg, 1.0 mmol) afforded **4h** as a white solid (226 mg, 0.6 mmol, 63%). mp 123.4 °C, *t*_r 8.87 min purity 98.8%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.28–1.47 (m, 2H), 1.44–1.75 (m, 4H), 1.97 (t, *J* = 7.3 Hz, 2H), 3.92 (t, *J* = 6.4 Hz, 2H), 7.15 (dd, *J* = 5.1, 3.6 Hz, 1H), 7.54–7.71 (m, 2H), 8.25 (s, 1H), 8.67 (s, 1H), 10.35 (s, 1H), 12.12 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.9, 25.0, 27.4, 32.2, 75.5, 118.0, 125.4, 126.8, 128.1, 136.8, 150.3, 156.4, 161.8, 169.0.

HRMS (*m/z*): MH⁺ calcd for C₁₄H₁₇N₃O₄S₂ 356.0733; found, 356.0735.

4.1.2.23. 4i *N*-((6-(Hydroxyamino)-6-oxohexyl)oxy)-4-(thiophen-3-yl)thiazole-2-carboxamide. Synthesis according to general procedure C using **3i** (598 mg, 1.0 mmol) afforded **4i** as a white solid (198 mg, 0.6 mmol, 56%). mp 140.4 °C, *t*_r 8.79 min purity 98.6%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.38 (tt, *J* = 11.3, 6.3 Hz, 2H), 1.58 (dp, *J* = 22.1, 7.0 Hz, 4H), 1.97 (t, *J* = 7.3 Hz, 2H), 3.92 (t, *J* = 6.4 Hz, 2H), 7.42–7.81 (m, 2H), 8.01 (dd, *J* = 2.7, 1.6 Hz, 1H), 8.25 (s, 1H), 8.67 (s, 1H), 10.35 (s, 1H), 12.12 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.8, 25.0, 27.3, 32.1, 75.5, 118.7, 122.9, 126.3, 127.3, 135.7, 151.7, 156.5, 161.4, 169.0.

HRMS (*m/z*): MH⁺ calcd for C₁₄H₁₇N₃O₄S₂ 356.0733; found, 356.0734.

4.1.2.24. 4j *N*-((6-(Hydroxyamino)-6-oxohexyl)oxy)-4-(*p*-tolyl)thiazole-2-carboxamide. Synthesis according to general procedure C using **3j** (606 mg, 1.0 mmol) afforded **4j** as a white solid (212 mg, 0.6 mmol, 58%). mp 134.3 °C, *t*_r 10.20 min purity 97.8%.

¹H NMR (600 MHz, DMSO-*d*₆): δ 1.37 (tt, *J* = 9.5, 6.2 Hz, 2H), 1.54 (p, *J* = 7.5 Hz, 2H), 1.61 (p, *J* = 6.7 Hz, 2H), 1.97 (t, *J* = 7.4 Hz, 2H), 2.35 (s, 3H), 3.92 (t, *J* = 6.5 Hz, 2H), 7.22–7.34 (m, 2H), 7.90–8.00 (m, 2H), 8.35 (s, 1H), 8.67 (s, 1H), 10.35 (s, 1H), 12.14 (s, 1H).

¹³C NMR (151 MHz, DMSO-*d*₆): δ 20.9, 25.0, 25.0 (d, *J* = 9.3 Hz), 27.4, 32.2, 75.6, 118.5, 126.2, 129.4, 130.7, 138.0, 155.6, 156.6, 161.4, 169.0.

HRMS (*m/z*): MH⁺ calcd for C₁₇H₂₁N₃O₄S 364.1326; found, 364.1330.

4.1.2.25. 4k *N*-((6-(Hydroxyamino)-6-oxohexyl)oxy)-4-(4-(trifluoromethyl)phenyl)thiazole-2-carboxamide. Synthesis according to general procedure C using **3k** (382 mg, 0.6 mmol) afforded **4k** as a light brown solid (134 mg, 0.3 mmol, 56%). mp 127.2 °C, *t*_r 11.278 min purity 98.5%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.37 (qd, *J* = 8.7, 5.5 Hz, 2H), 1.48–1.68 (m, 4H), 1.97 (t, *J* = 7.3 Hz, 2H), 3.93 (t, *J* = 6.4 Hz, 2H), 7.86 (d, *J* = 8.2 Hz, 2H), 8.23–8.33 (m, 2H), 8.66 (s, 2H), 10.36 (s, 1H), 12.25 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.9, 25.0, 27.4, 32.2, 75.6, 121.7, 122.4, 125.8 (d, *J* = 3.2 Hz), 126.0, 126.9, 127.9–129.4 (m), 137.1, 153.7, 156.4, 162.1, 169.0.

HRMS (*m/z*): MH⁺ calcd for C₁₇H₁₈F₃N₃O₄S 418.1043; found, 418.1041.

4.1.2.26. 4l 4-(Benzofuran-2-yl)-*N*-((6-(hydroxyamino)-6-oxohexyl)oxy)thiazole-2-carboxamide. Synthesis according to general procedure C using **3l** (316 mg, 0.5 mmol) afforded **4l** as a off-white solid (112 mg, 0.3 mmol, 58%). Yield 58%, mp 150.4 °C, *t*_r 10.70 min purity 99.3%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.18–1.45 (m, 2H), 1.58 (dp, *J* = 22.2, 7.0 Hz, 4H), 1.97 (t, *J* = 7.3 Hz, 2H), 3.93 (t, *J* = 6.4 Hz, 2H), 7.25–7.43 (m, 3H), 7.66 (dq, *J* = 8.2, 0.9 Hz, 1H), 7.70–7.78 (m, 1H), 8.42 (s, 1H), 8.67 (d, *J* = 1.8 Hz, 1H), 10.35 (d, *J* = 1.8 Hz, 1H), 12.27 (s, 1H).

¹³C NMR (75 MHz, DMSO-*d*₆): δ 24.9, 25.0, 27.4, 32.2, 75.6, 104.1, 111.2, 121.3, 121.8, 123.5, 125.3, 128.1, 146.5, 150.5, 154.3, 156.3, 163.1, 169.0.

HRMS (*m/z*): MH⁺ calcd for C₁₈H₁₉N₃O₅S 390.1118; found, 390.1117.

4.1.2.27. 4m *N*-((6-(Hydroxyamino)-6-oxohexyl)oxy)-4-(4-(pyrrolidin-1-yl)phenyl)thiazole-2-carboxamide. Synthesis according to general procedure C using **3m** (500 mg, 0.8 mmol) afforded as **4m** a yellow solid (93 mg, 0.2 mmol, 29%). mp 136.2 °C, *t*_r 9.29 min purity 98.9%.

¹H NMR (300 MHz, DMSO-*d*₆): δ 1.28–1.47 (m, 2H), 1.58 (dp, *J* = 21.9, 6.9 Hz, 4H), 1.86–2.07 (m, 6H), 3.23–3.34 (m, 4H), 3.92 (t, *J* = 6.4 Hz, 2H), 6.48–6.81 (m, 2H), 7.75–8.01 (m, 2H), 8.05 (s, 1H), 8.67 (s, 1H), 10.35 (s, 1H), 12.07 (s, 1H).

¹³C NMR (151 MHz, DMSO-*d*₆): δ 24.9, 25.0, 25.0, 27.4, 32.2, 47.3, 48.6, 75.5, 111.5, 114.7, 120.7, 127.4, 147.8, 156.6, 156.8, 160.8, 169.0.

HRMS (*m/z*): MH⁺ calcd for C₂₀H₂₆N₄O₄S 419.1748; found, 419.1746.

4.2. Biological Evaluation. 4.2.1. Enzyme Assay.

HDAC1, 2, 3, 4, 6, and 8 human recombinant enzymes were purchased from Reaction Biology Corp. (Malvern, PA). HDAC 11 was purchased from Sigma-Aldrich (Saint Louis, MO). The HDAC activity assay of HDAC1 (catalog no. KDA-21-365), 2 (catalog no. KDA-21-277), 3 (catalog no. KDA-22-278), 4 (catalog no. KDA-21-279), 6 (catalog no. KDA-21-213), 8 (catalog no. KDA-21-481), and 11 (catalog no. SRP0113) was performed in 96-well plates (Corning Incorporated, New York City, NY). Briefly, 20 ng of HDAC1, 2, 3, and 8, 2 ng of HDAC4, 17.5 ng of HDAC6, and 30 ng of HDAC11 per reaction were used. Recombinant enzymes were diluted in assay buffer (50 mM Tris–HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, and 1 mg/mL BSA). 80 μL of this dilution was incubated with 10 μL of different concentrations of inhibitors in assay buffer. After a 5 min incubation step, the reaction was started with 10 μL of 400 μM (HDAC1), 300 μM (HDAC2), 500 μM (HDAC3), 150 μM (HDAC6) Boc-Lys(Ac)-AMC (Bachem, Bubendorf, Switzerland) or 100 μM (HDAC4), 60 μM (HDAC8), 500 μM (HDAC11) Boc-Lys(TFA)-AMC (Bachem, Bubendorf, Switzerland). The reaction was stopped after 90 min by adding 100 μL stop solution 16 mg/mL trypsin, 8 μM vorinostat for HDAC1, 2, and 3, 8 μM panobinostat for HDAC4, 8, and 11, and 4 μM tubastatin for HDAC6 in 50 mM Tris–HCl, pH 8.0, and 100 mM NaCl. Fifteen min after the addition of the stop solution, the fluorescence intensity was measured at an excitation of 355 nm and emission of 460 nm in a NOVOstar microplate reader (BMG Lab-Tech, Ortenburg, Germany).

4.2.2. Cell Culture. All leukemic cell lines were cultured at 37 °C with 5% CO₂ in RPMI 1640 GlutaMax medium supplemented with 10–20% fetal bovine serum (FBS), depending on the recommendation of the German collection of microorganisms and cell culture (DSMZ). PDX samples were generated by (intravenously) injecting leukemia cells, isolated from patients into immune-deficient NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ or NSG mice aged 8–12 weeks.^{45,72} The transplanted leukemia cells were isolated from the spleen or the bone marrow of the mice. If the proportion of human cells was below 90%, a mouse cell depletion kit (Miltenyi Biotec) was used to enrich the human cells. Subsequently, leukemia cells (≥90% human) derived from the bone marrow and spleen of the mice were utilized to conduct a short-term ex vivo drug sensitivity assay. PDX cells were short-term cultured in RPMI 1640 GlutaMax with 15% FBS, 0.1 mM 2-Mercaptoethanol, 1 mM Sodium Pyruvate, and Gentamicin 0.5 μg/mL. All animal experiments were conducted in accordance with the regulatory guidelines of the official committee at LANUV (Akt. 81-02.04.2017.A441), under the authorization of the animal research institute (ZETT) at the Heinrich Heine University Düsseldorf. Patient samples were received after obtaining informed consent in accordance with the Declaration of Helsinki. The experiments were approved by the ethics committee of the medical faculty of the Heinrich Heine University (Study no.: 2019-566).

4.2.3. High Throughput Drug Screening. All compounds were used in 10 mM stock solutions and dissolved in DMSO.⁴⁵ Drug screening plates were preprinted with the Tecan D300e in 384 or 1536 well plates. To avoid plate effects, all plates were randomized, and all wells were further on normalized to the highest DMSO volume on the plate. Plates were stored at -80°C and thawed 1 h prior to the experiment. Cells concentration and viability was determined via the Vi-CELL BLU cell counter. Only if cells exceeded 90% viability they were deemed acceptable for seeding. Cell lines were seeded at a concentration of 0.04×10^6 cells/mL in 384 well plates or at 0.5×10^6 cells/mL in 1536 well plates, while PDX samples were seeded in 1536 well plates at 1.5×10^6 cells/mL via the Multidrop reagent dispenser. The seeded plates were incubated for 72 h and subsequently evaluated via CellTiter-Glo. A Tecan Spark microplate reader was used to measure the emerging luminescence.

Initial IC_{50} determination of all experimental compounds was done in 11 concentrations (from 0.005 to 25 μM) three times with the three cell lines, K562 (CML), HL60 (AML), and HPBALL (T-ALL). The viability was determined in relation to the DMSO controls, and IC_{50} was calculated via Prism (log(inhibitor) vs normalized response – Variable slope). Screening with commercially available inhibitors was done in 6 concentration from 0.005–25 μM . Viability and inhibition curves were determined in the same way. DSS was calculated via the R package DSS. The dDSS was calculated by subtracting the DSS value of five healthy controls from the leukemia value. Significance was calculated by comparing all dDSS values of two drugs in a one-way Anova test. Heatmaps were generated via the complex heatmap package.

4.2.4. Synergy Drug Screening. Drug synergy was investigated by a matrix screening approach. Plates were designed by combining each concentration of drug A with each concentration of drug B. All drugs were printed in 8 concentrations on 384 well plates. (4d: 0.025–0.75 μM , 4m: 0.005–0.35 μM , decitabine and clofarabine 0.005–5 μM). HL60 were seeded with a concentration of 0.1×10^6 cells/mL and afterward processed by the previously described protocol.²⁶ After the viability calculation, ZIP scores were generated through the “SynergyFinder” package.

4.2.5. Immunoblotting. Leukemia cells were treated with the indicated concentrations for 24 h. Afterward, the cells were collected and washed three times with cold PBS and, in the end, snap frozen with liquid nitrogen. Cells were lysed in Pierce RIPA buffer (with cOmplete Protease Inhibitor cocktail, PhosSTOP & Dithiothreitol), and DNA was removed via high speed centrifugation. The protein concentration was determined via the BCA assay. Protein lysates were diluted via 5X laemmli buffer and denatured at 95°C for 5 min. Into every pocket of a 10% acryl amid gel, a volume with 20 μg of protein was added. SDS-Page were run for 90 min at 100 mV in 1X running buffer. The proteins were transferred with 1X transfer buffer with 10% methanol onto nitrocellulose membranes at 100 mV for 90 min.

The membranes were blocked with 3% BSA in Tris buffered saline with Tween for 1 h. The membranes were incubated overnight at 4°C with primary antibodies, including antiacetyl- α -tubulin (catalog no. 5335), antiacetyl-histone H3 (catalog no. 9677S), anticlaved PARP (catalog no. 9541), and anti-GAPDH (catalog no. 97166), following the manufacturer's guidelines (Cell Signaling Technology). Signals were quantified via ImageJ and divided by the signal of the housekeeper.

4.2.6. Caspase 3/7 and Trypan Blue Assay. At a concentration of 0.25 μM , either 4d or 4m was introduced to wells containing HL60 leukemia cells. Additionally, compounds were coadministered with a caspase inhibitor Q-VD at a concentration of 10 μM in separate wells. Control conditions encompassed wells treated solely with DMSO or exclusively with Q-VD. Each experimental condition was executed in quadruplicate. Following a 24 or 48 h incubation period, the cells underwent treatment with a Caspase 3/7 Kit (Promega), and luminescence was quantified using the Tecan Spark microplate reader. In parallel, the identical cell solutions was dispensed into 24-well plates, and cell enumeration was conducted

employing the Vi-CELL BLU (Beckman Coulter) cell counter after 24 or 48 h.

4.2.7. Cell Cycle Analysis. HL60 cells were plated in a concentration of 0.1×10^6 cells/mL in 12 well plates with a volume of 1.5 mL. Both 4d and 4m were added to the cultures at 0.15 or 0.20 μM . After 24 h of incubation, the cells were centrifuged and incubated with 0.2 mL Nicoletti assay buffer (0.1% trisodium citrate dehydrate, 0.1% Triton X-100, 50 μg /mL propidium iodide, 0.5 mg/mL RNase A). The solution was incubated 15 min at 4°C and afterward measured via CytoFLEX (Beckman Coulter).

4.3. Molecular Modeling Studies. **4.3.1. Molecular Docking.** The cryo-EM structures of HDAC2 (PDB-ID: 7KBG),⁴² HDAC4 (PDB-ID: 2VQM),⁷³ and HDAC8 (PDB-ID: 1T69)⁷⁴ were prepared for molecular docking using the Protein Preparation Wizard as implemented in the Maestro GUI of the Schrödinger Suite version 2024-1. Protonation states for Asp, Glu, His, and Lys, tautomers for His, and chi flips for Asp, Glu, and His were calculated at $\text{pH } 7.0 \pm 2.0$ using the PROPKA⁷⁵ implementation within Maestro. A restrained energy minimization was performed focusing only on hydrogen atoms. Three-dimensional structures of the ligands 4a–4m and vorinostat were generated and prepared using the LigPrep module in Maestro. Docking studies were performed with Glide (Schrödinger Release 2024-1) using the Glide XP docking protocol,⁴⁰ generating 75 poses for each ligand.

4.3.2. Effective Binding Energy Computations. To estimate relative affinities of compounds 4d and 4m, the best scoring pose of each ligand in HDAC2 was postprocessed using the MM-GBSA method in Prime with the VSGB 2.1 solvation model.⁷⁶ Side-chain flexibility of the protein was considered for all residues within 8 Å of the ligand pose.

4.4. Structure Determination. **4.4.1. Protein Preparation.** CD2 from DrHDAC6 was recombinantly expressed using His₆-MBP-TEV-HDAC-pET28a(+) vector and purified as previously described with minor modifications.⁷⁷ Briefly, HDAC6 was expressed using the *Escherichia coli* strain BL21(DE3) grown in 2X yeast extract tryptone media (2YT) supplemented with 0.02 M glucose and kanamycin (50 mg/mL). Expression was induced at an optical density at 600 nm (OD_{600}) of 1.5 with 0.1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) along with the addition of 0.2 mM zinc sulfate. Cultures were then incubated at 20°C overnight and harvested by centrifugation.

To isolate the protein, cells were resuspended in wash buffer (50 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ pH 8.0, 300 mM NaCl, 5% Glycerol, 1 mM TCEP) and lysed by sonication. The crude lysate was clarified by centrifugation and then applied to a Protino Ni-NTA 5 mL column (Macherey-Nagel, Düren, Germany) using an ÄKTA Go system (Cytiva, Marlborough, Massachusetts, United States). The His₆-MBP-TEV-HDAC6 fusion protein was eluted using 150 mM imidazole. The fractions containing protein were combined, and the buffer exchanged with wash buffer. To remove the maltose binding protein and the affinity tag from the fusion protein TEV cleavage was performed. TEV cleavage was performed by adding the protease in a ratio of 1:10 in a buffer containing 1 mM DTT and 0.5 mM EDTA at 4°C overnight. Subsequently, the solution was applied to an HisTrap Excel 5 mL column (Cytiva). Fractions were collected while washing the column with 10 mM imidazole. The volume of the combined fractions was reduced, and the solution loaded onto an HiLoad 16/600 Superdex 75 pg column (Cytiva). The column was washed with buffer (50 mM HEPES, 100 mM KCl and 5% Glycerol, pH 7.5). The purity of the target protein was monitored throughout using SDS-PAGE.

4.4.2. Crystallization. All HDAC6–inhibitor complexes were crystallized in sitting drops by the vapor diffusion method at 18°C . For cocrystallization of the inhibitors with HDAC6, the protein solution (14.7 mg/mL in 50 mM HEPES, 100 mM KCl, and 5% Glycerol, pH 7.5) was incubated with 2 mM inhibitor. Then, 1 μL of the HDAC6–inhibitor complex solution was mixed with 1 μL of a reservoir solution. The structure of the HDAC6–4d complex was determined from a crystal obtained in 0.1 M sodium acetate, pH 8.7, 0.1 M sodium formate, and 28% PEG3350. The structure of

the HDAC6–4m complex was determined from a crystal obtained in 0.1 M sodium acetate, pH 8.7, 0.1 M sodium formate, and 22% PEG3350. Crystals were harvested after 6 weeks and flash frozen in liquid nitrogen using a 50% aqueous PEG3350 solution as the cryoprotectant.

4.4.3. Data Collection and Structure Determination. X-ray diffraction data was collected at 100 K using synchrotron radiation on beamline MASSIF-3 (ID30A-3) at the European Synchrotron Radiation Facility, Grenoble, France.^{78,79} The data was processed using XDS,⁸⁰ molecular replacement, and structure refinement were performed using CCP4.⁸¹ Data reduction was performed with AIMLESS,⁸² and molecular replacement was performed using Phaser⁸³ with the coordinates of CD2 of HDAC6 from *D. rerio* in complex with trichostatin A (TSA) (PDB ID: 5WGI).⁷⁷ Model building and refinement were performed with Coot⁸⁴ and Refmac.⁸⁵ PyMOL⁸⁶ was used to prepare the figures and to calculate the root-mean-square deviation (RMSD) of the Ca atoms using the align command with the number of cycles set to 0, thus, not including outlier rejection. The atomic coordinates have been deposited with the Protein Data Bank, Research Collaboratory for Structural Bioinformatics at Rutgers University (PDB ID: 9GGH and 9GGK). Authors will release the atomic coordinates upon article publication.

4.5. In Vitro Pharmacokinetics. 4.5.1. LC–MS/MS Method.

For the determination of in vitro pharmacokinetics of 4d and 4m, a tailored liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) assay was developed. Chromatographic separation was performed using a Luna Pentafluorophenyl (PFP (2)) column (100.0 × 2.0 mm; 3 μm; Phenomenex Ltd., Aschaffenburg, Germany). For the mobile phase, 0.1% FA in water (v/v) and 0.1% FA in methanol (v/v, B) were applied. Gradient separation at a flow rate of 0.4 mL/min was used, involving the following steps: 0.0–1.5 min: 5% B, 1.5–2.5 min: 5%–20% B, 2.5–4.5 min: 20%–95% B, 4.5–7.5 min: 95% B, and 7.5–8.0 min: 95%–5% B. The injection volume was set to 20 μL, and the column oven was maintained at 60 °C. A TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts) with an ESI interface was used for the mass spectrometric detection. A spray voltage of 4000 V and a capillary temperature of 300 °C were applied. The vaporizer temperature was maintained at 380 °C, the sheath gas at 50 au, aux gas pressure at 5 au and ion sweep gas at 1.0 au. Argon was utilized as the collision gas with a pressure of 1.6 mTorr. 4j was used as the internal standard for 4d and 4m, while deuterated standards were used for the control compounds in the respective in vitro pharmacokinetic assays. The following multiple reaction monitoring transitions with their collision energy and tube lens voltage (TL) were monitored in the positive ion mode: 4d: 380.23 → 175.0 mass-to-charge ratio (*m/z*) (CE: 40 V, TL: 96 V), 4m: 419.19 → 229.1 *m/z* (CE: 26 V, TL: 100 V), 4j: 364.15 → 217.1 *m/z* (CE: 17 V, TL: 158 V), Carvedilol: 407.04 → 224.07 *m/z* (CE: 22 V, TL: 150 V), Carvedilol d5 CE: 412.28 → 229.1 *m/z* (CE: 23 V, TL: 116 V), Propranolol 260.0 → 155.0 *m/z* (CE: 24 V, TL: 86 V), Propranolol d7 267.18 → 116.12 *m/z* (CE: 20 V, TL: 88 V), Itraconazol 705.4 → 392.18 *m/z* (CE: 34 V, TL: 154 V), Itraconazol d4 709.6 → 396.23 *m/z* (CE: 36 V, TL: 165 V).

4.5.2. Distribution Coefficient (log *D*). The log *D* determination was based on the shake flask method optimized for mass spectrometric analysis.⁸⁷ The distribution of test compounds was investigated between buffer presaturated octanol and octanol-presaturated 0.1 M potassium phosphate buffer pH 7.4. A 10 mM solution of the test compounds in DMSO and the control carvedilol were diluted 1:100 in buffer. A 200 μL aliquot was directly taken and 1:1 diluted in acetonitrile (v/v, standard). To the residual buffer volume, octanol was added in different v/v ratios: 1:0.1, 1:0.01, 3:0.01, and 5:0.01. Samples were shaken for 1 h to reach equilibrium before the two phases were separated by centrifugation at 13,200g for 10 min. Determinations were performed in triplicate for each compound from the aqueous phase. For the calculation of log *D*, the buffer/octanol v/v ratio was used in which the amount of

drug was most similar in order to obtain the most precise measurement. The log *D* was then calculated by the following eq 1:

Calculation of log *D*. *A*_{St}: area ratio standard, *A*_w: area ratio after partition, *V*_w: volume aqueous phase, and *V*_o: volume octanol.

$$\log D = \log \left(\left(\frac{A_{St}}{A_w} - 1 \right) \frac{V_w}{V_o} \right) \quad (1)$$

4.5.3. Plasma Stability. Ex vivo plasma stability was studied in fresh human plasma at 37 °C. Plasma was prewarmed to 37 °C, and reactions were started by spiking 4d and 4m to the plasma to a final concentration of 1 μM, 50 nM, and 2.5 nM. Sample aliquots of 100 μL were taken at 0, 30, 60, 120, 240, and 360 min and after 24 h. Each aliquot was mixed with 300 μL ice-cold acetonitrile containing the internal standards and directly vortexed, followed by 15 min shaking at 800 rpm at room temperature. Then, samples were centrifuged for 5 min at 13,200g. 300 μL of the supernatant was evaporated to dryness under heated nitrogen stream at 50 °C. Samples were stored at 4 °C, until completion of the assay and reconstituted in 100 μL 50/50 acetonitrile/water (v/v). This customized sample preparation protocol was characterized by good recoveries of 104.7 ± 6.7% for 4d and 104.5 ± 6.6% for 4m and low matrix effects of less than 11% for both compounds. The assay was conducted in triplicate. As a control, the compounds were incubated in 4% BSA to identify potential enzyme-related degradation in plasma. In vitro plasma half-life (*t*_{1/2}) was calculated by *t*_{1/2} = ln 2/*k*_e, where *k*_e is the slope in the linear fit of the natural logarithm of the fraction remaining of the parent compound vs incubation time.

4.5.4. Blood-To-Plasma Ratio. The blood-to-plasma ratio (*K*_{B/P}) was determined to investigate the drug binding to erythrocytes. Therefore, freshly drawn whole blood was spiked to a final concentration of 1 μM, 50 nM, and 2.5 nM and cautiously shaken to avoid lysis and incubated at 37 °C for 30 min. Spiked whole blood was centrifuged at room temperature for 10 min at 2,000g, and the obtained plasma was used for analysis. Additionally, plasma was directly spiked to a final concentration of 1 μM, 50 nM, and 2.5 nM and also incubated for 30 min at 37 °C (reference plasma; not considering distribution into the red blood fraction). 100 μL of reference plasma and of plasma separated from spiked whole blood were precipitated with 300 μL of ice-cold acetonitrile containing 3.33 ng/mL of the internal standards. Following immediate vortexing, samples were shaken for 15 min at 800 rpm at room temperature. Subsequently, samples were centrifuged for 5 min at 13,200g and 300 μL of the supernatant was evaporated to dryness under a heated nitrogen stream at 50 °C. The residue was reconstituted in 100 μL 50/50 acetonitrile/water (v/v). Whole blood and plasma of two donors (male and female) in three independent replicates were used for the analysis. Carvedilol was used as a control compound with known *K*_{B/P}. The hematocrit was determined volumetrically. The blood-to-plasma-ratios were calculated according to eqs 2 and 3;^{88,89}

Calculation of the Red blood cell partitioning coefficient. *H*: hematocrit; *K*_{e/p}: red blood cell partition coefficient; pref: reference plasma; and p: plasma separated from spiked whole blood.

$$K_{e/p} = \frac{1}{H} \times \left(\frac{\text{Area ratio}_{pRef}}{\text{Area ratio}_p} - 1 \right) + 1 \quad (2)$$

Calculation of the Blood-to-plasma ratio (*K*_{B/P}). *H*: hematocrit; *K*_{e/p}: red blood cell partition coefficient.

$$K_{B/P} = (K_{e/p} \times H) + (1 - H) \quad (3)$$

4.5.5. Plasma Protein Binding. Plasma protein binding was determined using equilibrium dialysis. A 20% ethanol regenerated dialysis membrane with a molecular weight cutoff of 6 kDa (Reichert Chemietechnik GmbH + Co, Heidelberg, Germany) was placed into a 96-well dialysis apparatus to obtain two chambers. 150 μL of plasma spiked to a final concentration of 1 μM, 50 nM, and 2.5 nM

of the test compounds and 150 μL of 0.9% saline were added to the respective sides of the 96-well dialysis plate; wells were sealed and the plate was placed into an incubator at 37 $^{\circ}\text{C}$ to dialyze for 24 h. Following 24 h of incubation, 100 μL of plasma were removed, directly precipitated with ice-cold acetonitrile containing the internal standards, and prepared as described above. 100 μL of methanol with internal standards was added to the saline at the acceptor chamber to keep the compounds dissolved before taking the respective aliquot. Plasma protein binding was analyzed in three independent replicates and itraconazole was used as a control in each chamber to account for potential leaking of the dialysis membrane. Plasma protein binding was calculated according to eq 4, where f_b is the fraction bound, c_{te} is the total plasma drug concentration at equilibrium, c_f is the free drug concentration of the acceptor side, V_{pi} is the initial plasma volume, and V_{pe} is the equilibrium plasma volume.⁹⁰

Calculation of drug fraction bound.

$$f_b [\%] = \frac{(c_{te} - c_f) \times \frac{V_{pe}}{V_{pi}}}{\left[(c_{te} - c_f) \times \frac{V_{pe}}{V_{pi}} \right] + c_f} \times 100 \quad (4)$$

To determine plasma protein binding >99% more accurately, the dilution method was applied.⁹¹ For this purpose, a 1:10 and a 1:20 dilution of plasma in 0.9% saline was additionally used as the donor. The diluted plasma was spiked to a final concentration of 1 μM , 50 nM, and 2.5 nM of **4d** and **4m** and incubated with 0.9% saline in the acceptor chamber for 24 h at 37 $^{\circ}\text{C}$. Sample purification was performed as for the classical approach. Calibration curves from 1:10 and 1:20 diluted plasma were used to determine the corresponding donor concentrations. Plasma protein binding was analyzed in triplicates. As a control, the plasma protein binding of itraconazole was determined. Plasma protein binding was calculated according to eqs 5, 6, and 7 where f_u is the fraction unbound and D is the dilution level of the plasma.

Calculation of diluted fraction unbound. $f_{u,d}$: free fraction from diluted plasma.

$$f_{u,d} [\%] = \left(1 - \frac{(c_{te} - c_f) \times \frac{V_{pe}}{V_{pi}}}{\left[(c_{te} - c_f) \times \frac{V_{pe}}{V_{pi}} \right] + c_f} \right) \times 100 \quad (5)$$

Calculation of undiluted fraction unbound. D : plasma dilution factor, f_u : fraction unbound, $f_{u,d}$: fraction unbound from diluted plasma.

$$\text{Undiluted } f_u = \frac{\frac{1}{D}}{\left[\left(\frac{1}{f_{u,d}} \right) - 1 \right] + \frac{1}{D}} \quad (6)$$

Calculation of undiluted fraction bound.

$$\text{Undiluted } f_b [\%] = (1 - f_u) \times 100 \quad (7)$$

4.5.6. Microsomal Stability. HLMs were used to determine microsomal stability using the cosolvent method. For this purpose, 905 μL of 0.1 M potassium phosphate buffer pH 7.4 and 25 μL of 20 mg/mL pooled (from 150 donors) HLMs (Corning, New York, USA) were added and gently inverted for mixing. 10 μL of a 100 μM working solution for **4d**, **4m**, and the control compound propranolol in acetonitrile/DMSO 80/20 (v/v) was added and gently inverted for three times. 47 μL of this mix was aliquoted into three reaction tubes per time point. To start the reaction, 3 μL of a freshly prepared mix of 125 μL NADPH Regenerating System Solution A and 25 μL NADPH Regenerating System Solution B (Corning, New York, USA) was added. The tubes were incubated at 37 $^{\circ}\text{C}$ and 300 rpm, and reactions were stopped at the time points 0, 15, 30, 45, and 60 min by adding 150 μL of ice-cold acetonitrile (containing 100 ng/mL of the internal standards). Each sample was

directly vortexed and placed on ice. Following, samples were centrifuged at 13,200g for 10 min, and the supernatant was diluted 1:10 with acetonitrile/water 50/50 (v/v). A negative control (no HLMs) and a blank (no analytes) were used as controls besides propranolol. First-order kinetics was used to calculate half-life ($t_{1/2}$) and intrinsic clearance (CL_{int}) (eqs 8 and 9).

Calculation of half-life ($t_{1/2}$). k_e : elimination rate constant.

$$t_{1/2} = \frac{\ln 2}{k_e} \quad (8)$$

Calculation of intrinsic clearance. $t_{1/2}$: half-life.

$$CL_{int} [\mu\text{L}/\text{min}/\text{mg protein}] = \frac{\ln(2)}{t_{1/2}} \times \frac{\text{volume of incubation } [\mu\text{L}]}{\text{protein amount in incubation } [\text{mg}]} \quad (9)$$

The elimination rate constant (k_e) was determined as the negative slope of the plotted natural logarithm of the test compound peak area ratio versus time.

To correct for nonspecific binding in the HLM incubation, plasma protein binding of **4d** and **4m** to HLMs was determined by equilibrium dialysis. The procedure was conducted as described above, replacing plasma by a 0.5 mg/mL HLM mixture (no NADPH). The unbound intrinsic clearance, which is solely influenced by the activity of metabolizing enzymes, was calculated (eq 10).

Calculation of unbound intrinsic clearance. CL_{int} : intrinsic clearance and $f_{u,mic}$: unbound fraction microsomal incubation.

$$\text{Unbound } CL_{int} = \frac{CL_{int}}{f_{u,mic}} \quad (10)$$

4.5.7. In Vitro–In Vivo Extrapolation of Hepatic Clearance. The in vivo hepatic clearance of **4d** and **4m** was estimated using the in vitro determined $K_{B/P}$, unbound intrinsic clearance, and plasma protein binding at 50 nM according to eq 11. A hepatic blood flow of 1500 mL/min was used for calculation, displaying the normal value in the healthy male.⁹² In addition, the hepatic extraction ratio was calculated according to eq 12. Drugs with an extraction ratio <0.3 were classified as low hepatic extraction drugs, 0.3–0.7 as intermediate hepatic extraction drugs, and >0.7 as high hepatic extraction drugs.

Calculation of hepatic clearance. CL_H : hepatic clearance, $CL_{int,u}$: unbound intrinsic clearance, f_u : fraction unbound in plasma, $K_{B/P}$: blood-to-plasma ratio, and Q_H : hepatic blood flow.

$$CL_H = Q_H \times \frac{\frac{f_u}{K_{B/P}} \times CL_{int,u}}{Q_H + \frac{f_u}{K_{B/P}} \times CL_{int,u}} \quad (11)$$

Calculation of the hepatic extraction ratio (E_H). CL_H : hepatic clearance and Q_H : hepatic blood flow.

$$E_H = \frac{CL_H}{Q_H} \quad (12)$$

4.5.7.1. Preclinical Pharmacokinetic Study and Assessment. The pharmacokinetics of **4d** were assessed (by Pharmacelsus GmbH) following a single intraperitoneal dose (10 mg/kg) in C57BL/6 mice. Blood samples were collected from the tail vein of C57BL/6 mice at 0.25, 0.5, 1, 2, 4, 7, and 24 h post dose. The pharmacokinetic parameters were calculated based on the concentration–time profiles of three mice using a standard NCA method. The maximum concentration (C_{max}) and the time to reach C_{max} (t_{max}) were directly acquired from the concentration–time curves. The apparent elimination half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$, where λ_z was the elimination rate constant calculated by linear regression of the terminal linear portion of the ln-concentration–time curve. The area under the plasma concentration–time curve (AUC) from time 0 to the last time point (AUC_{last}) was calculated

using the linear trapezoidal rule method. The AUC from 0 to infinity (AUC_{inf}) was calculated as $AUC_{last} + Ct/\lambda_z$, where Ct is the last measurable concentration. Oral clearance (CL/F) was calculated as $dose/AUC_{inf}$ and apparent volume of distribution (V_d/F) was obtained by dividing CL/F by λ_z .

4.5.7.2. Animal Experiment. The periclinical animal experiments were conducted to evaluate the therapeutic efficacy of **4d** on two in vivo leukemic mouse models encompassing cell line -derived xenograft (CDX) and mouse tumor allografts.

A CDX model was constructed by transplanting MV4-11 (DSMZ, no. ACC 102), a human AML cell line, into immunodeficient mice. MV4-11 cells were maintained in RPMI 1640 medium supplemented with 20% FBS. Luc-GFP-expressing MV4-11 cells were generated by lentiviral transduction with the luciferase reporter gene.⁹³ MV4-11 cells were maintained in RPMI 1640 medium supplemented with 20% FBS. MV4-11 GFP-Luc (0.5×10^6) cells were intravenously injected into 6 to 8 weeks old immunodeficient NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, Jackson laboratory). Engraftments of the leukemia cells was verified by the in vivo imaging system (IVIS Spectrum In Vivo Imaging System, PerkinElmer) 14 days after injection. The leukemia bearing mice were randomly divided to two treatment groups (4 mice/group), vehicle control (10% DMSO + 18% PEG300 + 5% TWEEN 20 + 68% dH₂O), and compound **4d** (10 mg/kg). Starting from day 16, a total of 8 daily doses were administered through intraperitoneal (i.p.) injections.

A murine cell line-derived syngeneic model was constructed by transplanting C1498 (ATCC, TIB-49), an AML cell line that originated from a female C57BL/6 mouse, into immunocompetent mice.⁷⁰ C1498 cells were maintained in RPMI 1640 medium supplemented with 20% FBS. Luc-GFP-expressing C1498 cells were generated according to methods described above. C1498 GFP-Luc (0.5×10^6) cells were intravenously injected into 6 weeks old male C57BL/6J mice (Janvier). Verification of leukemic engraftments was done with IVIS imaging 5 days after tumor inoculation. The tumor-bearing mice were randomly divided into three treatment groups (4 mice/group), namely vehicle control, vorinostat (HDACi) (20 mg/kg), and compound **4d** (20 mg/kg). From day 6 to 10, daily treatments were administered i.p. for 5 consecutive days, followed by an IVIS imaging on day 11 and another successive 5 day treatment course. Body weight from each mouse was recorded every day. Treatment efficacy and tumor progression were monitored by means of IVIS and quantification of ROI values (radiance, p/s/cm²/sr). The animal experiments were conducted in accordance with the regulatory guidelines of the official committee at LANUV, under the authorization of the animal research institute (ZETT) at the Heinrich Heine University Düsseldorf.

4.5.7.3. Flow Cytometry. In order to investigate changes in intracellular expression levels of histone 3 acetylation upon compound **4d** treatment, C1498 tumor-bearing C57BL/6J mice were treated with vehicle or **4d** (20 mg/kg). For preparation of mice liver cells, liver tissue of vehicle or **4d** (20 mg/kg) treated mice were excised 12 h post treatment administration. The liver cells were isolated by smashing and filtering the liver tissue through a 40 μ m cell strainer filter (Greiner cat. no. S42040). Erythrocyte lysis was performed by washing the cells once in ammonium chloride isotonic buffer. The liver cells acquired from both vehicle control or **4d** treated mice were harvested and fixed with fixation/permeabilization concentrate (Invitrogen cat. no. 00-5123-43) for 30 min at room temperature in the dark and were washed with permeabilization buffer (Invitrogen cat. no. 00-8333-56). Afterward, the cells were stained with Acetyl-Histone H3 (Lys9) antibody (Cell signaling, cat. no. 9649) for 3 h at room temperature in the dark. After incubation, the cells were washed again with the permeabilization buffer and stained with the 1:300 diluted Cyanine3 Donkey antirabbit IgG antibody (BioLegend, cat. no. 406402), and were then suspended in FACS buffer (PBS, 1% FCS, 5 mM EDTA) for flow cytometric analysis. Flow cytometry was performed by utilizing the Beckman Coulter CytoFLEX Cytometer, and the results were analyzed with FlowJo software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.4c02024>.

Simulated annealing omit map of **4d** and **4m** in the CD2 of *D. rerio* HDAC6 data collection and refinement statistics, comparison of the cytotoxic data of compounds **4a–4m** in a heatmap format effectiveness of **4d** in a MV4-11 leukemia xenograft mouse model, Western blot showing induction of ac-H3 in the C1498 cell line Histogram plot of ac-H3 intracellular staining in liver cells from C1498 tumor bearing mice, and NMR spectra and HPLC traces of synthesized compounds Raw IC₅₀, DSS, and dDSS values (PDF)

Molecular formula strings (CSV)

Atomic coordinates of HDAC2_4a_noH (PDB)

Atomic coordinates of HDAC2_4m_noH (PDB)

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raphy. J.S.-D., J.-W.T., K.S., M.K., P.S., A.A.P., and T.W. performed the biological evaluation. L.B. and N.H. provided the HDAC enzyme data. T.G. and B.B.B. performed the bioanalytical determination and evaluation of in vitro and in vivo pharmacokinetics. Y.L.H. performed molecular modeling. Y.L.H. and H.G. analyzed the results. A.B., H.G., M.U.K., B.B.B., S.B., and T.K. acquired funding and provided resources. F.F., J.S.-D., S.B., and T.K. designed the study. The manuscript was written through the contributions of all authors. All authors have given approval to the final version.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AML, acute myeloid leukemia; AUC_{inf} , area under the curve from 0 to infinity; AUC_{last} , area under the curve from 0 to the time of the last quantifiable concentration; BCP-ALL, B cell precursor acute lymphoblastic leukemia; BSA, bovine serum albumin; CE, collision energy; CL/F, apparent total body clearance following extravascular administration; C_{max} (ng/mL), maximum observed concentration; CML, chronic myeloid leukemia; dDSS, differential DSS; DSMZ, German collection of microorganisms and cell culture; DSS, drug sensitivity scores; ESI, electrospray ionization; FBS, fetal bovine serum; HAT, histone acetyltransferases; HBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluroniumhexafluorophosphat; HDAC, histone deacetylases; HDACi, HDAC inhibitors; HLM, human liver microsomes; HRMS, high-resolution mass spectra; HSCs, hematopoietic stem cells; HTDS, high throughput drug screening; Hz, Hertz; $K_{B/P}$, blood-to-plasma ratio; mp, melting points; MRM, multiple

reaction monitoring; PDX, patient-derived xenograft; ppm, parts per million; $t_{1/2}$, Terminal elimination half-life or apparent terminal elimination half-life; T-ALL, T cell acute lymphoblastic leukemia; TBST, tris buffered saline with Tween; TFA, trifluoroacetic acid; TL, Tube lens; TLC, thin-layer chromatography; t_{max} (h), time to reach the maximum concentration; V_z/F , apparent volume of distribution following extravascular administration; λ_z , first order terminal elimination rate constant; ZIP, zero interaction potency

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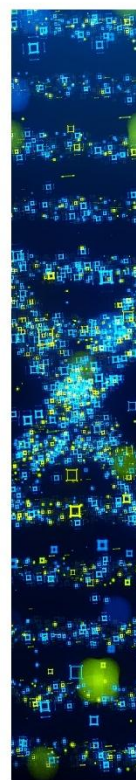
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3.4 - Epigenetic remodeling via HDAC6 inhibition amplifies anti-tumoral immune responses in myeloid leukemia cells

Due to the highly circumstantial effects of selective HDAC6 inhibition, this study tried to identify the leukemia subtype that could benefit from HDAC6i as a synergistic addition to the therapy protocol. A database-driven approach identified pediatric AML as a leukemia subtype in which high HDAC6 expression is associated with poor survival as compared to ALL. Next, a CRISPR-Cas9-mediated KO approach in myeloid leukemia cells to assess the effects of HDAC6 loss was employed. After generating HDAC6 KO cell lines from K562 (CML) and MV4-11 (AML), no significant difference in proliferation rate was observed *in vitro*; however, differences were evident in an *in vivo* mouse model. The effect of HDAC6 loss was characterized through mass spectrometry of both proteome and secretome. This revealed significant shifts in the cellular metabolism and differential expression of functional components like the lysosome, immune functions, and DNA damage repair. A promising candidate that was upregulated in both techniques was RNase T2. This lysosome-associated protein acts as a tumor suppressor by attracting macrophages and inducing the anti-tumor pro-inflammatory M1 phenotype. The upregulation was observed across all myeloid lineage HDAC6 KO models, but not in ALL. The most clinically advanced HDAC6 preferential inhibitor, ricolinostat, was able to induce the same effect in multiple myeloid cell lines. To investigate the mechanism that leads to the upregulation of RNase T2, the K562 HDAC6 KO was analyzed both via ATAC-seq and RNA-seq. A general increase in chromatin accessibility was observed, which included the promotor region of RNase T2. In addition to its potential to stimulate the innate immune system through RNase T2, HDAC6 inhibition can increase the activation of cytotoxic T cells both *in vitro* and *in vivo*. In single-compound mouse model treatments, ricolinostat showed a promising trend. High-throughput drug screening established that the highly relevant AML chemotherapeutics cytarabine and clofarabine synergize with HDAC6 inhibition. In conclusion, several traits of HDAC6 inhibition in myeloid lineage leukemia were identified that could be relevant in the clinical context.

Contributions: In general, writing of the manuscript and experimental design, execution, evaluation, and visualization of the following data:

- K562 HDAC6 KO generation / *in vitro* characterization (see Figure 1A-D)
- Survival analysis of AML and ALL of patients with high and low HDAC6 expression (see Supplement 1A-H)
- Dependency of leukemia cell lines of the HDAC family (see Supplement 1I)
- Sample preparation for the Mass spectrometry & secondary analysis via gene set expression analysis or string analysis (see Figure 2 A-D & Supplement 2 A-D)

- Generation and Validation of KCL22 HDAC6 KO (see Supplement 2E)
- Immunofluorescence staining of LAMP1 in K562 and KCL22 HDAC6 KO (see Figure 2E)
- Preparation of ATAC-seq and RNA-seq samples of K562 HDAC6 KO samples and subsequent analysis (see Figure 3 A-E & Supplement 3A-H)
- Western blot of RNase T2 for K562/KCL22 HDAC6 KO (see Figure 4 A-B), K562 HDAC6 KO supernatant (see Supplement 4A), and K562/THP1/HL60/PDX-CML/PDX-AML1 treated with ricolinostat (see 4D-H)
- Immunofluorescence staining of RNaseT2 in K562/HL60/THP1 treated with ricolinostat (see Figure 4I-K)
- Expression and correlation analysis of RNase T2 and HDAC6 in AML/ALL patients (see Supplement 4G-J)
- Survival analysis and immune signature correlation of low/high HDAC6/RNase T2 ratios in AML/ALL patients (see Figure 4N-Q)
- Initial Treatment of C1498 cells with ricolinostat in co-culture (see Figure 5D-H & Supplement 5E/H-I)
- High throughput drug screening of K562 HDAC6 KO (see Figure 6A)
- Apoptosis assays for Cytarabine & Clofarabine in K562 HDAC6 KO (see Figure 6B-E & Supplement 6A-C)
- Matrix synergy drug screening of ~50% of samples in the matrix synergy screening of ricolinostat with cytarabine or clofarabine (see 6F-6I & Supplement 6D-E)
- Correlation analysis of ZIP area scores from the matrix synergy scores with pathway/transcription factor activation scores inferred from RNA-seq data (see Figure 6J & Supplement 6F)
- Expression analysis of HDAC6 across AML and B-/T-ALL (Supplement 6I)

ARTICLE OPEN



Epigenetic remodeling via HDAC6 inhibition amplifies anti-tumoral immune responses in myeloid leukemia cells

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Histone deacetylase 6 (HDAC6) has emerged as a promising therapeutic target in cancer due to its immunomodulatory effects. While its prognostic significance remains debated, we demonstrate that HDAC6 loss significantly impairs myeloid leukemia progression in vivo, despite having no functional impact on leukemia cell proliferation in vitro. Global proteome and secretome profiling of HDAC6-knockout (KO) cells revealed upregulation of several immune-related modulators, including RNase T2, a tumor suppressor known to modulate the tumor microenvironment. Notably, RNase T2 upregulation upon HDAC6 loss was observed in myeloid leukemia cells but not in lymphoblastic leukemia cells. Moreover, pharmacological inhibition of HDAC6 recapitulated this phenotype, leading to RNase T2 upregulation in myeloid leukemia cells. ATAC-seq revealed increased chromatin accessibility of RNase T2 following HDAC6 loss, highlighting a functionally epigenetic regulatory contribution. Further functional assays conducted in an immunocompetent setting, both ex vivo and in vivo, demonstrated that HDAC6 inhibition sensitized murine myeloid leukemia cells to broad CD8⁺ T cell activation as evidenced by increased TNFα and CD107a expression. Consistently, in a syngeneic murine model, HDAC6 inhibition restricted the growth of myeloid leukemia cells. Moreover, an extended drug screening analysis identified Cytarabine and Clofarabine as significantly synergizing with HDAC6 inhibitor (Ricolinostat) in myeloid leukemia cell lines and in patient-derived xenograft (PDX) cells, while showing limited synergy in lymphoid leukemia cell lines, PDX, or healthy control cells. These findings suggest that HDAC6 represents a promising therapeutic target in myeloid lineage-derived leukemia cells by simultaneously enhancing immune activation and increasing chemosensitivity.

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INTRODUCTION

Aberrant epigenetic modifications, including histone deacetylation, play a key role in the leukemogenesis of acute myeloid leukemia (AML) and contribute to chemotherapy resistance by silencing tumor suppressor genes regulating chemosensitivity [1]. Dysregulation of HDACs has been linked to impaired hematopoietic differentiation, cell cycle defects, DNA damage accumulation, and reduced cell viability [2]. Although mutations in HDACs are uncommon in myeloid malignancies, dysregulated expression and aberrant recruitment by leukemia-associated fusion proteins have been reported [3, 4]. HDAC inhibitors (HDACi) have recently emerged as a promising class of therapeutics for various malignancies, including AML [5–7]. However, the approved HDACi for treating hematological malignancies are predominantly pan-HDACi, which broadly inhibit multiple HDAC isoforms involved in

essential cellular functions [8, 9]. This lack of specificity leads to a range of adverse effects, including cardiac toxicity and severe gastrointestinal disturbances, some of which can be life-threatening [10–12]. These toxicities often require the discontinuation of HDACi-based therapies to prevent severe complications. Additionally, in clinical practice, HDACi are rarely used as monotherapy and are typically combined with other therapeutic agents, adding complexity to treatment regimens [13, 14]. Selective targeting of specific HDAC isoforms may offer a promising approach to restricting their activity to particular cellular processes, potentially reducing toxicity and enhancing therapeutic tolerability.

Among the various HDAC isoforms, HDAC6 has emerged as a particularly compelling therapeutic target due to its distinct biological roles and relevance in oncogenesis [15]. HDAC6 is

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primarily localized in the cytoplasm and regulates a range of cellular functions through substrate deacetylation [16–19]. However, HDAC6-deficient mice remain viable and develop normally, supporting its potential as a safe therapeutic target [20]. HDAC6 is found to be overexpressed across various hematologic malignancies [21–23]. Pharmacological HDAC6 inhibition has been shown to target leukemic stem cells (LSCs) in chronic myeloid leukemia (CML) [22, 24, 25]. A recent study from a zebrafish model demonstrates that inhibition of HDAC6 suppresses LSC expansion and reduces the proliferation of AML cell lines [26], underscoring its potential as a therapeutic target across myeloid malignancies [27]. However, the prognostic relevance of HDAC6 as a therapeutic target remains debated, as HDAC6 inhibition alone has been reported to exert minimal or no cytotoxic effects on leukemia cells in ex vivo studies [28–30].

Through a database-driven approach, we identified HDAC6 as a promising candidate in AML. Using genetic models and employing multi-omics characterization, we identified RNase T2, a known activator of pro-inflammatory signaling, to be upregulated upon targeting HDAC6. Further, our ex vivo and in vivo studies demonstrated that HDAC6 inhibition enhanced broad CD8⁺ T cell activation and, therefore, anti-leukemic responses. Beyond its immunomodulatory role, we found that HDAC6 inhibition sensitized myeloid leukemia cells to standard chemotherapeutics. These findings highlight the potential of HDAC6 as a target in myeloid leukemia, underscoring the need for further investigation into its clinical applicability.

RESULTS

HDAC6 ablation impairs in vivo growth of (K562 and MV4-11) myeloid leukemia cells

We data-mined clinical data using TARGET [31] and TCGA-LAML [32] datasets, and identified prognostic implications. High HDAC6 expression significantly correlated with poorer survival in AML, whereas in acute lymphoblastic leukemia (ALL), elevated HDAC6 levels were associated with improved survival outcomes (Supplementary Fig. S1A–H). These findings contrast with earlier (ex vivo) studies suggesting that selective HDAC6 inhibition has minimal or no cytotoxic effects on the leukemia cells [28–30]. Further, in vitro CRISPR-based dependency analysis (using data from the DepMap portal) also suggests that HDAC6 exhibits the lowest dependency among HDAC family members in leukemia cells (Supplementary Fig. S1I).

To address the observed discrepancies between the in vitro and in vivo effects of HDAC6 inhibition, we generated CRISPR-Cas9-mediated HDAC6-knockout (KO) models. Specifically, HDAC6 was deleted in (*KMT2A*-rearranged) MV4-11 AML cells and, in parallel, in (*BCR::ABL1*⁺) K562 chronic myeloid leukemia (CML) cells, given prior reports identifying HDAC6 as a key regulator in CML biology [22, 24, 25], to investigate HDAC6 function across distinct myeloid contexts (Fig. 1A–H). Loss of HDAC6 resulted in increased α -tubulin acetylation, confirming functional impairment of HDAC6 in these models (Fig. 1A, E). First, to evaluate the differences in the cellular growth, a proliferation assays were performed, which revealed no significant differences in doubling time between HDAC6-KO and control cells (Fig. 1B, F). Similarly, a colony formation assay showed no alterations in clonogenic potential upon HDAC6 loss (Fig. 1C, G). Cell cycle analysis further demonstrated minimal changes in the distribution of cells across different phases (Fig. 1D, H). However, we observed an increased susceptibility of K562 HDAC6-KO cells to NK cell-mediated killing in an ex vivo PBMC-derived NK cell killing assay, compared to the respective controls (Supplementary Fig. S1J). Notably, in an in vivo setting, HDAC6-KO (K562 and MV4-11) leukemia models exhibited a significant ($p < 0.05$) reduction in leukemia progression when transplanted into NSG mice compared to controls (Fig. 1I–L). This led to a significant ($p < 0.01$) increase in overall survival in mice

injected with HDAC6-KO K562 cells, while no significant differences in survival were observed in the MV4-11 HDAC6-KO model. (Supplementary Fig. S1K–L). This discrepancy in survival outcomes may be attributed to the more aggressive disease kinetics of AML-derived MV4-11 cells relative to CML-derived K562 cells, potentially diminishing the observable survival benefit at later stages despite early and significant reductions in leukemic burden. Taken together, our data, supported by publicly available datasets, suggest that while HDAC6 loss has minimal impact on in vitro proliferation, it significantly impairs the progression of myeloid leukemia cells in vivo.

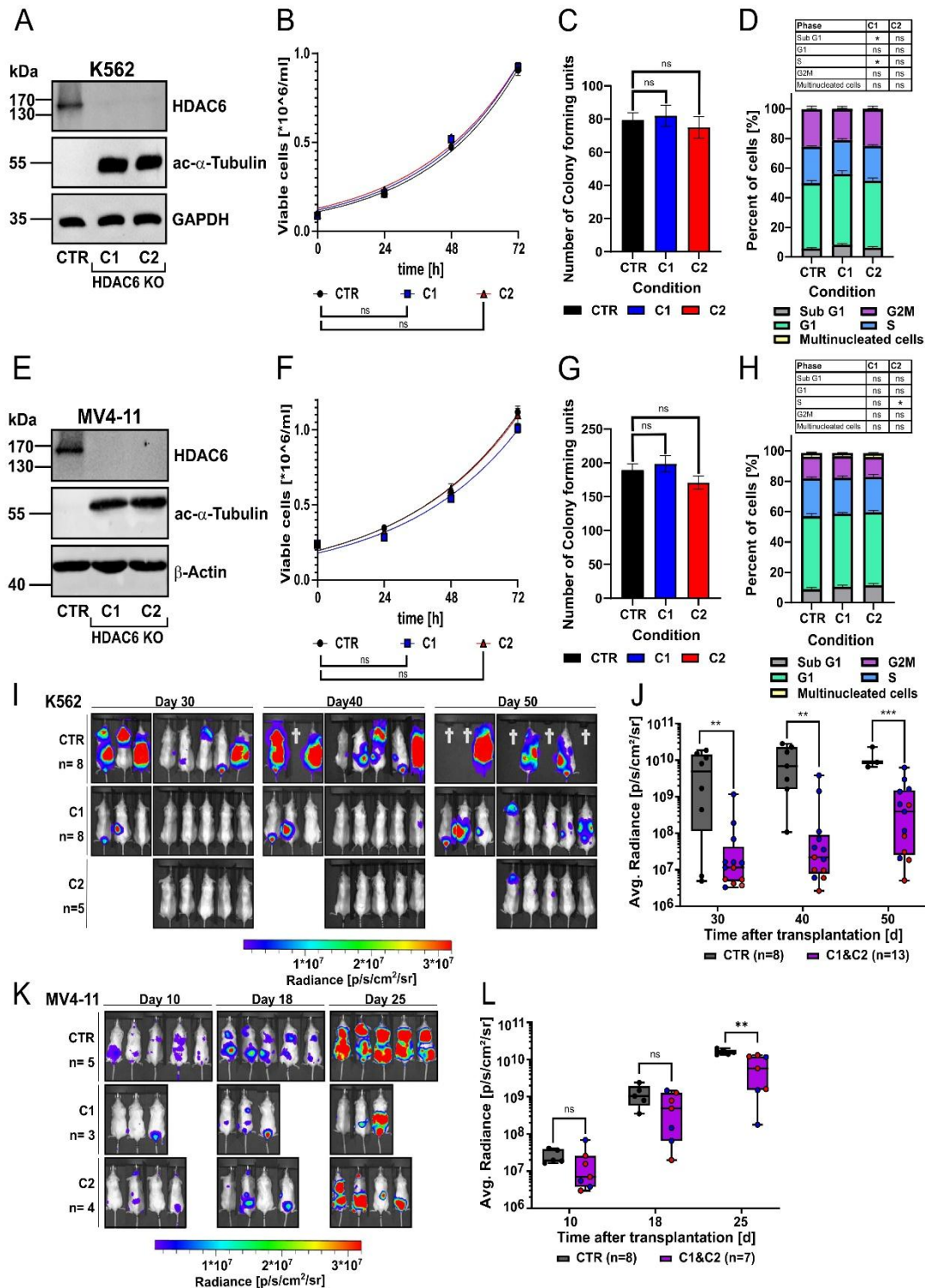
Targeting HDAC6 upregulates lysosomal-related protein accumulation

Building on the impaired leukemia progression observed upon HDAC6 loss, we employed quantitative mass spectrometry (MS) to profile global changes in the cellular proteome and secretome (Fig. 2A, B). Gene set enrichment analysis from the proteome analysis identified multiple dysregulated functional pathways upon HDAC6 loss in K562 cells (Fig. 2C and Supplementary Fig. S2A, B). Among the most significantly ($FDR < 0.05$) enriched functional clusters in the proteomic analysis were lysosome-associated proteins, including LAMP1, LAMP2, and RNase T2. Secretome analysis further validated an upregulation of lysosomal enzymes, suggesting increased extracellular release of lysosomal content (Fig. 2B). Additionally, HDAC6-KO cells exhibited enrichment of pathways related to immune activation, inflammatory responses, MAPK signaling and cellular motility (Fig. 2C). Functional enrichment analysis using STRING confirmed significant ($FDR < 1.E-15$) changes in lysosomal and immune-related pathways, including neutrophil degranulation and innate immune system processes, among the overlapping proteins between the proteome and secretome profiles (Fig. 2D and Supplementary Fig. S2C, D). Using fluorescence microscopy, a significant ($p < 0.01$) increase in LAMP1 expression was validated across HDAC6-KO generated models, including K562, (CML-derived) KCL22, and MV4-11 (Fig. 2E and Supplementary Fig. S2E). In addition, HDAC6-KO K562 and MV4-11 cells showed elevated LAMP2 expression (Supplementary Fig. S2F). We further evaluated the lysosomal protease Cathepsin H (CTSH) in the MV4-11 HDAC6-KO models; however, CTSH levels were not significantly elevated upon HDAC6 loss relative to the corresponding control cells (Supplementary Fig. S2G).

To evaluate whether HDAC6 targeting consistently influences the expression of lysosomal proteins in primary disease models, we incorporated (*BCR::ABL1*⁺) PDX-CML cells into our experimental framework. While HDAC6-KO was achievable in established leukemia cell lines, it could not be performed in PDX cells due to their inability to withstand the prolonged culture required for clonal editing. Accordingly, HDAC6 dependency in PDX-CML cells was assessed using short-term pharmacological inhibition with the HDAC6 inhibitor Ricolinostat [33, 34]. Short-term treatment with Ricolinostat also resulted in a concentration-dependent increase in the LAMP1 expression in the PDX-CML cells (Fig. 2F). Together, these results demonstrate that targeting HDAC6 inhibition impacts lysosomal and immune-related pathways.

HDAC6 loss enhances the accessibility at the LAMP1 and RNase T2 gene loci

Although HDAC6 primarily resides in the cytoplasm, it can localize to the nucleus and participate in chromatin regulation [35]. To examine genome-wide changes in chromatin accessibility associated with HDAC6 loss, we performed ATAC-seq analysis in K562 HDAC6-KO cells (Fig. 3A, B). In total, 10,577 promoter-associated ATAC-seq peaks were identified, and their accessibility is depicted in the violin plot shown in Fig. 3B. While only a limited number of genomic loci exhibited chromatin compaction following HDAC6 loss, a substantial number of loci and gene sets demonstrated



increased accessibility (Supplementary Fig. S3A, B), including loci encoding LAMP1 and RNase T2 (Fig. 3C, D). Next, to assess how increased chromatin accessibility influences global gene expression, we performed RNA-seq on HDAC6-KO (K562) cells

(Supplementary Fig. S3C). Gene set enrichment analysis (GSEA) on the RNA-seq data identified upregulation of pathways related to protein acetylation regulation, exocytotic vesicle secretion, and MAPK signaling, while processes associated with HDAC6 function,

Fig. 1 HDAC6 ablation impairs the in vivo growth of myeloid leukemia cells. **A** Representative western blot for HDAC6 and acetylated α -tubulin protein levels in two clones (C1 and C2) of K562 HDAC6-knockout (KO) and a non-targeting control (CTR) ($n = 3$). **B** Proliferation curve determined by trypan blue assay of K562 HDAC6-KO. Statistical significance was calculated by comparing the doubling time of the growth curve (unpaired t -test, ns not significant, $n = 3$). **C** Colony-forming unit (CFU) assay performed by seeding K562 HDAC6-KO cells in a semi-solid medium (unpaired t -test, ns not significant, $n = 3$). **D** Bar plot showing cell cycle of K562 HDAC6-KO analysis (unpaired t -test, ns not significant, $n = 3$). Similarly, analysis was performed in the MV4-11 HDAC6-KO model, including western blot (**E**), proliferation assay (**F**), CFU assay (**G**), and cell cycle analysis (**H**) (unpaired t -test, ns not significant, $*p < 0.05$, $n = 3$). **I–K** In vivo growth kinetics of K562 (**I**) and MV4-11 (**K**) leukemia cells in NSG mice, monitored at the indicated time points post-injection using bioluminescence imaging. **J–L** Quantification of the average radiance of (**I–K**) for K562 (**J**) and MV4-11 (**L**) models (Mann–Whitney U -test, ns not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

including aggresome and autophagosome formation, were down-regulated (Supplementary Fig. S3D, E) [36, 37]. To integrate our findings, RNA-seq data were overlaid onto the ATAC-seq dataset, revealing a substantial (24.2%) overlap between differentially accessible chromatin regions and transcriptionally upregulated genes (Supplementary Fig. S3F, G). Furthermore, integrated multi-omics analysis, incorporating ATAC-seq, RNA-seq, proteomic, and secretomic data, revealed significant enrichment of gene sets associated with the cell periphery and plasma membrane (Fig. 3E and Supplementary Fig. S3H). These findings suggest that HDAC6 inhibition may regulate surface receptor expression and influence leukemia cell interactions with the surrounding micro-environment [38].

Targeting HDAC6 induces the expression of tumor suppressor RNase T2 in myeloid leukemia cells

Next, we selected the lysosomal lumen-associated endoribonuclease RNase T2 as a potential target downstream of HDAC6, based on its well-established role as a tumor suppressor [39, 40]. Beyond its enzymatic activity, RNase T2 can trigger immune responses within the tumor microenvironment, particularly by promoting the recruitment of M1-polarized macrophages [41, 42].

Notably, all HDAC6-KO models (K562, KCL22, and MV4-11) demonstrated a consistent and significant ($p < 0.05$) upregulation of RNase T2 (Fig. 4A–C). Consistent with the MS-based secretome analysis, HDAC6-KO K562 cells exhibited elevated levels of RNase T2 in the culture supernatant (Supplementary Fig. S4A). Next, to determine whether RNase T2 upregulation upon HDAC6 inhibition is reversible, we performed knock-in (KI) experiments by reintroducing HDAC6 into HDAC6-KO (K562) cells. Functional validation of HDAC6 re-expression was confirmed by the reversal of α -tubulin hyperacetylation (Supplementary Fig. 4B). HDAC6 restoration in HDAC6-KO cells also led to a significant ($p < 0.05$) reduction in RNase T2 expression, supporting the role of HDAC6 in regulating RNase T2 expression.

Next, to determine whether this effect was specific to myeloid leukemia cells or extended to ALL cells, we assessed RNase T2 protein levels in HDAC6-KO or knockdown (KD) models using B-cell acute lymphoblastic leukemia (B-ALL) cell lines (NALM6 and NALM20) and in (*BCR::ABL1*⁺) PDX-B-ALL2 cells (Supplementary Fig. S4C–F and Supplementary Table 3 for PDX information). Specifically, RNase T2 expression remained unchanged or even downregulated upon HDAC6 loss in the tested B-ALL models. Consistently, correlation analysis (using the St. Jude PeCan database) revealed that in myeloid leukemia patients, HDAC6 and RNase T2 expression levels are negatively correlated ($r = -0.13$, $p < 0.05$), whereas in lymphoid leukemia patients, a strong positive correlation ($r = 0.53$, $p < 0.0001$) was observed (Supplementary Fig. S4G–J).

Furthermore, short-term treatment with the HDAC6 inhibitor Ricolinostat at sub-cytotoxic concentrations (selected based on the differential sensitivity of each cell model and validated by increased acetylated α -tubulin levels as a readout of HDAC6 inhibition) resulted in a significant ($p < 0.05$) upregulation of RNase T2 across multiple myeloid leukemia cell lines, including K562, THP-1, and HL60 (Fig. 4D–F). Additionally, we included (*BCR::ABL1*⁺) PDX-CML and (*RUNX1*-mutated) PDX-AML1 cells in the assay, which also

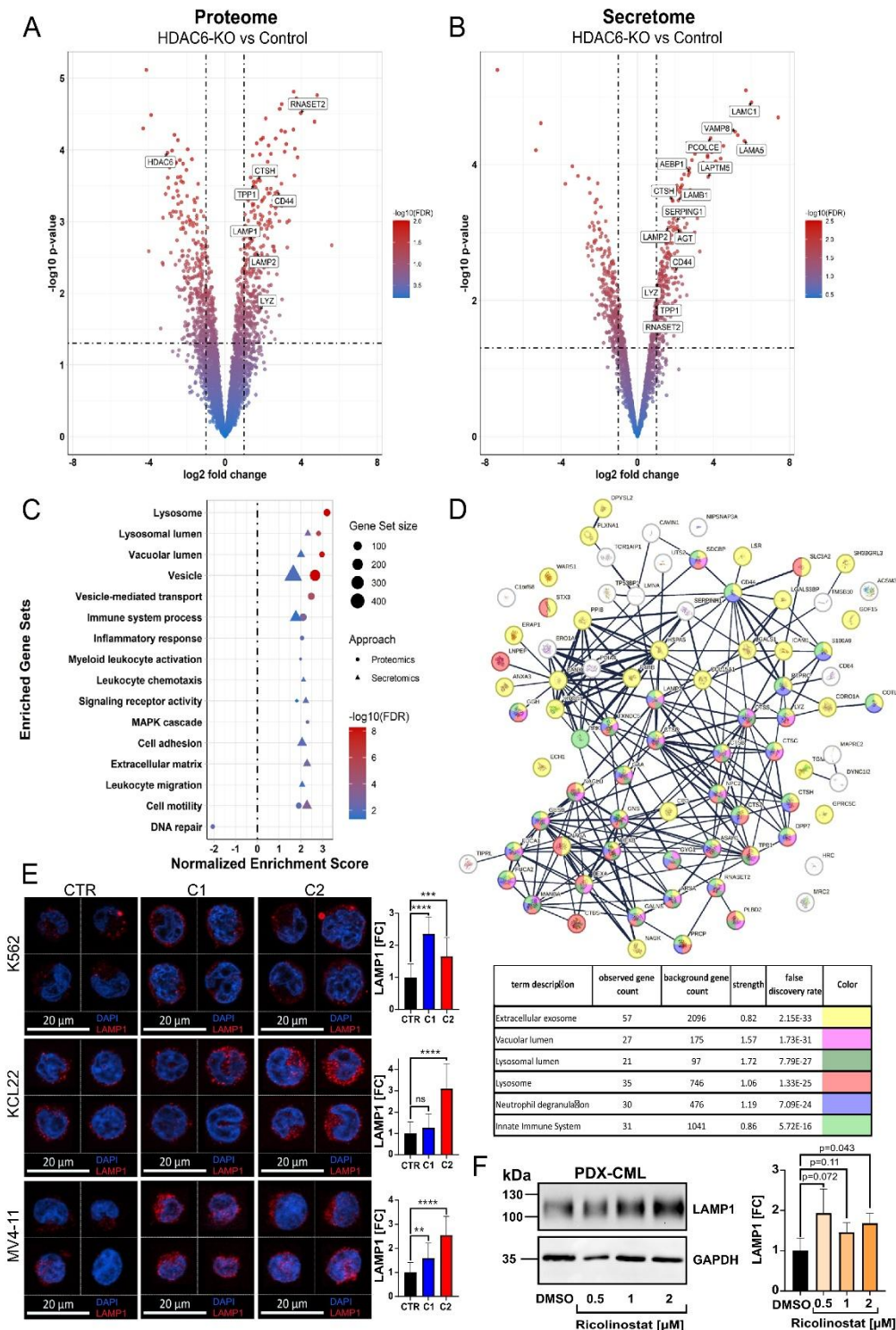
revealed a concentration-dependent increase in RNase T2 levels upon treatment with Ricolinostat (Fig. 4G, H). Immunofluorescence microscopy further confirmed that RNase T2 accumulated in a punctate pattern upon Ricolinostat treatment, in myeloid leukemia (K562, MV4-11, THP-1, and HL60) cells (Fig. 4I–K and Supplementary Fig. S4K). Consistently, short-term treatment with Ricolinostat or the other HDAC6 inhibitor Citarinostat resulted in increased RNase T2 transcription in AML-derived (*KMT2Ar*) THP-1 cells, as well as in (*BCR::ABL1*⁺) PDX-CML and (*KMT2Ar*) PDX-AML7 cells (Supplementary Fig. S4L, M).

These findings suggest that HDAC6 inhibition effectively induces a phenotype characterized by a low HDAC6/RNase T2 ratio. Notably, a survival analysis (using the TARGET dataset) showed that AML patients with a low HDAC6/RNase T2 ratio had significantly ($p = 0.0003$) improved survival outcomes in comparison to those with a high ratio, whereas the opposite trend was observed in the ALL dataset. (Supplementary Fig. S4N, O). Additionally, better survival in AML was associated with lower levels of anti-inflammatory monocytes and M2 macrophages (Supplementary Fig. S4P, Q). Taken together, HDAC6 regulates RNase T2 expression in myeloid leukemia cells.

HDAC6 inhibition sensitizes myeloid leukemia cells to CD8⁺ T cells

Based on our multi-omics and in vivo data suggesting that targeting HDAC6 affects immune-related signaling, we aimed to further explore its immunomodulatory role in a more immunocompetent in vivo setting. We therefore employed a syngeneic mouse model using C1498 cells, derived from C57BL/6 mice and classified as acute myelomonocytic leukemia [43]. Briefly, murine C1498-Luciferase-GFP+ cells were injected into immunocompetent C57BL/6 wild-type mice (Fig. 5A). After confirming engraftment, the mice were treated with four doses of Ricolinostat (50 mg/kg). Bone marrow, spleen, and lymph nodes were collected after the last treatment. A significant increase ($p < 0.05$) in TNF α production was observed in bone marrow-infiltrating T cells from Ricolinostat-treated mice, whereas only a modest (non-significant) elevation was detected in splenic T cells, and no changes were observed in lymph node T cells (Fig. 5B and Supplementary Fig. S5A, B). Similarly, CD107a (LAMP1) expression was upregulated in bone marrow T cells and unchanged in spleen- and lymph node-derived T cells (Fig. 5C and Supplementary Fig. S5C, D), a marker of CTL lytic granule release involved in target-cell killing [44].

To validate these findings, an ex vivo co-culture model was established with syngeneic murine CD8⁺ T cells (Fig. 5D). Treatment of C1498 cells with Ricolinostat induced a significant ($p < 0.01$) upregulation of GZMB, IL-2, and notably TNF α in CD8⁺ T cells (Fig. 5E). Moreover, the supernatant from Ricolinostat-treated C1498 cells was enough to elicit the same response in T cells, while Ricolinostat treatment alone did not significantly affect the expression of these markers (Fig. 5F and Supplementary Fig. S5E). Notably, treatment with Ricolinostat consistently enhanced CD8⁺ T cell-mediated cytotoxicity of C1498 cells (Fig. 5G). Consistent with observations in human myeloid leukemia cells, Ricolinostat treatment led to an upregulation of RNase T2 expression in murine C1498 cells (Supplementary Fig. S5F).



To further validate the involvement of HDAC6 in eliciting T cell responses, a C1498 HDAC6-KO model was generated (Supplementary Fig. S5G). While the cytokine response was less pronounced with the C1498 HDAC6-KO model, we still observed

a significant ($p < 0.05$) increase in CD8⁺ T cell susceptibility to cytotoxicity and cytokine responses, similar to the (wild-type) C1498 cells upon Ricolinostat treatment (Fig. 5H and Supplementary Fig. S5H, I). Next, to assess the therapeutic benefit of

Fig. 2 Targeting HDAC6 upregulates lysosome-related protein accumulation. Volcano plot depicting the cellular (A) or secreted (B) protein log2 fold change against the negative log₁₀(*p*-value) determined from five independent replicates of K562 HDAC6-KO (C1). Cut-off for the log2 fold change was set to >1, while for the $-\log_{10}(p\text{-value}) > 1.301$ (equals <0.05) was used. The color code represents the $-\log_{10}(\text{FDR})$ (Significance analysis of microarrays or SAM method, $n = 4-5$). **C** Dot plot showing the manually curated enriched gene sets obtained by a *clusterprofiler* analysis for both proteome and secretome results. The FDR cut-off for inclusion of protein entries was set to < 0.1 (Kolmogorov–Smirnov test, Benjamini–Hochberg correction). **D** Protein interaction network of the overlapping proteins that were enriched both in the proteomics and secretomics analysis via string. Inclusion criteria were a shared positive direction of enrichment and an FDR < 0.1 (hypergeometric test, Benjamini–Hochberg correction). **E** Fluorescence microscopy analyzing the localization and amount of LAMP1 in K562, KCL22, and MV4-11 HDAC6-KO models. Representative cells are shown, while in the right panel, the bar graph compares the fold change of fluorescence signal across all analyzed cells (unpaired *t*-test, ns not significant, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 20$ cells). **F** Western blot analysis of LAMP1 and GAPDH expression of PDX-CML cells treated with Ricolinostat, and in the right panel, quantification results normalized to GAPDH (unpaired *t*-test, $n = 3$).

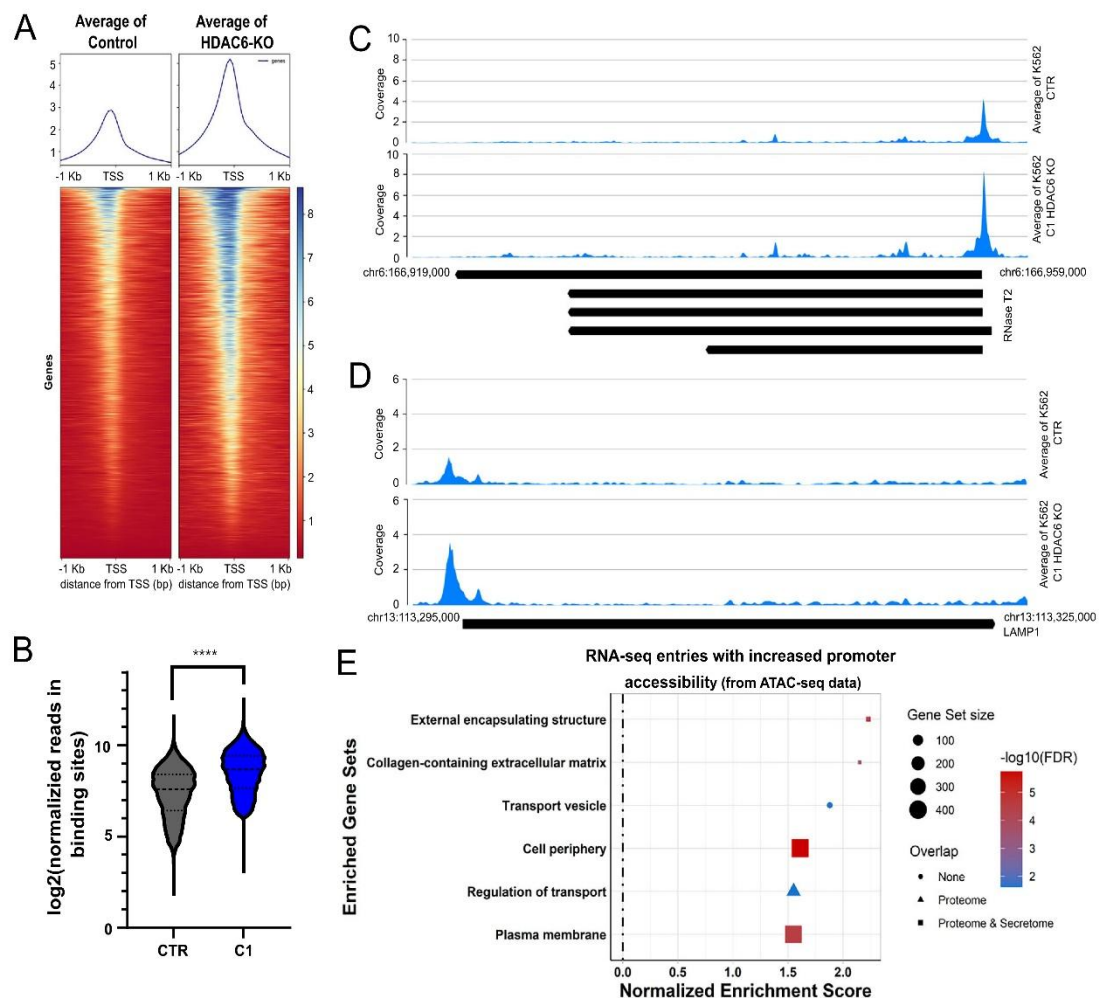
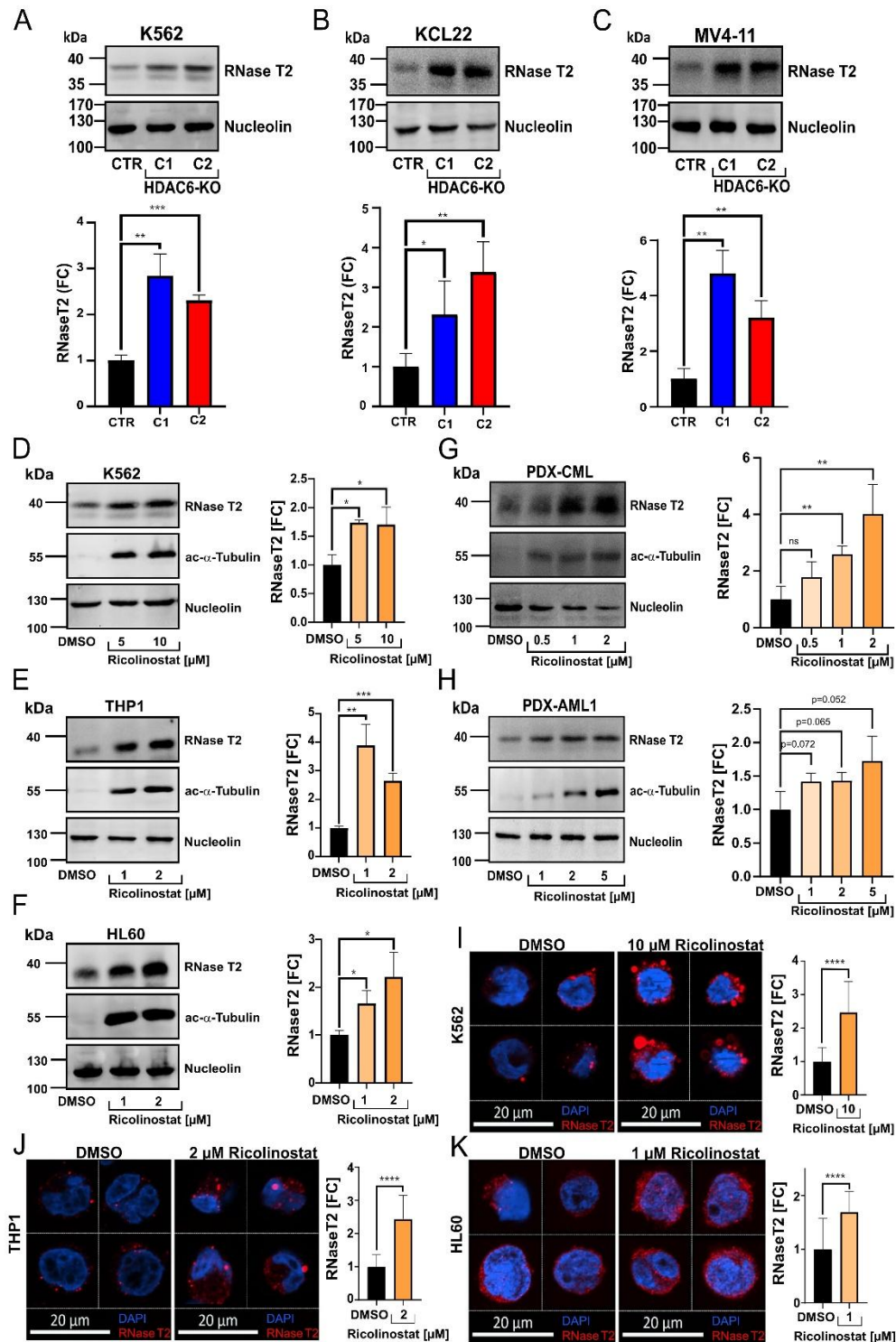


Fig. 3 HDAC6 inhibition enhances the accessibility at the LAMP1 and RNase T2 gene loci. **A** Heatmap illustrating the distribution of chromatin accessibility relative to the transcription start site (TSS) in control (CTR) and HDAC6-KO (C1) K562 cells, as determined by ATAC-seq ($n = 2$). **B** Violin plot comparing the log₂-normalized read density at the binding sites of all significantly enriched genes between CTR and HDAC6-KO (Mann–Whitney *U*-test, **** $p < 0.0001$, $n = 10,577$). ATAC-seq track plots illustrating chromatin accessibility at the RNase T2 (C) and LAMP1 (D) loci in CTR and HDAC6-KO K562 cells. **E** Dot plot showing the manually curated enriched gene sets obtained by a *clusterprofiler* analysis for the significant RNA-seq entries with significantly increased accessibility in the promoter region (FDR < 0.05). The shape of the dot indicates whether the term was also significantly upregulated in the Proteome or Secretome (Kolmogorov–Smirnov test, Benjamini–Hochberg correction).

Ricolinostat as a monotherapy, we treated C57BL/6J mice following engraftment with C1498-Luc-GFP+ cells over a prolonged period (3 weeks) and monitored leukemia progression (Fig. 5I). Although Ricolinostat treatment showed a trend toward

restricting leukemia progression, the results were not statistically significant, indicating that while HDAC6 inhibition enhances T cell-mediated immune responses (Fig. 5J, K), and its therapeutic benefit in vivo requires additional combination partners.



Targeting HDAC6 sensitizes myeloid leukemia cells toward standard chemotherapeutics, Cytarabine and Clofarabine

To enhance the therapeutic impact of HDAC6 inhibition and identify potential anti-cancer combination partners, we conducted

a synthetic lethality screen using HDAC6-KO (K562) cells (Fig. 6A). HDAC6-KO cells showed significantly ($p < 0.05$) increased sensitivity to several drug classes, including tyrosine kinase inhibitors (TKIs) and anti-metabolites such as Clofarabine, Cytarabine, and

Fig. 4 Targeting HDAC6 induces the expression of tumor suppressor RNase T2 in myeloid leukemia cells. Western blot analysis of RNase T2 expression in HDAC6-KO myeloid leukemia models: K562 (A), KCL22 (B), and MV4-11 (C), and in the lower panel, quantification results in the form of a bar graph (unpaired *t*-test, ***p* < 0.01, ****p* < 0.001, *n* = 3). Western blot analysis of RNase T2 and acetylated α -tubulin levels in K562 (D), THP-1 (E), HL60 (F), PDX-CML (G), and PDX-AML1 (H) cells following 24 h of treatment, or 48 h for HL60, with the HDAC6 inhibitor Ricolinostat at the indicated concentrations. The right panel shows the results of the quantification normalized to housekeeper Nucleolin (unpaired *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *n* = 3). Fluorescence microscopy analysis of RNase T2 localization and expression in K562 (I), THP-1 (J), and HL60 (K) cells treated with Ricolinostat for 24 h at the indicated concentrations. Representative cells are shown, while in the right panel, the bar graph compares the fold change of fluorescence signal across all analyzed (*n* = 20) cells (unpaired *t*-test, ns not significant, *****p* < 0.0001).

5-Azacytidine. Given the clinical relevance of Cytarabine in treating AML, we examined its effects alongside Clofarabine. Both drugs exhibited enhanced cytotoxicity in HDAC6-KO cells, with Clofarabine showing a particularly strong reduction in proliferation (Fig. 6B and Supplementary Fig. S6A). HDAC6-KO cells also displayed increased apoptosis induction (*p* < 0.01) and sub-G1 accumulation following treatment with Cytarabine or Clofarabine (Fig. 6C, D, and Supplementary Fig. S6B, C). Elevated levels of PARP cleavage (*p* < 0.05) following treatment with Cytarabine or Clofarabine further confirmed enhanced apoptotic responses in HDAC6-KO cells compared to corresponding controls (Fig. 6E). These findings suggest that HDAC6 loss can enhance the sensitivity to nucleoside analogs.

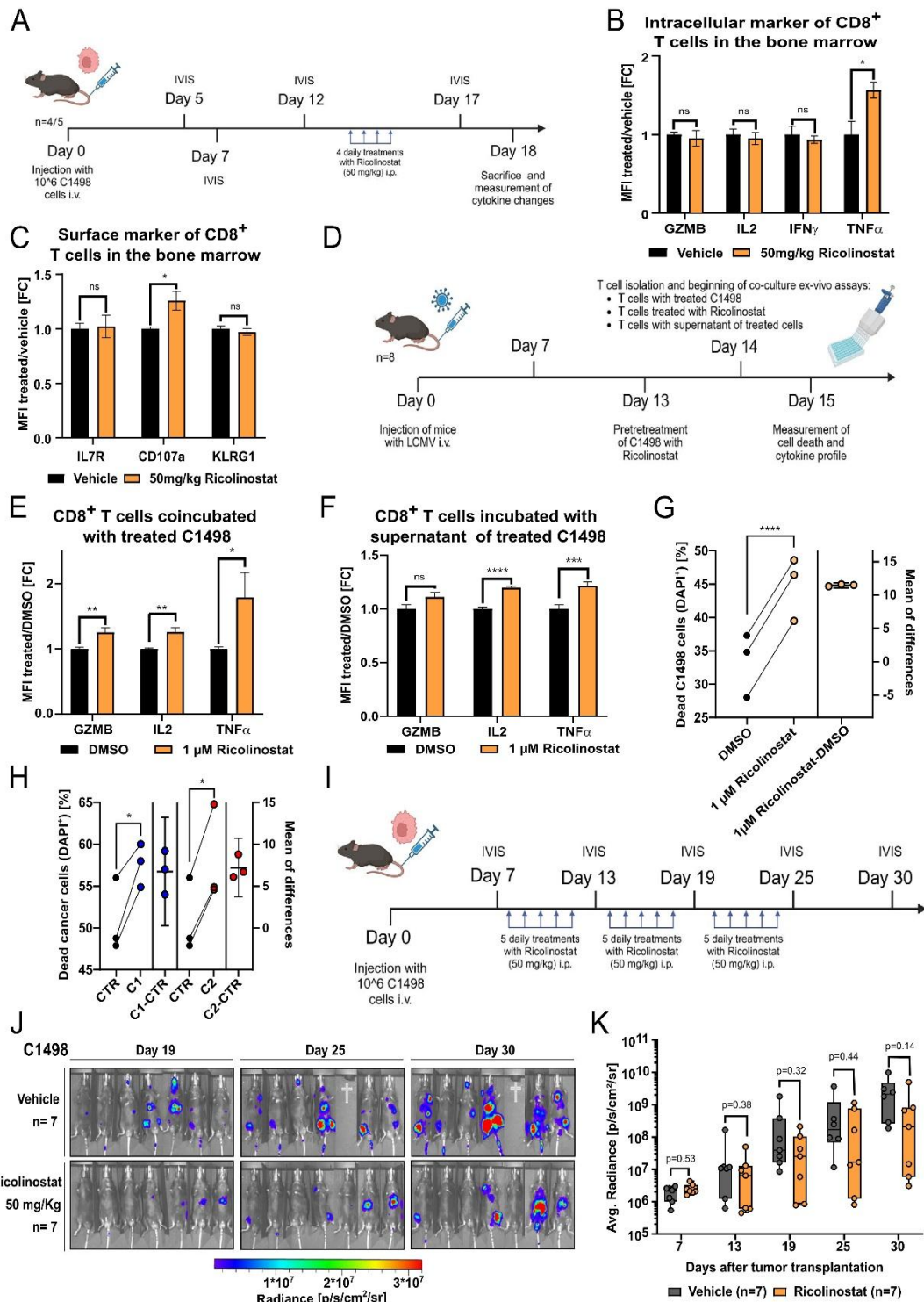
To validate these findings, we investigated potential synergistic interactions between Ricolinostat and Cytarabine or Clofarabine. Both Cytarabine and Clofarabine exhibited strong synergistic effects with Ricolinostat in K562 and MV4-11 cells (Fig. 6F, G). Encouraged by these findings, we extended our investigation to a broader cohort, including (*n* = 10) AML, (*n* = 4) B-ALL, and (*n* = 4) T-ALL cell lines. Additionally, we included PDX-AML/CML cells derived from initial or relapsed pediatric/adult patients (*n* = 8), along with PDX-B-ALL (*n* = 5), PDX-T-ALL (*n* = 7), and healthy donor-derived PBMCs (*n* = 4), fibroblasts (*n* = 2), mesenchymal stem cells (MSCs), and cord blood-derived CD34⁺ cells as controls (Fig. 6H, I, and Supplementary Fig. S6D, E). Notably, myeloid lineage-derived leukemia cells demonstrated heightened sensitivity to the HDAC6 inhibitor Ricolinostat, when combined with Clofarabine or Cytarabine, compared to B-ALL, T-ALL, and healthy control samples. Next, to investigate mechanisms associated with the observed synergy between HDAC6 inhibitor (Ricolinostat) and Clofarabine or Cytarabine, we correlated publicly available transcriptomic profiles (from DepMap portal, 25Q2) of AML and CML cell lines with experimentally determined (ZIP) synergy scores (Fig. 6J and Supplementary Fig. S6F). This analysis revealed a positive correlation between synergistic response and gene sets involved in the negative regulation of DNA double-strand break repair via non-homologous end joining, as well as cellular oxidant detoxification pathways. These findings suggest that impaired DNA repair capacity and elevated oxidative stress may enhance the efficacy of combination therapy. Conversely, a negative correlation was observed with TP73-mediated transcription factor activation, a member of the p53 family involved in DNA damage response and apoptosis regulation. To experimentally interrogate these findings, HDAC6-KO (K562 and MV4-11) cells were treated with Cytarabine and Clofarabine (Supplementary Fig. 6G, H). In both models, HDAC6 ablation resulted in a pronounced induction of TP73 protein levels relative to control cells, consistent with activation of a compensatory DNA damage response upon genotoxic stress [45, 46]. Together, these data point to a potential mechanistic link between HDAC6 inhibition and DNA damage response to enhance chemotherapeutic efficacy upon HDAC6 inhibition in myeloid leukemias.

DISCUSSION

Despite substantial progress in elucidating the molecular pathogenesis of AML, therapeutic strategies have remained

largely unchanged, relying predominantly on conventional chemotherapy and allogeneic hematopoietic stem-cell transplantation [3, 47–49]. Therefore, there is a pressing need to identify and develop novel therapeutic strategies. HDAC6 has emerged as a promising anti-cancer target [15]. The present study reveals a critical discrepancy between the *in vitro* and *in vivo* functions of HDAC6 in myeloid leukemia, challenging the prevailing understanding of HDAC6's role in hematologic malignancies. While previous *ex vivo* studies have demonstrated minimal cytotoxic effects of selective HDAC6 inhibition on cancer cells [28–30], our comprehensive analysis using public datasets, CRISPR-mediated knockout models, and *in vivo* xenograft studies provides compelling evidence that HDAC6 plays a significant role in (myeloid) leukemia progression within the physiological context of the tumor microenvironment. The absence of significant differences in proliferation rates, colony formation capacity, and cell cycle distribution between HDAC6-KO and control cells *in vitro* corroborates previous findings and explains why *ex vivo* screening approaches (like DepMap Portal) may have overlooked HDAC6 as a therapeutic target. However, the significant reduction in leukemia progression observed in NSG mice transplanted with HDAC6-KO leukemia cells demonstrates that HDAC6's essential functions become apparent only within the complex *in vivo* environment, potentially via modulation of innate immune pathways that remain active in the NSG mice model [50].

Our proteomic analyses reveal that HDAC6 loss results in significant enrichment of lysosome-associated proteins, including LAMP1, LAMP2, and RNase T2, consistent with HDAC6's established role in coordinating aggresome transport and autophagosome-lysosome fusion through cactactin and cytoskeletal interactions [51]. HDAC6 facilitates lysosomal transport along microtubules via dynein-mediated mechanisms, enabling lysosome-autophagosome co-localization and fusion [52, 53]. Loss of HDAC6 can therefore lead to lysosomal accumulation, as reported in this study showing increased LAMP2 levels and reduced autophagic activity upon HDAC6 knockdown [54]. The disruption of these interconnected pathways upon HDAC6 loss may contribute to altered lysosomal protein expression and secretion, where lysosomal accumulation could arise from both impaired trafficking and compensatory biogenesis. Further, our ATAC-seq analyses demonstrate increased chromatin accessibility at LAMP1 and RNase T2 loci, potentially providing a supplementary transcriptional mechanism for lysosomal protein upregulation. However, we did not observe a significant increase in the lysosomal protease CTSH in MV4-11 HDAC6-KO cells, as seen in contrast to proteomics studies in K562 cells, indicating that HDAC6 loss exerts selective rather than uniformly affecting all lysosomal components, which may vary across genetically distinct myeloid leukemia contexts. Importantly, the extracellular release of lysosomal enzymes, as evidenced by secretome analysis, could potentially alter the local microenvironment and enhance immune cell function. Supporting this, our STRING analysis revealed enrichment of neutrophil degranulation and innate immune pathways among overlapping proteome-secretome proteins, indicating potential immunomodulatory effects of HDAC6 loss [55]. Collectively, the integrated multi-omics analysis reveals



enrichment of cell periphery and plasma membrane-associated gene sets, suggesting that HDAC6 inhibition may modulate surface receptor expression and leukemia-microenvironment interactions.

RNase T2, a lysosomal endonuclease and evolutionarily conserved component of the innate immune system that functions as an alarmin-like molecule within the tumor microenvironment to promote macrophage recruitment and pro-

Fig. 5 HDAC6 inhibition sensitizes myeloid leukemia cells to CD8⁺ T cells. **A** Experimental timeline of Ricolinostat (50 mg/kg) treatment following the injection of murine C1498-Luc-GFP + AML cells into wild-type C57BL/6 mice to determine CD8⁺ T cell marker level. Bar graph comparing the fold change in mean fluorescence intensity (MFI) of intracellular (**B**) or surface (**C**) marker of CD8⁺ T cells from the bone marrow after Ricolinostat treatment against the control (unpaired *t*-test, ns not significant, **p* < 0.05, *n* = 4–5). **D** Experimental timeline for the harvest of primed CD8⁺ T cells from C57BL/6 mice following Lymphocytic Choriomeningitis Virus (LCMV) injection, followed by ex vivo co-culture with C1498 cells. Bar graph showing the fold change in MFI of quantified cytokines from CD8⁺ T cells co-incubated with C1498 cells treated for 24 h with 1 μM Ricolinostat (**E**) or the supernatant from Ricolinostat-treated C1498 cells (**F**) (unpaired *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, *n* = 16). **G** Line plot showing the fraction of dead C1498 cells when co-incubated with CD8⁺ T cells, pretreated with Ricolinostat or DMSO (paired *t*-test, *****p* < 0.0001, *n* = 3). **H** Similar to (**G**), but using C1498 HDAC6-KO cells (in two clones, C1 or C2) vs non-targeting control (CTR). **I** Experimental timeline of Ricolinostat (50 mg/kg) treatment following the injection of murine C1498-Luc-GFP + AML cells into wild-type C57BL/6 mice to determine disease progression. **J** In vivo growth kinetics of C1498 cells in C57BL/6 mice treated with Ricolinostat, assessed at 19-, 25-, and 30-days post-injection via luminescence detection. **K** Quantification of the average radiance from (**J**) (Mann–Whitney *U*-test, *n* = 7).

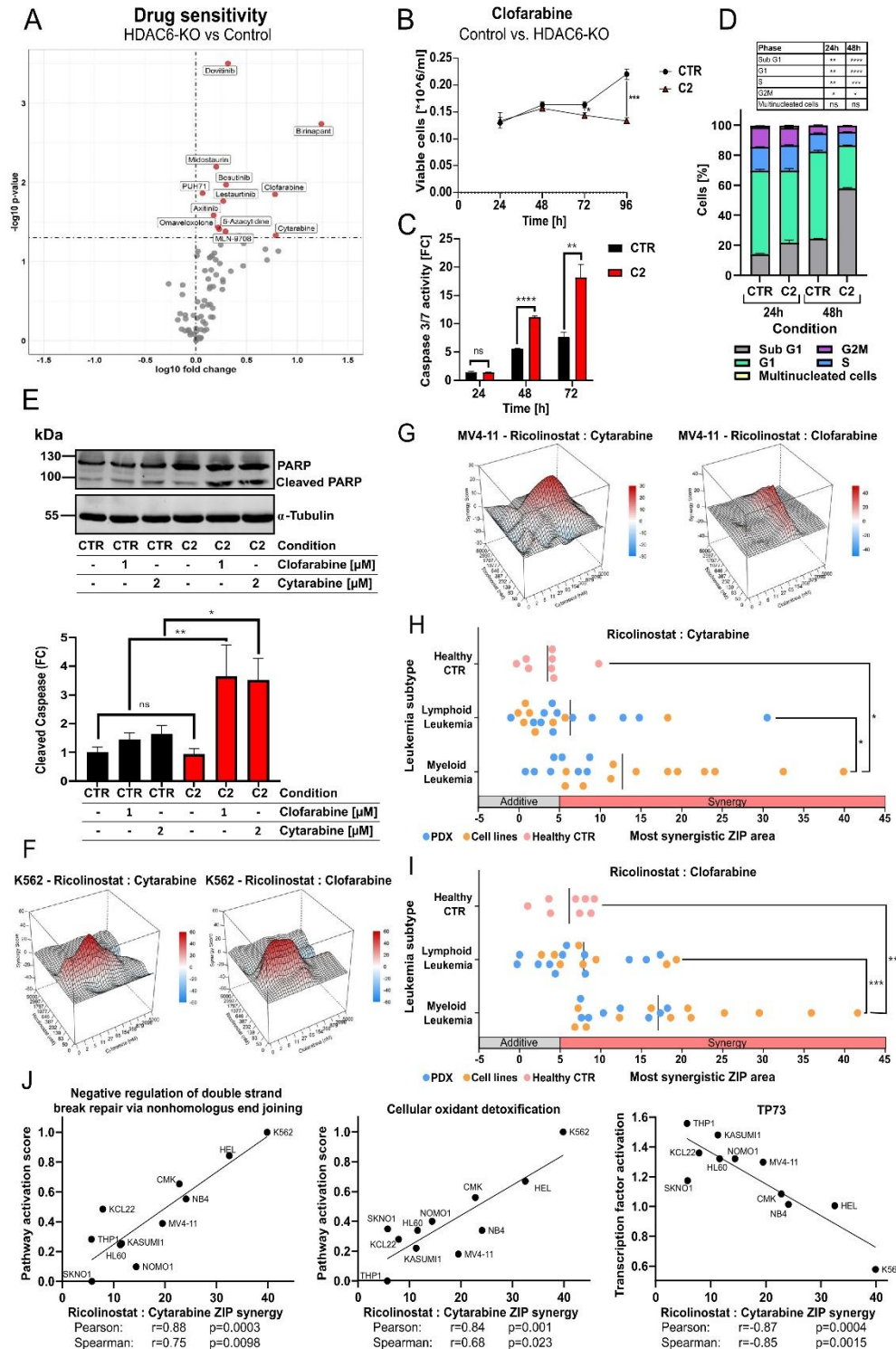
inflammatory activation [41, 42], emerges as a novel mediator in the anti-leukemic effects of HDAC6 targeting. We observed consistent upregulation of RNase T2 across all HDAC6-KO myeloid leukemia models, coupled with its elevated secretion into the extracellular environment, suggesting that HDAC6 normally functions to suppress this tumor suppressor. The reversibility of RNase T2 upregulation upon HDAC6 reintroduction in knock-in experiments confirms the direct regulatory relationship between these proteins. Notably, this regulatory axis is lineage-specific, as evidenced by differential responses observed when modeling HDAC6 loss or knockdown in ALL versus AML cells, which was further supported by distinct correlation patterns between HDAC6 and RNase T2 expression in myeloid versus lymphoid leukemias from public datasets. Supporting this, survival analysis revealed that AML patients with a low HDAC6/RNase T2 ratio had significantly improved survival compared to those with a high ratio, with the opposite trend observed in ALL patients. Additionally, a better survival in AML was associated with lower levels of anti-inflammatory monocytes and M2 macrophages, consistent with previous findings where HDAC6 inhibition or RNase T2 upregulation promotes macrophage polarization toward a pro-inflammatory phenotype [40, 56]. However, the molecular basis underlying this lineage-specificity remains unclear and warrants further investigation to elucidate whether differential epigenetic landscapes or lineage-restricted cofactors contribute to the divergent HDAC6-RNase T2 regulatory dynamics between myeloid and lymphoid leukemias. Further, pharmacological validation with Ricolinostat across myeloid leukemia cell lines and PDX models supports the translational relevance of the HDAC6-RNase T2 axis. We also observed increased RNase T2 mRNA levels in PDX-AML/CML samples upon Ricolinostat treatment, although the magnitude of induction varied, likely reflecting underlying genetic and epigenetic heterogeneity among individual samples. However, it is important to note that Ricolinostat is not an HDAC6-selective inhibitor, and its effects may also result from off-target actions or inhibition of other HDACs, making it difficult to attribute all observed effects solely to HDAC6 inhibition [57, 58]. Accordingly, these findings should be interpreted with caution. To ensure that the observed effects were attributable to on-target HDAC6 inhibition rather than compound-specific artifacts, we included another HDAC6 inhibitor, Citarinostat, as an orthogonal pharmacological validation. Nevertheless, additional validation using highly selective HDAC6 inhibitors, together with acetylation readouts of proteins not regulated by HDAC6, would strengthen the specificity controls and help confirm whether the observed regulation of RNase T2, and the associated anti-leukemic effects, are indeed HDAC6-dependent.

Extending our findings to immunocompetent models, HDAC6 inhibition enhanced CD8⁺ T cell activation [59], through TNFα induction and upregulation of CD107a (or LAMP1). The significant increase in TNFα production and CD107a expression specifically in bone marrow-infiltrating T cells from Ricolinostat-treated mice indicates that HDAC6 inhibition enhances T cell activation and

cytolytic function in the primary site of leukemia development. Consistently, we have observed an increase in lysosome-associated proteins upon targeting HDAC6, which aligns with emerging evidence that the lysosome plays a crucial role in regulating the cytotoxic activity of CD8⁺ T cell immunity [60]. Moreover, in our ex vivo co-culture assays, Ricolinostat alone elicited only a modest, non-significant increase in TNF-α in CD8⁺ T cells, indicating limited direct activation. In contrast, markedly stronger cytokine and effector responses were observed when T cells were exposed to Ricolinostat-treated (C1498) leukemia cells or their supernatants, supporting a leukemia cell-mediated mechanism, while not fully excluding direct effects of HDAC6 inhibition on T cells [61, 62]. The enhanced CD8⁺ T cell-mediated cytotoxicity observed upon Ricolinostat treatment represents a potential therapeutic option by which HDAC6 inhibition may improve myeloid leukemia outcomes in vivo. However, while Ricolinostat monotherapy showed a trend toward reduced (C1498) leukemia progression in an immunocompetent mouse model, the lack of statistical significance suggests that HDAC6 inhibition alone is insufficient for therapeutic efficacy and requires combination strategies.

Next, to identify potential therapeutic partners for HDAC6 inhibition, we performed a synthetic lethality screen and validation experiments in HDAC6-KO K562 cells to establish proof-of-concept that loss of HDAC6 sensitizes cells to nucleoside analogs (Cytarabine and Clofarabine). Building on this, pharmacological validation combining Ricolinostat with these agents across multiple AML and ALL models demonstrated stronger and more consistent synergistic effects in AML compared to ALL, with minimal impact on healthy controls. These findings align with prior reports that dual HDAC1/HDAC6 inhibition enhances cytarabine responses in AML [34] and are further supported by our transcriptomics and proteomics showing downregulation of DNA repair pathways in HDAC6-KO cells [45, 46]. Furthermore, we demonstrate that loss of HDAC6 enhances TP73 levels in response to Cytarabine and Clofarabine, which may reflect engagement of a protective, TP73-dependent stress response. This observation is consistent with our pathway analysis, revealing a negative association between TP73-mediated transcriptional programs and synergistic drug responses. However, as these findings were derived from limited cell line models, the mechanistic relationship between HDAC6 inhibition, TP73 activation, and DNA damage response remains preliminary. Future studies should extend these observations across additional myeloid models and incorporate functional assays to directly interrogate DNA repair pathways.

Further analysis of over 1500 leukemia patient samples (from the St. Jude PeCan database) revealed more heterogeneous HDAC6 expression patterns in AML compared to B-ALL and T-ALL (coefficient of variation: AML = 0.70 vs. B-ALL = 0.48 vs. T-ALL = 0.39) (Supplementary Fig. S6I). Given this heterogeneous HDAC6 expression across AML subtypes, future studies should prioritize identifying molecularly defined patient subgroups with enhanced



sensitivity to HDAC6 inhibition. Our data mining analyses demonstrated that high HDAC6 expression is particularly associated with poor prognosis in AML cases harboring NRAS mutations, as well as in FLT3- and IDH1-mutated subtypes

(Supplementary Fig. S1E–H). These findings suggest that specific genetic contexts may confer differential dependency on HDAC6, warranting further investigation to establish biomarker-driven therapeutic strategies for HDAC6 targeting in genetically stratified

Fig. 6 Targeting HDAC6 sensitizes myeloid leukemia cells toward standard chemotherapeutics Cytarabine and Clofarabine. **A** Volcano plot depicting the $\log_{10}(\text{IC}_{50} \text{ FC})$ and the $-\log_{10}(\text{p-value})$ between K562 CTR and K562 HDAC6-KO (C1 and C2). As cut-off was $-\log_{10}(\text{p-value}) > 1.301$ (equals < 0.05) used (unpaired *t*-test, $n = 3-6$). **B** Proliferation curve determined by trypan blue assay of K562 CTR and K562 HDAC6-KO C2 treated with 1 μM Clofarabine over the course of 96 h (unpaired *t*-test, $*p < 0.05$, $***p < 0.001$, $n = 3$). **C** Bar plot of the fold change of the caspase 3/7 activity of K562 CTR and K562 HDAC6-KO C2 treated with 1 μM Clofarabine over the course of 72 h (unpaired *t*-test, ns not significant, $**p < 0.05$, $****p < 0.0001$, $n = 3$). **D** Bar plot showing a cell cycle analysis of K562 HDAC6-KO models treated with Clofarabine. Statistical significance was determined via an unpaired *t*-test (ns not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, $n = 3$). **E** Western Blot analysis of PARP and cleaved PARP of K562 HDAC6-KO models treated with 1 μM Clofarabine or 2 μM Cytarabine. The lower panel bar graph shows the results of the quantification of cleaved PARP normalized to the housekeeper α -tubulin (unpaired *t*-test, $*p < 0.05$, $**p < 0.01$, $n = 3$). Matrix synergy plots for two leukemia cell lines. In K562 cells (**F**) and MV4-11 cells (**G**), the plots show the effects of increasing concentrations of Ricolinostat combined with increasing concentrations of Cytarabine or Clofarabine. Zero interaction potency (ZIP scores) indicate synergy for the pairings: Ricolinostat:Cytarabine in K562 (39.9) and MV4-11 (19.5); Ricolinostat:Clofarabine in K562 (41.6) and MV4-11 (21.1). Dot plot summarizing the most synergistic ZIP scores of AML/CML cell lines ($n = 12$), patient-derived PDX-AML/CML cells ($n = 8$), ALL cell lines ($n = 8$), PDX-ALLs ($n = 12$), and healthy controls ($n = 8$), when treated with Ricolinostat and Cytarabine (**H**) or Ricolinostat and Clofarabine (**I**) combination. Statistical significance was determined using an unpaired *t*-test (ns not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). **J** Pathway and transcription factor activities were inferred from gene expression data of AML and CML cell lines (from DepMap portal, 25Q2) and correlated with ZIP synergy scores of the Ricolinostat and Cytarabine combination.

AML patient populations. Together, our study provides compelling evidence that HDAC6 inhibition enhances immune-mediated leukemia cell clearance and sensitizes myeloid leukemia cells to chemotherapeutic agents. Given the minimal toxicity associated with HDAC6 inhibition, these findings support its potential use in combination with emerging immunotherapeutic strategies to enhance anti-leukemic efficacy.

MATERIALS AND METHODS

Cell culture

Leukemia cell lines were obtained from the DSMZ (Braunschweig, Germany) and cultured according to their recommendations. RPMI1640 GlutaMAX (Gibco) was supplemented with 10–20% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich). Cell lines were regularly authenticated by short tandem repeat profiling and tested for mycoplasma. A detailed list of cell lines used in the study is provided in Supplementary Table 1.

CRISPR-Cas9-mediated knockout (KO) or shRNA-mediated knockdown (KD) of HDAC6

HDAC6-KO models were generated either through lentiviral transduction or using nucleofection of CAS9-GFP (IDT) [63]. For monoclonal selection, cells were plated in semi-solid MethoCult media (Stemcell Technologies). See Supplementary Methods for sequences and more details.

Western blotting

Western blotting was performed as previously described [63]. For the list of antibodies and their respective concentrations, refer to the Supplementary Methods.

Proliferation, cell cycle, and apoptosis assays

Proliferation (via Trypan Blue staining), cell cycle (via Nicoletti method), and apoptosis assay (via measuring Caspase 3/7 activity) were performed as described earlier [63].

Colony-forming unit (CFU) assay

Leukemia cells were seeded in a methylcellulose-based medium. After seven days, colonies were counted and imaged, following previously described protocols [64].

Drug screening

Ex vivo high-throughput drug screening was performed as described earlier [63, 65]. The compound selection comprised FDA/EMA-approved chemotherapeutics and targeted agents commonly used in leukemia treatment protocols, along with inhibitors in various stages of clinical trials. Compounds were dispensed in a randomized layout using a digital dispenser (D300e, Tecan). A detailed list of drugs is provided in Supplementary Table 2. ZIP scores for the matrix synergy approach were calculated via the *Synergyfinder* R package.

qPCR

Total RNA was extracted using QIAzol and purified with the Maxwell RSC system. cDNA was synthesized from 2 μg RNA using the QuantiTect kit, and qPCR was performed on a Bio-Rad system with B2M and GAPDH as internal controls. For primer sequences and additional details, refer to Supplementary Methods.

Fluorescence microscopy

Cells were seeded on poly-D-lysine-coated coverslips, fixed, permeabilized, and stained with primary/secondary antibodies and DAPI. Imaging was performed with a Zeiss Axio Observer/Apoptome 3, and signal quantification was done using ImageJ. For more details, refer to Supplementary Methods.

Murine NSG transplantation

Prior to the injection, K562 and MV4-11 cells were transduced with a luciferase reporter plasmid to measure the leukemic burden via the in vivo imaging system IVIS (PerkinElmer) [63]. Cells were intravenously injected into 6–8 weeks old immunodeficient male or female NSG mice (Jackson Laboratory, #005557). For patient-derived xenograft (PDX) generation, leukemia cells from peripheral blood or bone marrow were injected into NSG mice (see Supplementary Table 3 for patient details), with engraftment monitored via FACS analysis of blood collected through orbital bleeding [63, 66]. Patient samples were obtained with informed consent in accordance with the Declaration of Helsinki, with approval from the local ethics committees at the sites in Düsseldorf, Vienna, and Lodz. All animal experiments were conducted in accordance with the regulatory guidelines of the official committee at LANUV (NRW), under the authorization of the animal research institute (ZETT) at the Heinrich Heine University Düsseldorf.

Murine C57BL/6J experiments

Wildtype male C57BL/6J (Charles River) mice were utilized to establish a murine C1498 AML (ATCC) derived syngeneic mouse model [67]. C57BL/6J wild-type mice were engrafted with (0.5×10^6) cells/mouse luciferase reporter gene transduced C1498 murine AML cell line at 6–7 weeks of age. Engraftment was confirmed 7 days after injection of C1498 leukemia cells using IVIS (PerkinElmer), and mice were then randomly assigned to treatment groups ($n = 7$ mice/arm). No blinding or randomization was performed. Group sizes were not predefined by statistical calculation and were selected based on previous experiments and pilot data. Monitoring of the leukemic burden and treatment with Ricolinostat followed the respective schedule. For more details, refer to the Supplementary Methods.

T cell co-culture assay

C57BL/6J mice were infected with the Lymphocytic Choriomeningitis Virus (LCMV) strain, which induces a strong effector CD8⁺ T cell response and is efficiently cleared in wild-type mice shortly after infection [67, 68]. LCMV-primed splenic T cells were isolated after two weeks post infection. C1498 cells were pretreated with Ricolinostat before co-culture with T cells. Supernatant-only and direct treatment conditions were also included. After

24 h, cells were analyzed by flow cytometry for viability and cytokine expression. For more details, refer to Supplementary Methods.

NK cell killing assay

PBMCs from healthy donors were cultured with IL-2 and IL-15. CFSE-labeled target cells were co-incubated with PBMC-derived NK cells for 4 h, followed by flow cytometric analysis to assess NK cell-mediated cytotoxicity. For more details, refer to the Supplementary Methods.

RNA-sequencing (RNA-seq), mass spectrometry (MS) based proteome and secretome analysis

RNA-seq and quantitative MS-based proteome and secretome analysis were performed as previously described [63]. For sample preparation, cultures were maintained at a medium density for several days to harmonize viability and growth rates.

ATAC-seq

The library preparation, sequencing, and initial consensus sequencing were performed according to the protocol of Active Motif. For more details, refer to Supplementary Methods.

Data mining

The HDAC6 dependency data was extracted from the Achilles gene dependency project of the DepMap (22Q1) database and visualized via a complex heatmap [69]. The expression data of pediatric patients was extracted from the PeCan platform of St. Jude [70]. Expression was either clustered by leukemia subtype or separated by the median of HDAC6 expression. The survival analysis was performed using the Survival Genie 2 platform [71], utilizing data generated by the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative (<https://www.cancer.gov/ccg/research/genome-sequencing/target>), phs000218 [31]. The data used for this analysis are available through the Genomic Data Commons (<https://portal.gdc.cancer.gov>). Samples were clustered by their optimal cutting point algorithm. Mutation clustered survival analysis was performed via Kaplan–Meier-plotter [32], which utilizes the TCGA-LAML dataset. Patients with IDH1, NRAS, or FLT3 mutation were split by a percentile-based best cut-off selection.

Pathway activity scores were inferred using Pathifier [72], and transcription factor activity was estimated using VIPER with the DoRothEA regulon [73], based on gene expression data from AML and CML cell lines in the DepMap (25Q2) dataset. The resulting activity profiles were then correlated with ZIP synergy scores of the Ricolinostat and Cytarabine or Clofarabine combination using both Pearson and Spearman correlation coefficients.

Statistics

Experiments were reproduced at least three times, unless specified otherwise in the respective section. Comparisons between two groups were evaluated using unpaired or paired two-tailed *t*-tests, whereas non-parametric comparisons of in vivo bioluminescence data were assessed using the Mann–Whitney *U*-test. Kaplan–Meier survival curves for patient datasets and mouse studies were analyzed using the log-rank test. For multi-omics datasets, significance thresholds were defined by false discovery rate (FDR) correction using the Benjamini–Hochberg method, including Significance Analysis of Microarrays (SAM) method for proteomics and secretomics, and quasi-likelihood *F*-tests for ATAC-seq differential accessibility. Gene set enrichment was assessed using Kolmogorov–Smirnov tests with FDR correction. For immunoblotting, band intensity values were normalized to the average intensity of each protein across the blot, adjusted to the corresponding loading control, and expressed as fold change relative to the mean of the respective control ($n \geq 3$). Data are presented as mean $\pm$ SD. For immunofluorescence imaging, twenty representative cells were randomly selected from at least three independent images and quantified using ImageJ. IC₅₀ values from the drug screening were derived using GraphPad Prism. Drug interaction analyses were performed using the Zero Interaction Potency (ZIP) model, and absolute synergy scores were summarized across all the tested samples. Statistical methods, cutoffs, and replicate numbers for analyses are provided in the respective figure legends.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

RNA-seq and ATAC-seq data have been deposited in the NCBI GEO database under accession ID: GSE297364 and GSE297366. Mass spectrometry-based proteomics and secretomics data are available via the ProteomeXchange Consortium through the PRIDE repository with dataset identifier PXD041871.

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AUTHOR CONTRIBUTIONS

JS-D, JT, and SB performed the study concept and design. JS-D, JT, PS, KS, MK, EV, NR, MV, DB, TL, AN, and SB performed the development of methodology, investigation, analysis, and interpretation of data. SS, PD, AS, KN, KS, UF, AAP, and AB contributed to resource provision, supervision, funding acquisition, and project administration. JS-D, JT, AB, and SB performed writing, reviewing, and editing of the paper. SB supervised this study. All authors discussed the results and commented on the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

Patient samples were collected after obtaining written informed consent in accordance with the Declaration of Helsinki and with approval from the respective local ethics committees of the Medical Faculty at University Hospital Düsseldorf (Germany), the Medical University of Vienna (Austria), and the Medical University of Lodz (Poland). All animal experiments were performed in compliance with institutional and governmental regulations and were approved by the responsible regulatory authority (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, LANUV, NRW, Germany) and the animal research institute (ZETT) of Heinrich Heine University Düsseldorf.

ADDITIONAL INFORMATION

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Epigenetic remodeling via HDAC6 inhibition amplifies anti-tumoral immune responses in myeloid leukemia cells

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Running Title:

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1. Supplemental Figures:

Figure S1

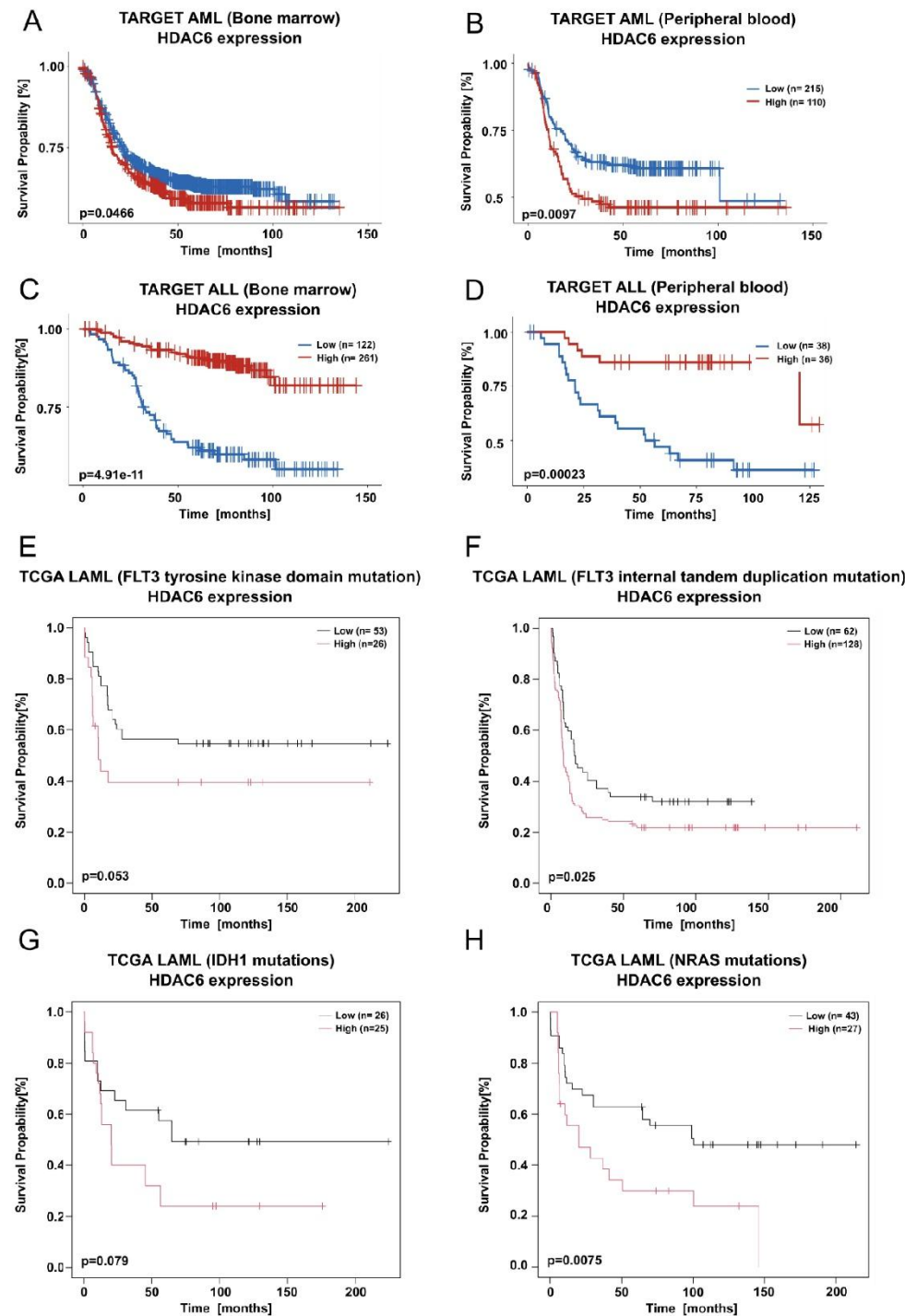
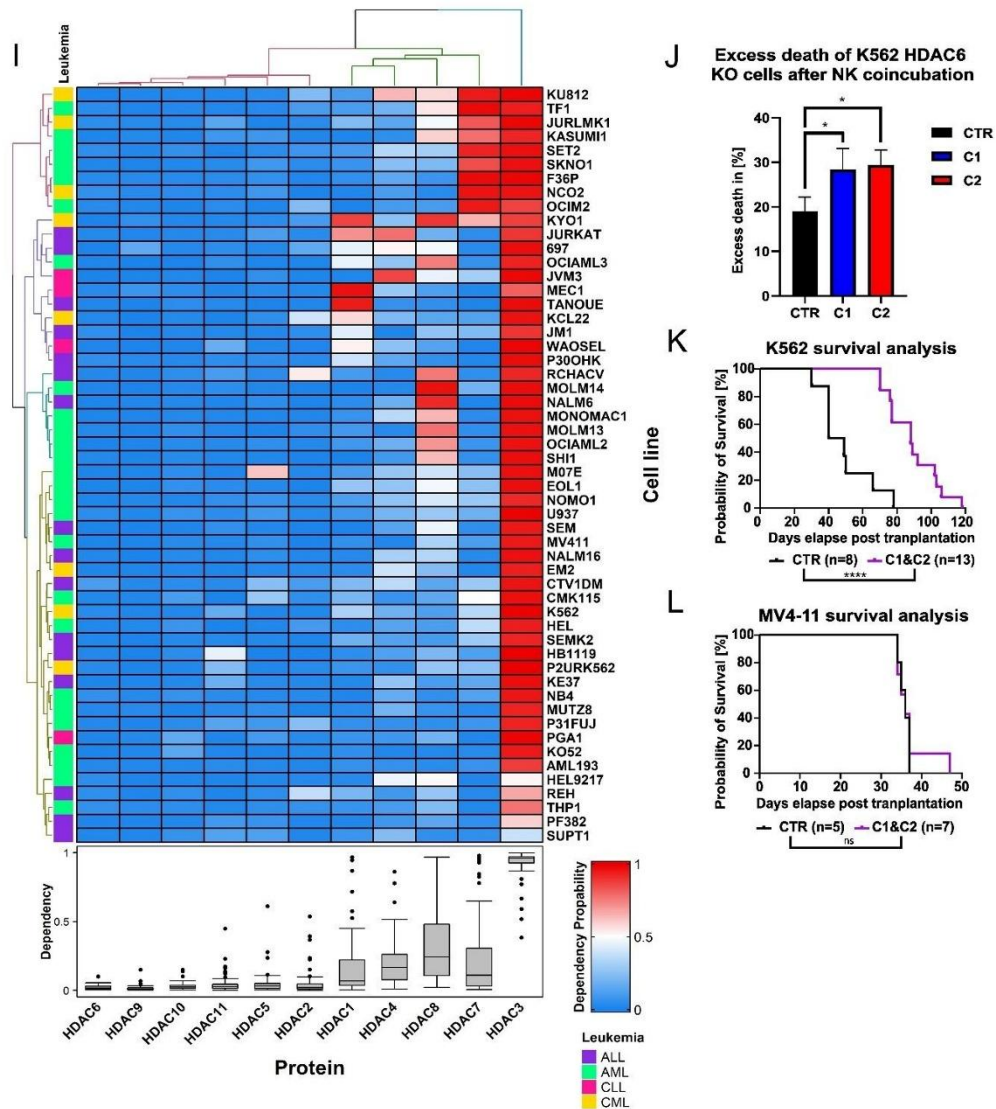


Figure S1 (continued)



Supplemental Figure 1. HDAC6 ablation impairs the in vivo growth of myeloid leukemia cells. (A-D) Kaplan-Meier survival plots of AML (A-B) or ALL (C-D) patients from the *TARGET* dataset, categorized by high or low HDAC6 expression levels in bone marrow (A or C) samples or peripheral blood (B or D) (log-rank test). (E-H) Kaplan-Meier survival plots of AML categorized by high or low HDAC6 expression levels from the TCGA-LAML dataset. Patients with FLT3, IDH1 or NRAS mutation were split by a percentile-based best cut-off selection (log-rank test). (I) Heatmap illustrating the dependency of leukemia cell lines from different subtypes on HDAC isoforms, based on data from the DepMap portal. (J) Bar graph comparing the cell death mediated by NK cells in K562 HDAC6 KO (C1 and C2) vs the non-targeting control (CTR) (unpaired t-test, *p < 0.05, n=3). (K-L) Kaplan Meyer survival plot of mice injected with K562 (K) or MV4-11 (L) HDAC6 KO models (log-rank test, ns= not significant, **p < 0.01, n=5-8).

Figure S2

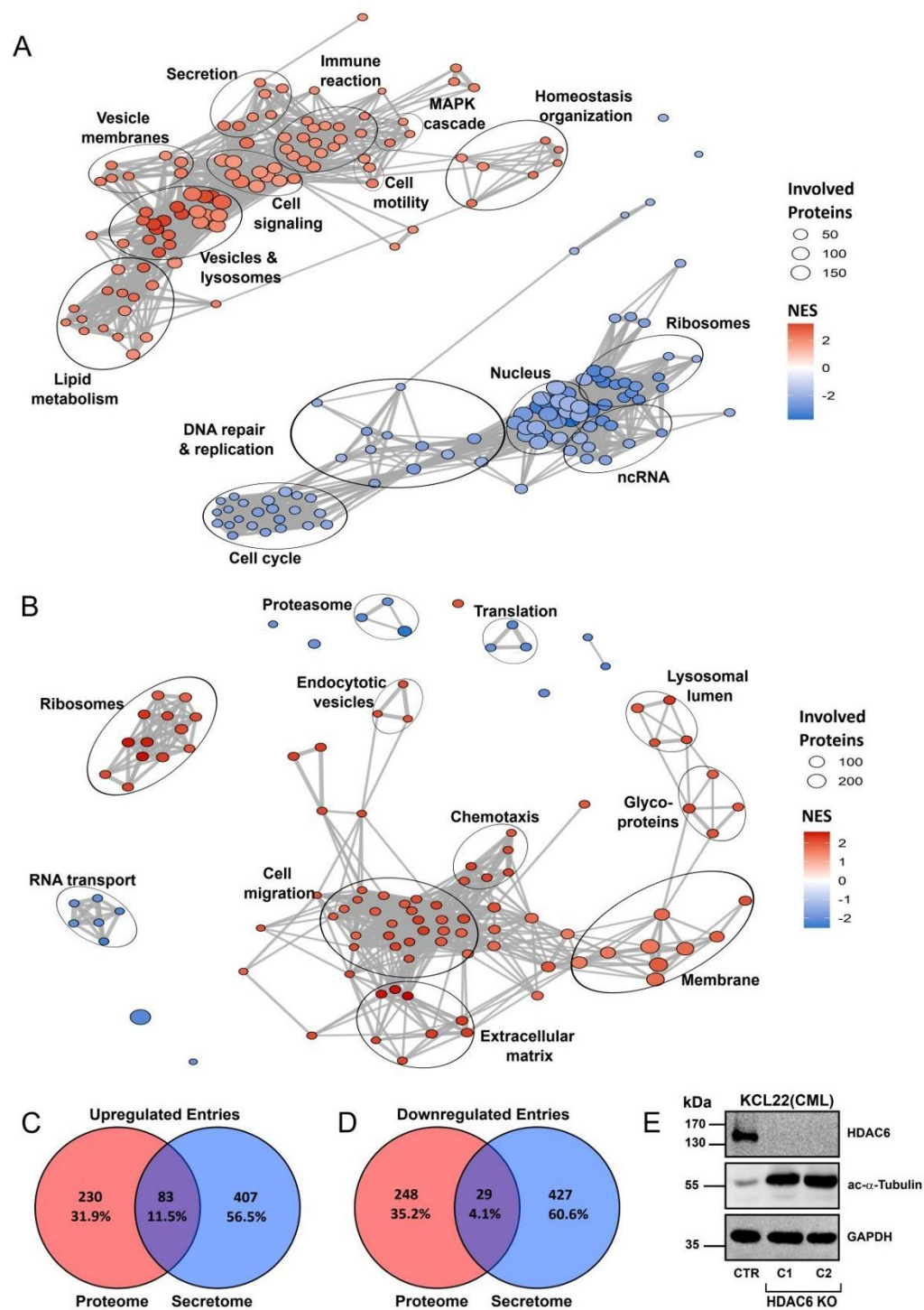
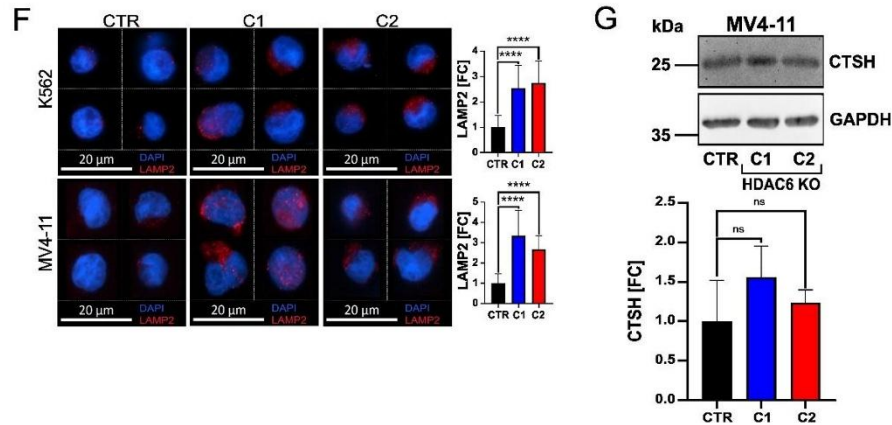


Figure S2 (continued)**Supplemental Figure 2. Targeting HDAC6 upregulates lysosome-related protein accumulation.**

(A-B) Enrichment map of gene sets from the proteome (A) or secretome (B) data of K562 HDAC6 KO models, generated using *clusterProfiler*, with a protein entry inclusion criterion of FDR < 0.1. Included gene sets possessed an FDR < 0.05 (Kolmogorov-Smirnov test, Benjamini-Hochberg correction). (C-D) Venn diagram showing the number of upregulated (C) and downregulated (D) overlapping and non-overlapping proteins from the proteome and secretome datasets with FDR < 0.1. (E) Representative western blot (WB) for HDAC6 and acetylated α -tubulin protein levels in two clones of KCL22 HDAC6 knock out (KO) (C1 & C2) and a non-targeting control (CTR, n=3). (F) Fluorescence microscopy analyzing the localization and amount of LAMP2 in K562 and MV4-11 HDAC6-KO models. Representative cells are shown while in the right panel the bar graph compares the fold change of fluorescence signal across all analyzed cells (unpaired t-test, ns= not significant, **p < 0.01, ***p < 0.001, ****p < 0.0001, n=20 cells). (G) Representative western blot for Cathepsin H (CTSH) protein levels in 2 clones (C1 and C2) of MV4-11 HDAC6-knockout (KO) and a non-targeting control (CTR). The lower panel shows the results of the quantification normalized to housekeeper GAPDH (unpaired t-test, n=3).

Figure S3

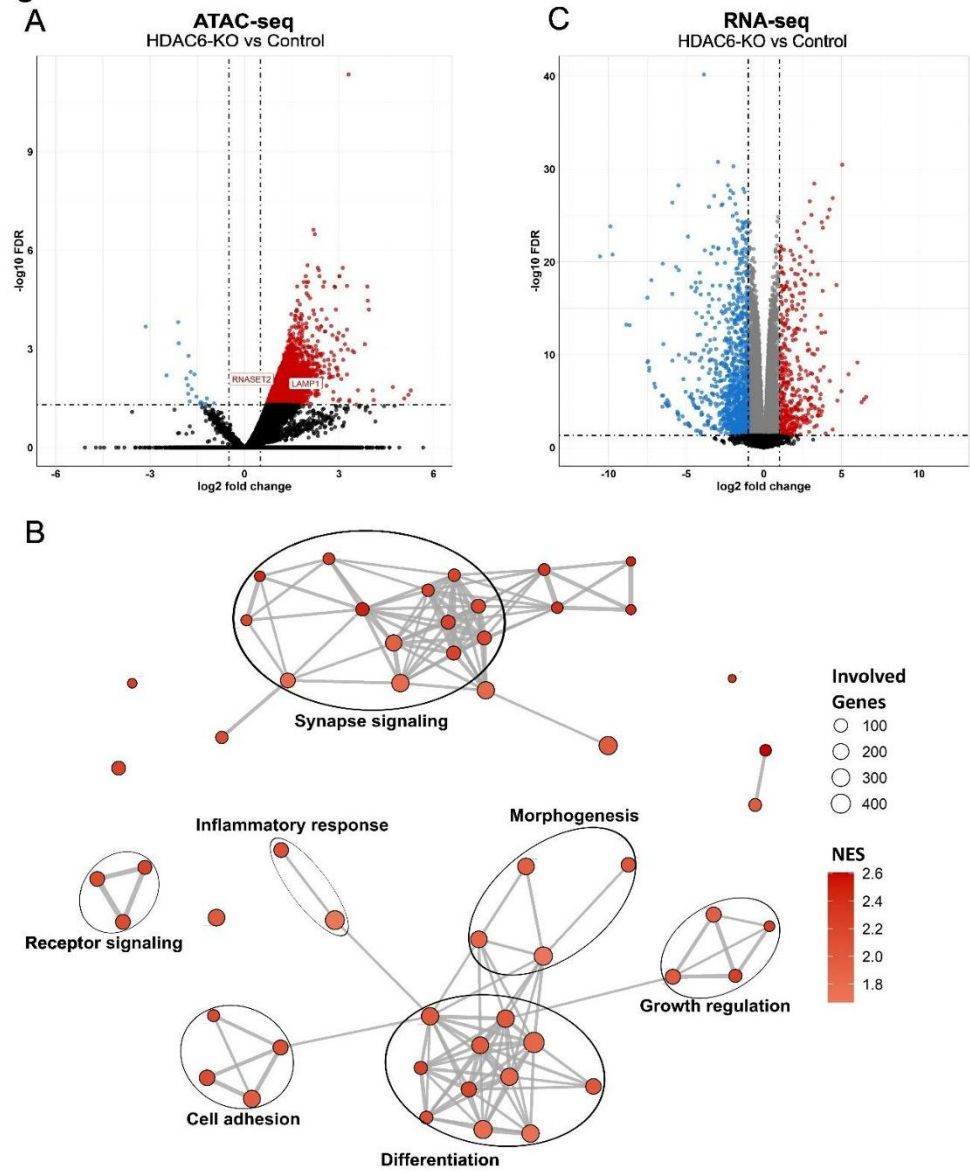
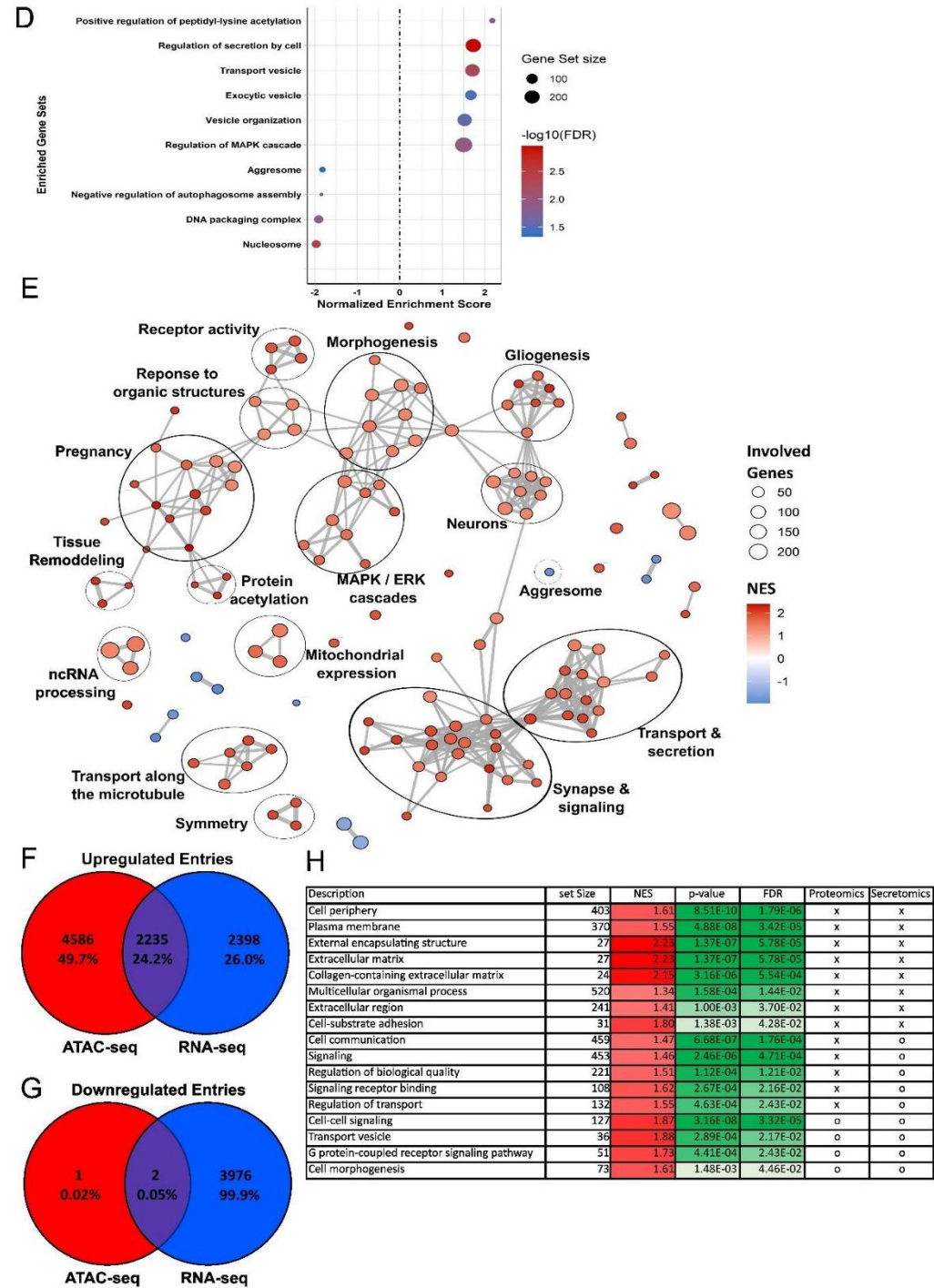


Figure S3 (continued)



Supplemental Figure 3. HDAC6 inhibition enhances the accessibility at the LAMP1 and RNase T2 gene loci. (A) Volcano plot depicting differentially accessible chromatin regions in HDAC6-KO C1 versus CTR cells, with the $\log_2$ fold change plotted against the $-\log_{10}(\text{p-value})$. The cut-off thresholds were set at $\log_2$ fold change > 0.5 and $-\log_{10}(\text{FDR}) > 1.301$ (quasi-likelihood F-test, Benjamini-

Hochberg correction, $n=2$). (B) Enrichment map of gene sets from the ATAC-seq data of differentially accessible promotor regions of K562 HDAC6-KO models, generated using *clusterProfiler*, with a gene entry inclusion criterion of $FDR < 0.05$. Included gene sets possessed an $FDR < 0.05$ (Kolmogorov-Smirnov test, Benjamini-Hochberg correction). (C) Volcano plot depicting differentially expressed genes from RNA sequencing HDAC6-KO C1 versus K562 control cells, with the $\log_2$ fold change plotted against the $-\log_{10}(p\text{-value})$. The cut-off thresholds were set at $\log_2$ fold change > 1 and $-\log_{10}(FDR) > 1.301$ (quasi-likelihood F-test, Benjamini-Hochberg correction, $n=3$). (D) Dot plot of selected enriched gene sets identified via *clusterProfiler* analysis of RNA-seq data. Significance cut-off for gene entry inclusion was set at $FDR < 0.05$. (Kolmogorov-Smirnov test, Benjamini-Hochberg correction). (E) Enrichment map of gene sets from the RNA-seq data, generated using *clusterProfiler*, with a gene entry inclusion criterion of $FDR < 0.05$. Included gene sets possessed an $FDR < 0.05$ (Kolmogorov-Smirnov test, Benjamini-Hochberg correction). (F-G) Venn diagrams illustrating the overlap between upregulated (F) and downregulated (G) genes identified by RNA-seq and ATAC-seq analysis. (H) Table containing the curated results of a clusterprofiler GSEA of significant RNA-seq entries ($FDR < 0.05$) with increased accessibility in the promotor region and overlap in the proteome or secretome analysis (Kolmogorov-Smirnov test, Benjamini-Hochberg correction).

Figure S4

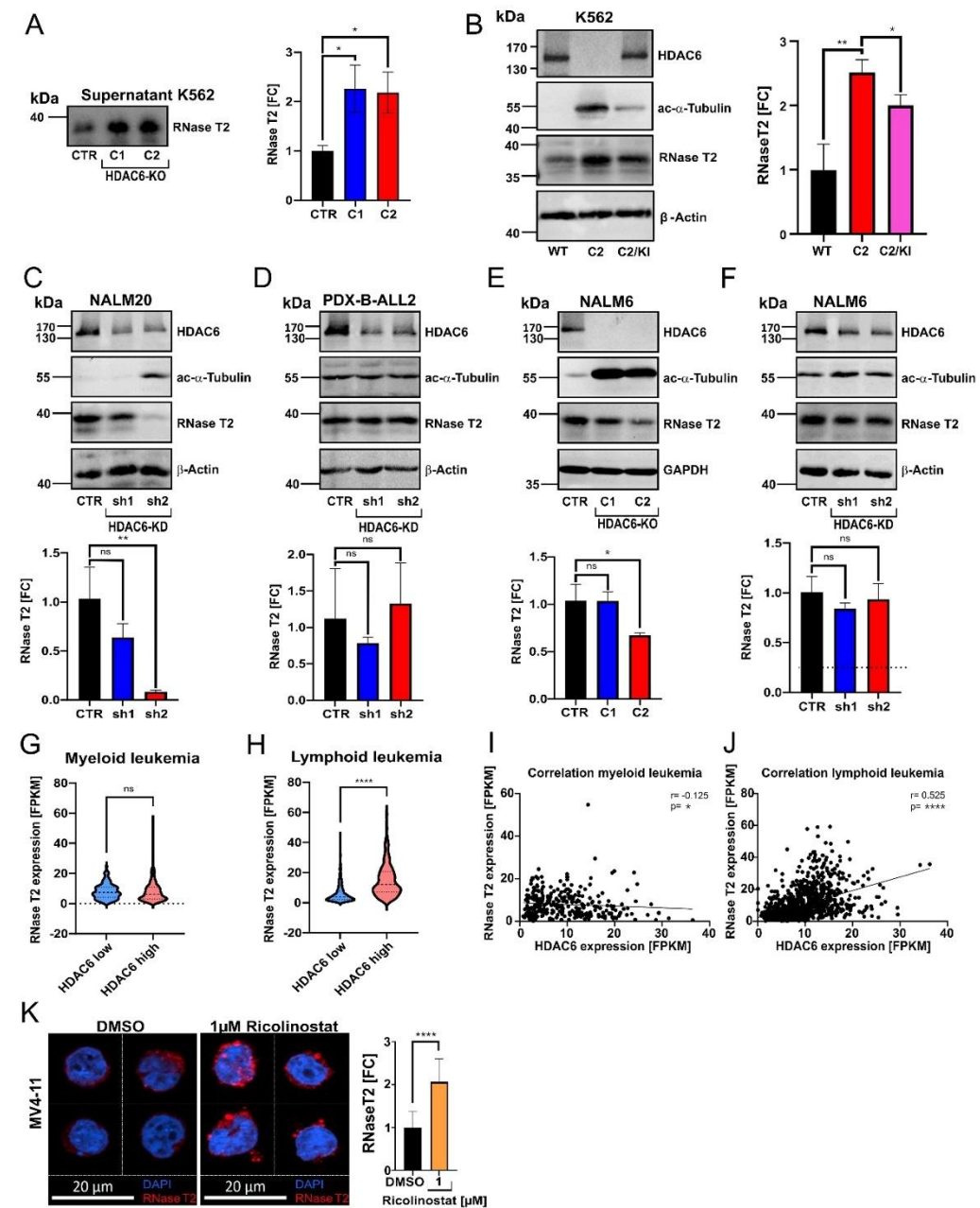
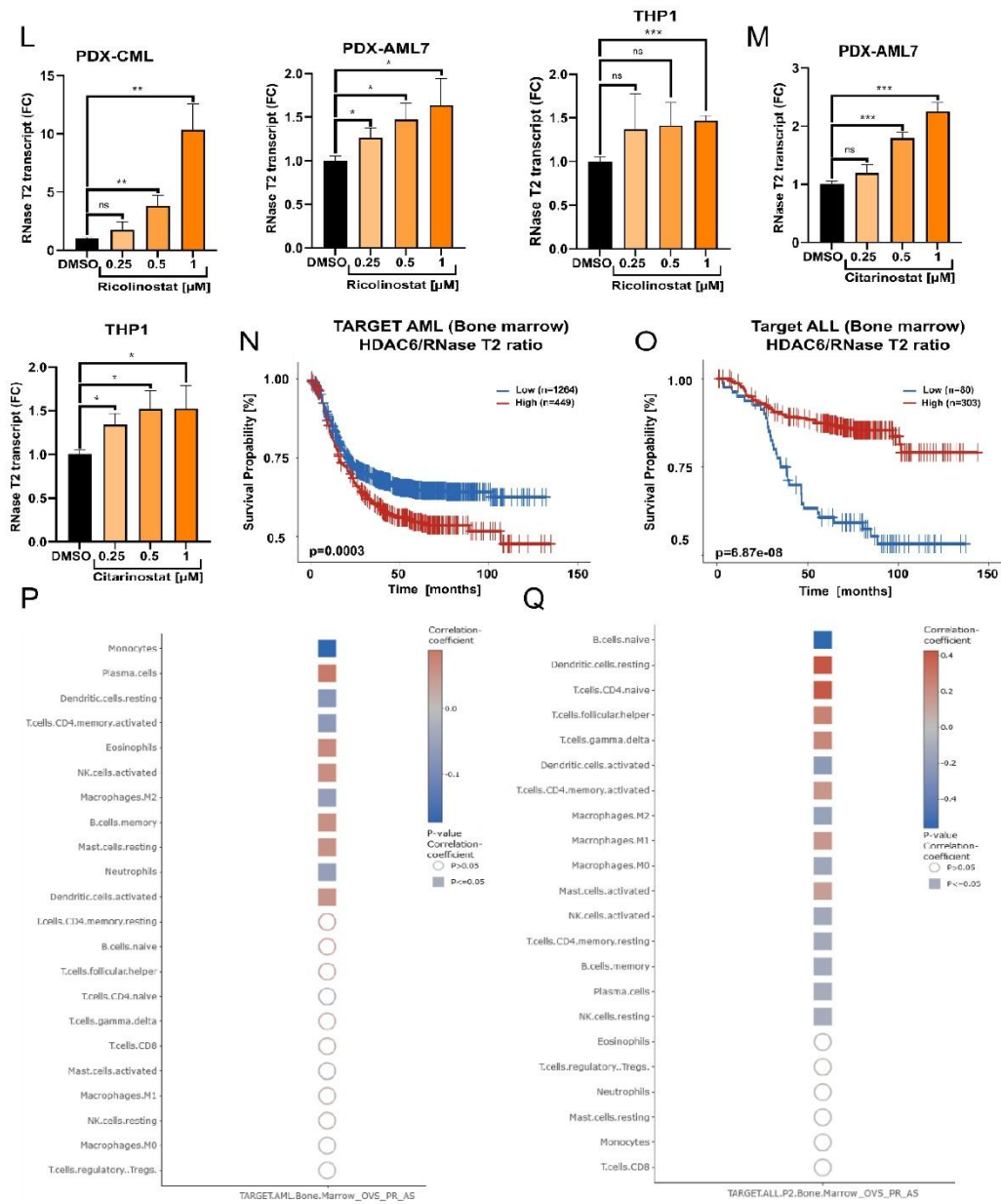
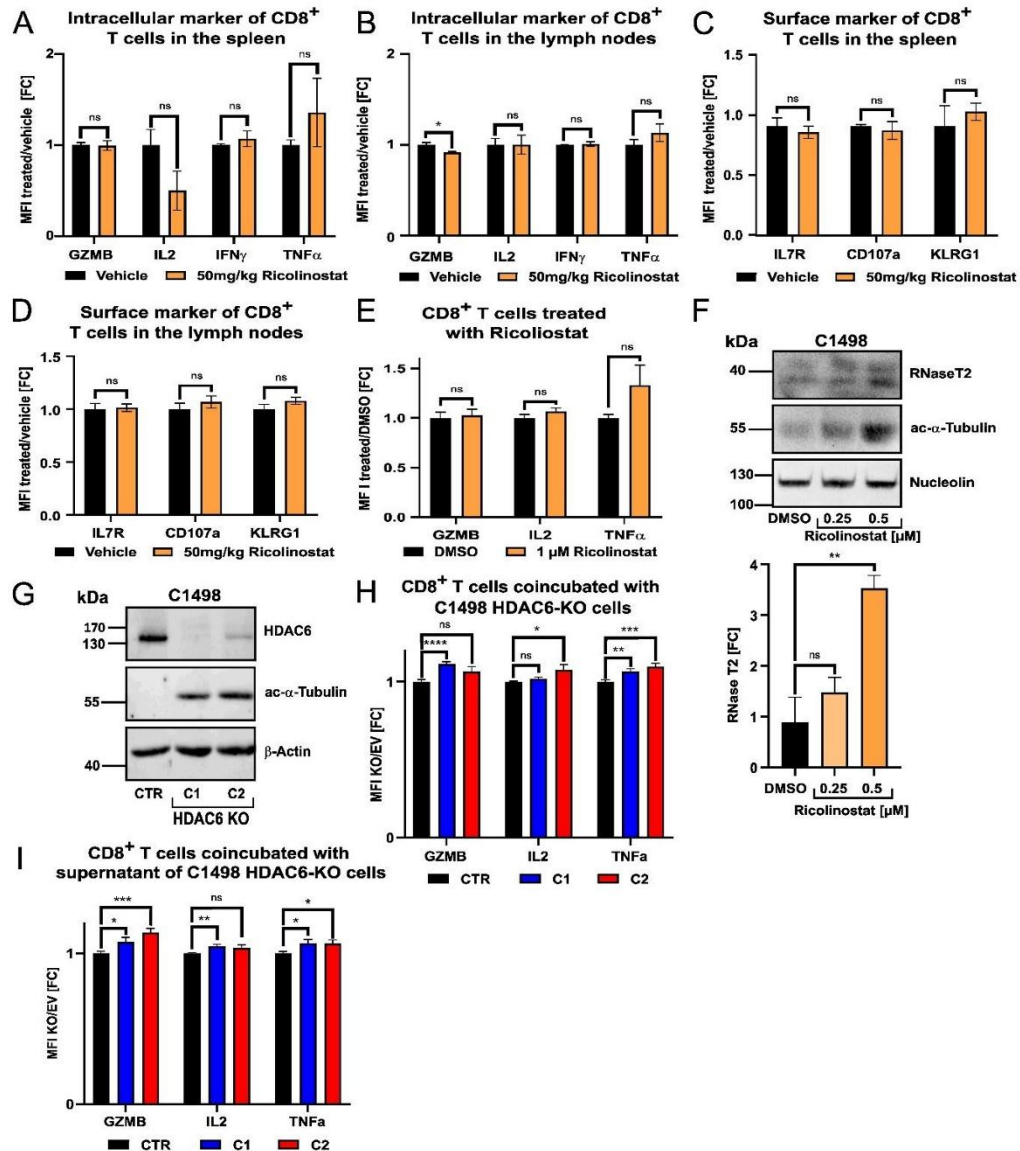


Figure S4 (continued)



Supplemental Figure 4. Targeting HDAC6 induces the expression of tumor suppressor RNase T2 in myeloid leukemia cells. (A) Western blot analysis of RNase T2 levels in the supernatant of HDAC6-KO K562 cells compared to the respective control, and in right panel quantification results in form of bar graph (unpaired t-test, ns = not significant, * $p < 0.05$, $n = 3$). (B) Western blot analysis of RNase T2 acetylated α -tubulin levels in K562 control (CTR), HDAC6-KO (C2) and HDAC6-KO (C2) with HDAC6 knock-in (C2/KI). The right panel (bar graph) shows the results of the quantification normalized to housekeeper β -actin (unpaired t-test, * $p < 0.05$, ** $p < 0.01$, $n = 3$). (C-F) Western blot analysis of HDAC6, acetylated α -tubulin, and RNase T2 levels in NALM20 HDAC6-knockdown (KD) (C), (*BCR::ABL1**) PDX-B-ALL2 HDAC6-KD (D) NALM6 HDAC6-KO (E) and NALM6 HDAC6-knockdown (KD) models. The lower panels show the results of the quantification, normalized to housekeeper GAPDH or β -Actin (unpaired t-test, ns = not significant, * $p < 0.05$, ** $p < 0.01$, $n = 3$). (G-H) Violin plots comparing the expression levels of RNase T2 in HDAC6 high and low expressing myeloid (G) or lymphoid leukemia samples (H) from the St. Jude *PeCan* database, clustered based on the mean value (Mann-Whitney U-test, ns = not significant, **** $p < 0.0001$, $n = 160$ -161 for myeloid, $n = 479$ for lymphoid). (I-J) Dot plots showing the correlation analysis of the (HDAC6 and RNase T2 expression) data from (G-H) (Spearman correlation, * $p < 0.05$, **** $p < 0.0001$, $n = 321$ for myeloid, $n = 958$ for lymphoid). (K) Fluorescence microscopy analysis of RNase T2 localization and expression in MV4-11 cells treated with Ricolinostat for 24 hours at the indicated concentrations. Representative cells are shown, while in the right panel the bar graph compares the fold change of fluorescence signal across all analysed cells (unpaired t-test, **** $p < 0.0001$, $n = 20$ cells). (L-M) Quantitative PCR analysis of RNase T2 expression in THP-1, PDX-CML and PDX-AML7 samples treated with Ricolinostat (L) or Citarinostat (M) for 72 hours at the indicated concentrations. Expression levels were normalized to β -actin and B2M ($n = 3$). (N-O) Kaplan-Meier survival plots of patients from the TARGET AML (K) or TARGET ALL (L) datasets, categorized by high or low HDAC6/RNase T2 expression ratio in the bone marrow (log-rank test $n = 449$ -1264 for AML, $n = 80$ -303 for ALL). (P-Q) Correlation analysis of immune signatures from the data in (I-J) (univariate Cox proportional hazards regression model).

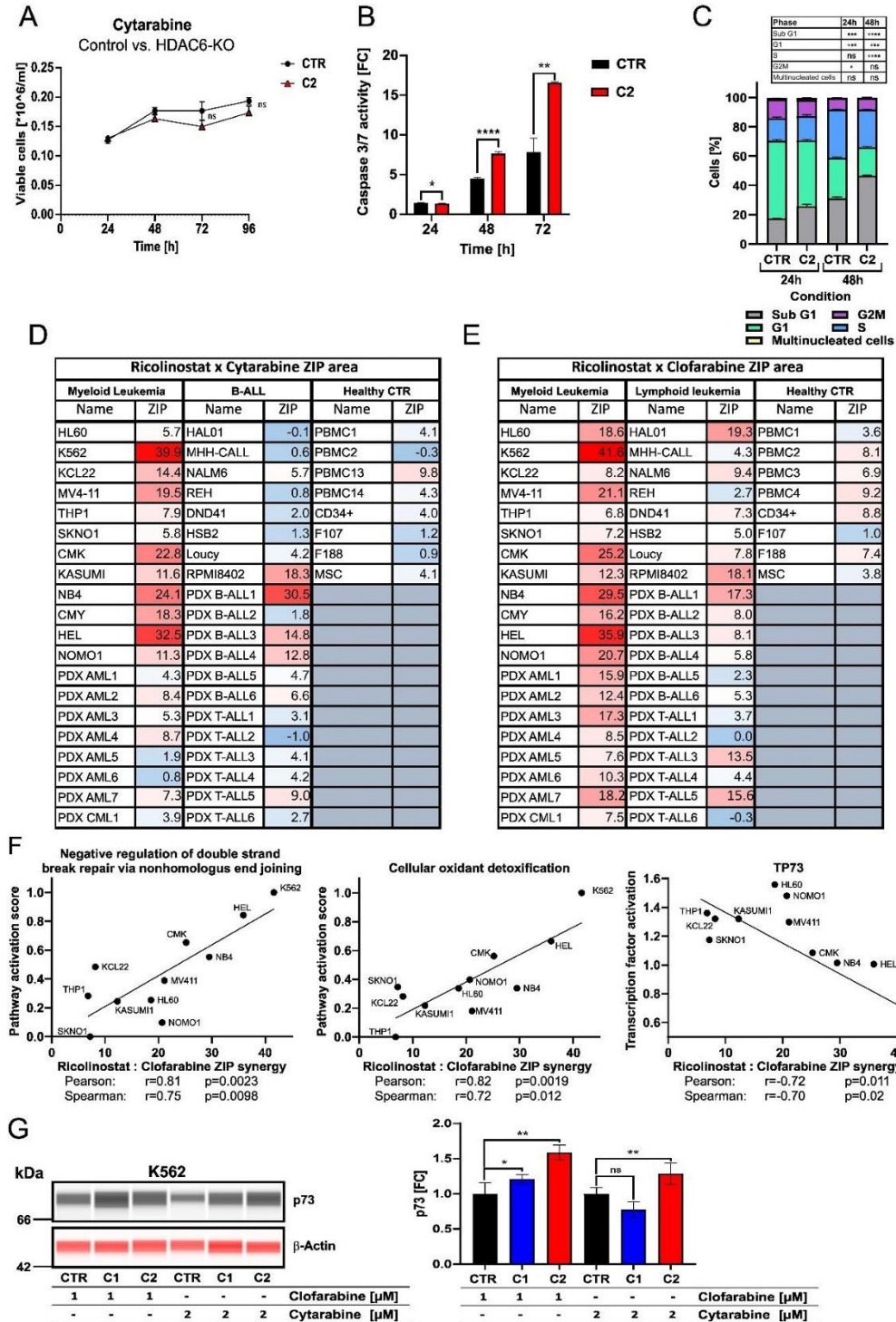
Figure S5

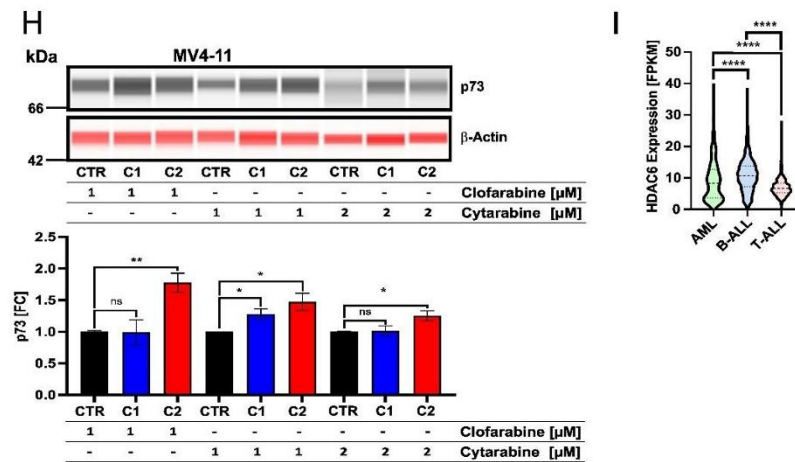
Supplemental Figure 5. HDAC6 inhibition sensitizes myeloid leukemia cells to CD8⁺ T cells. (A-B) Bar graph comparing the fold change in mean fluorescence intensity (MFI) of intracellular marker of CD8⁺ T cells from the spleen (A) or the lymph nodes (B) after Ricolinostat treatment against the control, following the injection of murine C1498-Luc-GFP⁺ AML cells into wild-type C57BL/6 mice (unpaired t-test, ns = not significant, * $p < 0.05$, $n = 4-5$). (C-D) Bar graph comparing the fold change in MFI of surface marker of CD8⁺ T cells from the spleen (C) or the lymph nodes (D) after Ricolinostat treatment against the control, following the injection of murine C1498-Luc-GFP⁺ AML cells into wild-type C57BL/6 mice (unpaired t-test, ns = not significant, $n = 4-5$). (E) Bar plot depicting fold change in MFI levels of intracellular marker of CD8⁺ T cells after 24-hour treatment with 1 μ M Ricolinostat (unpaired t-test, ns = not significant, $n = 3$). (F) Western blot analysis of RNase T2 and acetylated α -tubulin levels in C1498 cells treated for 24 hours with the indicated concentrations of Ricolinostat. The lower panel shows the results of the quantification normalized Nucleolin to as a bar graph (unpaired t-test, ** $p < 0.01$, $n = 3$). (G) Representative western blot (WB) for HDAC6 and acetylated α -tubulin protein levels in two clones of

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C1498 HDAC6 knockout (KO) (clone C1 & C2) and a non-targeting control (CTR), n=3. (H-I) Bar graph showing the fold change in MFI of intracellular marker of CD8⁺ T cells co-incubated with C1498 HDAC6 KO cells (H) or their supernatant (I) (unpaired t-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n = 16).

Figure S6





Supplemental Figure 6. Targeting HDAC6 sensitizes myeloid leukemia cells towards standard chemotherapeutics Cytarabine and Clofarabine. (A) Proliferation curve determined by trypan blue assay of K562 control (CTR) and K562 HDAC6-KO clone C2 treated with 2 μM Cytarabine over the course of 96 h (unpaired t-test, ns= not significant, n=3). (B) Bar plot of the fold change of the Caspase 3/7 activity of K562 CTR and K562 HDAC6-KO C2 treated with 2 μM Clofarabine over the course of 72h (unpaired t-test, *p< 0.05, **p< 0.01, ****p< 0.0001, n=3). (C) Bar plot showing a cell cycle analysis of K562 HDAC6-KO cells treated with Cytarabine (unpaired t-test, ns= not significant, *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001, n=3). (D-E) Tables summarizing the most synergistic area ZIP scores for the combination of Ricolinostat with Cytarabine (D) or Clofarabine (E) across various leukemia subtypes. (F) Pathway and transcription factor activities were inferred from gene expression data of AML and CML cell lines and correlated with ZIP synergy scores of the Ricolinostat and Clofarabine combination. (G-H) Representative western blot for p73 protein levels in 2 clones (C1 and C2) of K562 (G) or MV4-11 (H) HDAC6-knockout (KO) and a non-targeting control (CTR), following 48h treatment with Clofarabine or Cytarabine at the indicated concentrations. The side/below panel shows the results of the quantification normalized to housekeeper beta-actin (unpaired t-test, n=3). (I) Violin plot displaying HDAC6 expression across the most common childhood leukemia subtypes, from the St. Jude *PeCan* database (Mann-Whitney U-test, ****p< 0.0001, n=322-813).

Supplemental Table 1: List of cell lines used in the study

Cell Line	Leukemia Type / Origin	Medium	FBS (%)	Seeding Density	Passage Range	Source
K562	CML (blast crisis)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 10)
KCL22	CML (blast crisis)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 519)
MV4-11	AML (biphenotypic)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 102)
MOLM-13	AML (FLT3-ITD*)	RPMI-1640 + 1% Penicillin Streptomycin	20	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 554)
NB-4	AML (APL, PML-RARA*)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 207)
SKNO-1	AML (t(8;21))	RPMI-1640 + 10 ng/ml GM-CSF	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 690)
Kasumi-1	AML (t(8;21))	RPMI-1640 + 1% Penicillin Streptomycin	20	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 220)
NOMO-1	AML (M5 subtype)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 542)
HL-60	AML (promyelocytic)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 3)
NALM-6	B-ALL (pre-B)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 128)
NALM-20	B-ALL	RPMI-1640 + 1% Penicillin Streptomycin	20	0.1×10^6 cells/mL	P20–P35	DSMZ (ACC 681)
CMK	AMKL (megakaryoblastic)	RPMI-1640 + 1% Penicillin Streptomycin	20	0.1×10^6 cells/mL	P2–P4	DSMZ (ACC 392)
CMS	AMKL (Down syndrome)	RPMI-1640 + 1% Penicillin Streptomycin	20	0.1×10^6 cells/mL	P2–P4	DSMZ (ACC 985)
CMY	AMKL	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P2–P4	DSMZ (ACC 986)
LOUCY	T-ALL	RPMI-1640 + 1% Penicillin Streptomycin	20	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 394)
697	B-ALL (pre-B)	RPMI-1640 + 1% Penicillin Streptomycin	20	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 42)
MHH-CALL-2	B-ALL	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 341)
REH	B-ALL (t(12;21), ETV6-RUNX1*)	RPMI-1640 + 1% Penicillin Streptomycin	20	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 22)
HAL-01	T-ALL	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 610)
RPMI-8402	T-ALL	RPMI-1640 + 1% Penicillin Streptomycin	10	0.25×10^6 cells/mL	P10–P25	DSMZ (ACC 290)
THP-1	AML (KMT2A-MLLT3)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 16)
HEL	AML (JAK2 V617F)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 11)
DND41	AML (M6)	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 525)
HSB2	T-ALL	RPMI-1640 + 1% Penicillin Streptomycin	10	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 435)
Loucy	T-ALL (FAB L2)	RPMI-1640 + 1% Penicillin Streptomycin	20	0.1×10^6 cells/mL	P10–P25	DSMZ (ACC 394)
PDX cells	See Supplemental Table 3	RPMI-1640 + 0.1 mM 2-Mercaptoethanol + 1mM Sodium Pyruvate + 0.5 ug/ml Gentamicin Reagent Solution or StemSpan Leukemic Cell Culture kit (StemCell Technologies, Cat. No. 09720)	15	0.25×10^6 cells/mL	-	In house/ cooperation
PBMC	Healthy / non-malignant	Mononuclear Cell Medium (PromoCell, Cat. No. C-28030)	-	0.25×10^6 cells/mL	primary	In house
CD34+	Healthy cells (Hematopoietic stem cells)	StemSpan SFEM II with StemSpan CD34+ Expansion Supplement (StemCell Technologies, Cat. No. 09605 and 02691)	15	0.25×10^6 cells/mL	primary	Local cord blood bank (ITZ, UKD)
MSC/ Fibroblast	Healthy / non-malignant	RPMI-1640 + 1% Penicillin Streptomycin	10	0.033×10^6 cells/mL	P10–P25	In house/ cooperation

Supplemental Table 2: Compounds List in the Drug screening Library

Inhibitor	Mechanism of action / Target	Supplier	Reference
5-Azacytidine	Antimetabolites	Selleckchem	S1782
6-Mercaptopurine	Antimetabolites	Selleckchem	S4504
6-Thioguanine	Antimetabolites	Selleckchem	S1774
Clofarabine	Antimetabolites	Selleckchem	S1218
Cytarabine	Antimetabolites	Selleckchem	S5582
Cyclocytidine HCL	Antimetabolites	Selleckchem	S1973
Nelarabine	Antimetabolites	Selleckchem	S1213
Methotrexate	Antimetabolites	Selleckchem	S1210
Vinblastine	Antimitotics	Selleckchem	S4505
Vincristine	Antimitotics	Selleckchem	S1241
Mitoxantrone	Topoisomerase	Selleckchem	S2485
Daunorubicin	Topoisomerase	Selleckchem	S3035
Amsacrine	Topoisomerase	Selleckchem	S5627
Dexamethasone	GC/GCR complex	Selleckchem	S1322
Prednisolone	GC/GCR complex	Selleckchem	S2570
Belinostat	Histone deacetylases	Selleckchem	S1085
CI-994 (Tacedinaline)	Histone deacetylases	Selleckchem	S2818
Panobinostat	Histone deacetylases	Selleckchem	S1030
Romidepsin	Histone deacetylases	Selleckchem	S3020
Entinostat	Histone deacetylases	Selleckchem	S1053
Ricolinostat	Histone deacetylases	Selleckchem	S8001
GSK343	Histone methyltransferase	Selleckchem	S7164
Bortezomib	Proteasome	Selleckchem	S1013
MLN-9708	Proteasome	Selleckchem	S2181
Ganetespib	Heat shock protein 90	Selleckchem	S1159
PUH71	Heat shock protein 90	Selleckchem	S8039
AUY922 (LUMINESPIB)	Heat shock protein 90	Selleckchem	S1069
Alisertib	Aurora Kinase	Selleckchem	S1133
Aurora A Inhibitor I	Aurora Kinase	Selleckchem	S1451
Barasertib	Aurora Kinase	Selleckchem	S1147
Volasertib	Polo like kinases	Selleckchem	S2235
BI2536	Polo like kinases	Selleckchem	S1109
LY2835219	Cyclin dependent kinases	Selleckchem	S5716
Dinaciclib	Cyclin dependent kinases	Selleckchem	S2768
SY-1365-THZ1	Cyclin dependent kinases	Selleckchem	S7549
Axitinib	Tyrosine kinases	Selleckchem	S1005
Midostaurin	Tyrosine kinases	Selleckchem	S8064
Dovitinib	Tyrosine kinases	Selleckchem	S7765
Pexidartinib	Tyrosine kinases	Selleckchem	S7818
Lestaurtinib	Tyrosine kinases	Biomol	LKT-L1875
Quizartinib	Tyrosine kinases	Selleckchem	S1526
Decitabine	Antimetabolites	Selleckchem	S1200
Pacritinib	Tyrosine kinases	Selleckchem	S8057
Idelalisib	Tyrosine kinases	Selleckchem	S2226

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Dactolisib (BEZ235)	Tyrosine kinases	Selleckchem	S1009
Ro 08-2750	Tyrosine kinases	Medchem	HY-108466
ARQ-092 (Miransertib)	Tyrosine kinases	Selleckchem	S8339
Everolimus	mTOR	Selleckchem	S1120
Temsirolimus	mTOR	Selleckchem	S1044
AT9283	Tyrosine kinases	Selleckchem	S1134
CYT387 (Mometotinib)	Tyrosine kinases	Selleckchem	S2219
Ruxolitinib	Tyrosine kinases	Selleckchem	S1378
Fedratinib (TG101348)	Tyrosine kinases	Selleckchem	S2736
Tipifarnib	Farnesyltransferase	Selleckchem	S1453
Lonafarnib	Farnesyltransferase	Selleckchem	S2797
Sorafenib (Tosylate)	Tyrosine kinases	Selleckchem	S1040
Regorafenib	Tyrosine kinases	Selleckchem	S5077
Cobimetinib	Mitogen activated protein kinases	Selleckchem	S8041
MEK162 (Binimetinib)	Mitogen activated protein kinases	Selleckchem	S7007
Trametinib	Mitogen activated protein kinases	Selleckchem	S2673
Tirabrutinib	Bruton's tyrosine kinase	Medchem	HY-15771
Ibrutinib	Bruton's tyrosine kinase	Medchem	S2680
Imatinib	Tyrosine kinases	Medchem	S1026
Ponatinib	Tyrosine kinases	Medchem	S1490
Bosutinib	Tyrosine kinases	Medchem	S1014
Dasatinib	Tyrosine kinases	Medchem	S5254
Staurosporin	Tyrosine kinases	Medchem	S1421
BSI-201 (Iniparib)	Poly(ADP-ribose)-Polymerasen	Medchem	S1087
Olaparib	Poly(ADP-ribose)-Polymerasen	Medchem	S1060
ABT-199 (Venetoclax)	BCL2 apoptosis regulators	Medchem	S8048
Obatoclax	BCL2 apoptosis regulators	Medchem	S1057
QNZ (EVP4593)	NF- κ B	Medchem	S4902
Omaveloxolone	Inflammation modulation	Medchem	S7672
Birinapant	Cellular inhibitors of apoptosis	Medchem	S7015
Selinexor	Exportin	Medchem	S7252
AZD6738	Tyrosine kinases	Medchem	S7693
Homoharringtonine	Protein translation	Medchem	S9015
Bexarotene	Retinoid X receptors	Medchem	S2098
Nintedanib (BIBF1120)	Tyrosine kinases	Medchem	S1010
BAY 80-6946 (Copanlisib)	Tyrosine kinases	Medchem	S2802
Palbociclib	Cyclin dependent kinases	Medchem	S1116
Birabresib	BET bromodomain	Medchem	S7360
Tegaserod Maleate	5-HT ₄ receptor	Medchem	S5401
5-nonyloxy tryptamine	Serotonin receptor	Sigma Alderich	157798-13-5

Supplemental Table 3: Clinical characteristics of the leukemia patients xenografted in NSG mice

Related to Figure 2, 3, 4 and 6

PDXs	Samples used for xenograft	Age at first diagnosis (yrs)	Sex	FAB (AML)	Karyotype	Genetic lesions
CML	initial	14,9	F	-	46XX, t(9;22)(q34,q11); 47, idem, +21	BCR::ABL1, trisomy 21, trisomy 20
AML1	relapse	15,3	M	M0	46, XY	RARA, RUNX1
AML2	initial	14,3	M	M5	46, XY	FLT3-ITD
AML3	relapse	51,0	M	-	Normal Karyotype	FLT3-mut, NPM1
AML4	relapse	0,5	F	M0	46, XX, inv(9)(p11q12)	MLL::AFF1, NRAS
AML5	relapse	71	M	-	Normal Karyotype	ASXL1, DNMT3A, FLT3-ITD, FLT3-TKD, NPM1, TET2
AML6	initial	9,6	M	M4	46, XY, inv(16)(p13q24)	CBFB::MYH11, FLT3-TKD, NRAS, PTPN11
AML7	relapse	47,0	F	M4	46, XX, ins (10;11)(p12;q23q23)	KMT2A::AF10, KRAS:p.G12A, BCOR
B-ALL1	relapse	12,1	F	-	-	TCF3::PBX1
B-ALL2	relapse	4,5	M	-	46, XY, t(9;22)(q34;q11)	BCR::ABL1, IKZF1
B-ALL3	relapse	11,0	M	-	arr[GRCh37](Y)x0,(2-4)x1,5q23.2q31.3(125944131_142444598)x1,5q31.3q32(142804687_148958726)x1,(7,12,13,15-17)x1,(20)x1[0.9]	TP53 NM_000546.5:c.844C>T, p.Arg282Trp
B-ALL4	initial	3,1	M	-	SNP-array derived karyotype: 54,XY,+X,+Y,CN-LOH(1-6),del(6)(p22.2)/HIST1x2,CN-LOH(7,8),+9,+9,CN-LOH(10-13),+14,+14,CN-LOH(15-20),+21,+21,CN-LOH(22);	Mono-clonal non classical-high hyperdiploid ("masked near-haploid")
B-ALL5	relapse	5,4	M	-	SNP-array derived karyotype: 53,XY,+X,+Y,CN-LOH(1-6),del(6)(p22.2)/HIST1x2,CN-LOH(7,8),+9,CN-LOH(10-13),+14,+14,CN-LOH(15-19),del(19)(p13.3),CN-LOH(20),+21,+21,CN-LOH(22)	Mono-clonal non classical-high hyperdiploid ("masked near-haploid")
B-ALL6	initial	15,0	F	-	Karyotype by G-banding: 46,XX[10]; karyotype by SNP-array: 50,XX,+X,+X,CN-LOH(1-20,22),+21,+21	Mono-clonal non classical-high hyperdiploid ("masked near-haploid")
B-ALL7	relapse	7,0	M	-	29, XY	TP53 NM_000546.5:c.846_847insG CCGG,p.Arg283AlafsTer64
T-ALL1	initial	8,1	F	-	-	-
T-ALL2	initial	3,3	M	-	-	-
T-ALL3	initial	7,7	F	-	-	CDKN2A
T-ALL4	initial	1,5	F	-	-	Cornelia de Lange Syndrome
T-ALL5	initial	4,5	F	-	-	-
T-ALL6	initial	14,3	F	-	47, XX, t(5;14)(q35.1;q32.2), +8	IKZF1, trisomy 8

2. Supplemental Material and Methods:

CRISPR-Cas9 mediated knockout (KO): K562 wild type cells were transduced with a CAS9 expression plasmid (Addgene, Ref: 108100) via Lenti-X Packaging Single Shots (Takara, Ref: 631276) using HEK293T cells. gRNAs were cloned into the lentiviral expression plasmid with GFP or mCherry (Addgene, Ref: 108098 or 108099). Virus production was done in Lenti-X HEK293T cells using Lenti-X Packaging Single Shots (Takara). After 72 h in total, the lentiviral supernatant was harvested, filtered through a 0.45 µm filter and added to constitutively expressing Cas9 K562 cells (0.4×10^6 cells) that were previously generated. After transduction, positive cells were selected by the fluorescent marker, GFP or mCherry.¹

Generation of KCL-22, MV4-11, NALM6, and C1498 HDAC6-KO cells: The transfection was carried out using the Amaxa Nucleofection system SF Cell Line Kit (Lonza, Ref: V4XC-2032). For 2×10^5 cells, 100 pmol of Cas9-GFP protein (IDT, Ref: 10008100) was mixed with 120 pmol of gRNA (crRNA:tracrRNA 1:1) and assembled for 20 min at RT. Afterwards, the labelled ssODN was added and the mixture was combined with the cell suspension (resuspended cells in Nucleofector solution SF) and the electroporation enhancer (IDT). The complete volume was transferred to the Nucleocuvette module, placed in the 4D-Nucleofector system (Lonza) and electroporated with the program CA-137 for KCL22, DJ-100 for MV4-11, CV-104 for NALM6 and CM-138 for C1498. After 24h, the cells were sorted for GFP and monoclonal selected via semi-solid cloning. The following guide RNA (gRNA) sequences were utilized:

Construct	Sequence (5' – 3')
gRNA_HDAC6 human 1	GCA CAT CCC AAT CTA CGA TA
gRNA_HDAC6 human 2	CTA TTG CAT GTT CAA CCA CG
gRNA_HDAC6 murine 1	GAA CAT CCC AAT CCA CGA TTA GG
gRNA_HDAC6 murine 2	ACA CGT ATA ATA CAC TGC CAG GG

Western Blotting: Cells were harvested by centrifugation at 400 x g for 5 min at 4°C and washed three times with ice-cold PBS and then snap-frozen in liquid nitrogen.¹ 100-300 µL protein lysis (see below) buffer and was used for 5 million cells. Cells were lysed on ice for 30 minutes with periodically vortexing, centrifuged two times (10000 x g for 20 min at 4 °C) and protein quantification was performed by BCA-Assay (Thermo

Fisher Scientific, #23227). 10 - 20 µg lysates were separated by SDS-PAGE at 50 V for 30 mins during stacking phase with subsequent 100 V for 2h during separation phase and blotted onto 0.45 µm nitrocellulose membrane (Cytiva, #10600002) at 100 V for 1h or overnight (30 V, 16 h). Membranes were washed twice with TBS and analyzed for their protein content by ponceau staining (Sigma-Aldrich #P7170) staining. After three washes with TBS-T, membranes were blocked in 5% BSA (Sigma-Aldrich, #A3294) TBS-T solution, washed three times with TBS-T and incubated with primary antibody solution in 5% BSA solution overnight at 4°C. Membranes were washed three times with TBS-T, incubated for 1h with secondary HRP-conjugate (Cell Signaling Technologies, #7074 and #7076) at 1:2000 in TBS-T solution, washed again three times in TBS-T and lastly one time in TBS. For visualization ECL-Solution (Cytiva, #GERPN2106) was used as per manufacturer's instruction. Images were captured using conventional chemiluminescent blot imaging module of Jess western blotting system (Biotechne). Only p73 blots were performed using capillary-based automated western blot system (Bio-technne).

List of antibodies used in immunoblots:

Target	Dilution	Origin	Supplier	Reference
HDAC6 (Human)	1:1000	Rabbit	Cell signaling	7558S
HDAC6 (Mouse)	1:1000	Rabbit	Cell signaling	7612
ac-α-Tubulin	1:1000	Rabbit	Cell signaling	5335S
LAMP1	1:1000	Rabbit	Cell signaling	9091T
RNAse T2	1:1000	Rabbit	Abcam	ab 140191
RNAse T2	1: 500	Mouse	Santa Cruz	sc-393729
PARP	1:1000	Rabbit	Cell signaling	9542S
CTSH	1:250	Rabbit	Cell signaling	54007T
p73	1:10 (JESS)	Rabbit	Cell signaling	14620T
GAPDH	1:1000	Rabbit	Cell signaling	5174S
β-Actin	1:1000	Mouse	Cell signaling	12262S
β-Actin	1:50 (JESS)	Mouse	R&D systems	MAB8929
Nucleolin	1:1000	Rabbit	Cell signaling	14574S
α-Tubulin	1:1000	Rabbit	Cell signaling	2144S
Anti-rabbit HRP	1:2000	Goat	Cell signaling	7074S
Anti-mouse HRP	1:2000	Horse	Cell signaling	7076S

Anti-rabbit HRP	undiluted (JESS)	Goat	Bio-Techne	042-206
Anti-mouse NIR	1:20 (JESS)	Goat	Bio-Techne	043-821

NK-cell killing assay: PBMCs were isolated from healthy donors' buffy coats, provided by the blood bank of the University Hospital Düsseldorf, using Ficoll-Paque™ Plus GE (Sigma-Aldrich, Ref: GE17-1440-02) density gradient centrifugation. After red blood lysis, samples were frozen with a cryopreservation medium containing FBS with 10% DMSO (Sigma-Aldrich, St. Louis, USA). For the experiments, PBMCs were thawed in RPMI GlutaMAX medium with 10% human serum (Sigma-Aldrich, Ref: H4522), 200 U/ml IL-2 (Miltenyi Biotec, Ref. 130-097-743) and 0.3 ng/ml IL-15 (Miltenyi Biotec, Ref. 130-095-760) and incubated at 37°C with 5% CO₂ overnight. Target cells were harvested and stained with CFSE (Thermo Fisher Scientific, Ref. C34570) and seeded into a 96-well U-bottom with 1×10^5 cells per well. PBMCs were harvested and seeded into the same plate with 1×10^6 cells per well. Target cells and PBMCs were co-cultured to test the NK cell activity. Mono-cultures of the respective cells were used as controls to be able to calculate the excess killing and death rates. After 4 hours of culture, cells were stained for flow cytometry.

qPCR. For RNA extraction, cell pellets were first lysed using QIAzol Lysis Reagent (QIAGEN), followed by isolation with the Maxwell® RSC Viral Total Nucleic Acid Purification Kit (Promega) using the Maxwell® RSC 48 instrument. A total of 2 µg of purified RNA from each sample was used as a template for cDNA synthesis with the QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative PCR (qPCR) was performed using a real-time PCR system (Bio-Rad). The mean Ct values of the housekeeping genes B2M and β-actin were used as internal controls to normalize target gene expression levels. Human primer sequences are listed below:

Target	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
RNase T2	GGC ATA CTG GCC TGA CGT AAT	CTT TTC CCA CTC ATG CTT CCA GA
B2M	GTA TGC CTG CCG TGT GAA C	AAA GCA AGC AAG CAG AAT TTG G
β-actin	GCA CTC TTC CAG CCT TCC	CTC GAA GCA TTT GCG GTG

Fluorescence microscopy: Microscope slide cover slips were coated with poly-D-lysine (Gibco, Ref: A3890401) for 1 hour, washed with PBS, and seeded with treated

or genetically engineered cells for adhesion. Cells were fixed in 4% formaldehyde (Merck, Ref: 1.00496), quenched in Tris-buffered saline, and permeabilized with 0.01% Triton X (Roth, Ref: 3051.2).² Blocking was performed overnight with 10% BSA at 4°C. Primary and secondary antibodies were used per supplier recommendations (see supplementary) with 2-hour incubations, followed by washes. Nuclei were stained with DAPI (Thermo Fisher, Ref: 62248), and slides were mounted with ProLong™ Gold (Invitrogen, Ref: P10144), dried for 48 hours, and imaged using an Axio Observer and Apotome 3 (Zeiss). Twenty representative cells were randomly selected from at least three independent immunofluorescence images to ensure robust statistical sampling. Quantification was performed using *ImageJ* analysis software following a standardized protocol. Raw fluorescence images were first split into individual color channels and the mean fluorescence signal of each cell was measured using the measure function. Background correction was applied by determining the mean background intensity from equivalent cell-free areas, which was subsequently subtracted from the mean cellular signal. The background-corrected fluorescence signals from genetic knockout (KO) or Ricolinostat-treated samples were then normalized against their respective control groups to generate fold change values. Representative images are included in the figure for visualization purposes to complement the quantitative data presented in the bar graphs.

List of antibodies used in Fluorescence microscopy:

Target	Dilution	Origin	Supplier	Reference
LAMP1	1:50	Rat	Santa Cruz	19992
			Thermo	
LAMP2	1:50	Rabbit	Fisher	PA1-655
			Scientific	
RNase T2	1:50	Mouse	Santa Cruz	393729
Anti-Mouse IgG Alexa Fluor™ plus 488	1:1000	Mouse	Invitrogen	A32723TR
Anti-Rat Alexa Fluor™ plus 647	1:1000	Rat	Invitrogen	A78947

ShRNA mediated HDAC6-knockdown (KD): SMARTvector lentiviral shRNA constructs (Horizon Discovery) containing a human EF1α promoter and TurboRFP fluorescent reporter were used to knock down HDAC6 expression, following the manufacturer's instructions. The specific shRNA sequences used are listed below.

Target	Mature antisense sequence (5' – 3')	Cat. number
sh1	CAC ATG ATC CGC AAG ATG C	V3SVHS09-10351093
sh2	CCA CGT ACT CAG CAC TGT G	V3SVHS09-8678884
sh3	ATC CCA ATC CAC AAT CAG G	V3SVHS09-7082476
Non-targeting (NT) control		SVC17012404 (hEF1a-Turbo RFP)

ATAC-seq: The data was uploaded and analyzed using the galaxy platform. After an initial quality control with FastQC the adapters were trimmed with Cutadapt. The mapping was done via Bowtie2 and filtered afterwards for mapping quality (≥ 30), proper pairing and chromosomal origin (! = mitochondrial), while PCR amplified duplicates were flagged via MarkDuplicates. Subsequently the files underwent a conversion from BAM to BED and were used with MACS2 for peak calling. Differences in sequencing depth were normalized using standard library-size scaling, implemented in *DiffBind* by adjusting read counts to 1,000,000 divided by the total number of reads per sample (simple linear scaling). All samples were processed in a single batch by Active Motif following standardized ATAC-seq protocols, minimizing the likelihood of batch effects. Library-size normalization is widely used in ATAC-seq workflows to control for technical variation in sequencing depth while preserving biologically meaningful differences between conditions. Each sample was analyzed with two technical replicates to increase data robustness and statistical confidence. For the generation of the heatmap the bigwig files were averaged through BigWig average, given to computeMatrix and finally visualized with plotHeatmap tools. Track plots were generated with the help of the pyGenomeTracks package. Differential gene expression analysis was done via DiffBind. The differentially open genes were processed similar to the RNAseq and Proteome dataset. The cut-off for the overlap analysis was $FDR < 0.05$. As reference genome hg38 was used.

HDAC6-knockin (KI): For the HDAC6 knock-in experiments, the HDAC6 plasmid (Addgene, Ref: 13823) was subcloned into a pCDH-EF1 α -GFP lentiviral vector (System Biosciences). K562 HDAC6-KO cells were transduced with the lentiviral construct, and GFP-positive cells were FACS-sorted.

T cell co-culture activation assay: C57BL/6J 6-8 weeks old mice were infected I.V. with 5×10^5 PFU LCMV Armstrong strain.³ After 2 weeks mice were sacrificed and splenic pan T cells were isolated with Pan T Cell Isolation Kit (Miltenyi Biotec, 130-095-130) according to manufacturer instructions. After determination of the density via trypan blue counting the cells were resuspended in culture media with 100 U/ml IL2 (Gibco, Ref: 00-02-250) and with a concentration of 1×10^6 cells per ml. 0.05×10^6 C1498 cells were treated 24 h prior to the co-culture experiment with 1 μ M of Ricolinostat. Afterwards the treatment media was discarded and the cells co-incubated with 0.1×10^6 of the isolated T cells. For the killing curve cells were diluted in three 1:1 steps. Separately 1×10^6 T cells were incubated with a 1:1 ratio of supernatant from treated C1498 to fresh media. The supernatant was filtered through a 0.45 μ m strainer to exclude the cells. Also 1×10^6 T cells were treated with 1 μ M of Ricolinostat. After 24h the cells were either analyzed in the CytoFLEX to determine the viability via DAPI (Invitrogen, 62248) or fixed for intracellular staining. After 24 hours cells were stained for surface markers, next intracellular cytokines staining performed with Foxp3 / Transcription Factor Staining Buffer Set (Invitrogen, 00-5523-00) according to manufacturer instructions with Fixable Viability Dye (Invitrogen, 65-0863-14.). Flow cytometry was performed on Cytoflex (Beckman Coulter) and data analyzed with FlowJo_v10.7.2 software. For each of the 8 mice duplicates were used. Significance was determined via unpaired t test (n=16).

Murine C57BL/6J experiments: Mice were sacrificed and spleen, lymph node (LN) and bone marrow (BM) isolated. Cells were stimulated for 1 hour with 5 ng/ml PMA (Sigma-Aldrich, Ref: P1585) and 25 ng/ml Ionomycin (Invitrogen, Ref: I24222) for cytokine accumulation, next Brefeldin A (Merck, Ref: B5936) was added to the culture and cells were incubated for additional 5 hours. Next, cells were stained for surface markers and intracellular cytokines staining (see supplementary) performed with Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Ref. 00-5523-00) with Fixable Viability Dye (Invitrogen, Ref: 65-0863-14).³

Another cohort of mice was used to further investigate the antitumor efficacy of HDAC6i treatment. The engraftment of leukemia cells was confirmed 7 days post tumor inoculation via IVIS and divided randomly to different treatment groups. In total three cycles of treatments (successive 5-day daily treatment course followed by one day break) with either vehicle control (10% DMSO+ 40% PEG300+ 5% TWEEN80 + 45%

ddH₂O) or 50mg/kg Ricolinostat (Selleckchem) were administered i.p.. The tumor burden of each mouse was assessed by monitoring the bioluminescent signals and quantifying the region of interest (ROI) weekly. Health conditions and body weight from all animals were closely monitored throughout the experimental period. After the endpoint of the experiment, a survival analysis was performed.

List of antibodies used in FACS:

Target	Origin	Supplier	Reference
CD3 (Human)	Rat	Invitrogen	25-0038-42
CD56	Mouse	BD Pharmingen	557711
CD107a	Mouse	BioLegend	328630
PD-L1	Mouse	BioLegend	329732
ULBP2/5/6	Mouse	BD OptiBuild	748131
CD3 (Mouse)	Hamster	eBioscience	47-0031-82
CD8	Rat	BD Biosciences	563068
CD4	Rat	Invitrogen	63-0042-82
KLRG1	Hamster	BD Biosciences	740553
GZMB	Rat	eBioscience	12-8898-82
IL2	Rat	eBioscience	45-7021-82
IFN- γ	Rat	eBioscience	17-7311-82
TNF α	Rat	eBioscience	48-7321-82
CD107a	Rat	BD Biosciences	560647
IL7R/CD127	Rat	eBioscience	45-1271-82

3. Supplemental References:

1. Vogt M, Dienstbier N, Schliehe-Diecks J, et al. Co-targeting HSP90 α and CDK7 overcomes resistance against HSP90 inhibitors in BCR-ABL1+ leukemia cells. *Cell death & disease*. Dec 6 2023;14(12):799. doi:10.1038/s41419-023-06337-3
2. Gebing P, Loizou S, Hansch S, et al. A brain organoid/ALL coculture model reveals the AP-1 pathway as critically associated with CNS involvement of BCP-ALL. *Blood advances*. Oct 8 2024;8(19):4997–5011. doi:10.1182/bloodadvances.2023011145
3. Stachura P, Liu W, Xu HC, et al. Unleashing T cell anti-tumor immunity: new potential for 5-Nonloxytryptamine as an agent mediating MHC-I upregulation in tumors. *Mol Cancer*. Aug 15 2023;22(1):136. doi:10.1186/s12943-023-01833-8

RNAse T2	1:50	Mouse	Santa Cruz	393729
Anti-Mouse IgG Alexa Fluor™ plus 488	1:1000	Mouse	Invitrogen	A32723TR
Anti-Rat Alexa Fluor™ plus 647	1:1000	Rat	Invitrogen	A78947

List of antibodies used in FACS:

Target	Origin	Supplier	Reference
CD3	Rat	Invitrogen	25-0038-42
CD56	Mouse	BD Pharmingen	557711
CD107a	Mouse	BioLegend	328630
PD-L1	Mouse	BioLegend	329732
ULBP2/5/6	Mouse	BD OptiBuild	748131
CD80	Mouse	BD Horizon	567426
B2M	Mouse	BD	656645
HLA-ABC	Mouse	Invitrogen	12-9983-42
CD3	Hamster	eBioscience	47-0031-82
CD8	Rat	BD Biosciences	563068
CD4	Rat	Invitrogen	63-0042-82
KLRG1	Hamster	BD Biosciences	740553
GZMB	Rat	eBioscience	12-8898-82
IL2	Rat	eBioscience	45-7021-82
IFN-γ	Rat	eBioscience	17-7311-82
TNFα	Rat	eBioscience	48-7321-82
CD107a	Rat	BD Biosciences	560647
IL7R/CD127	Rat	eBioscience	45-1271-82

3. Supplementary References:

- 1 Vogt, M. *et al.* Co-targeting HSP90 alpha and CDK7 overcomes resistance against HSP90 inhibitors in BCR-ABL1+ leukemia cells. *Cell death & disease* **14**, 799, doi:10.1038/s41419-023-06337-3 (2023).
- 2 Gebing, P. *et al.* A brain organoid/ALL coculture model reveals the AP-1 pathway as critically associated with CNS involvement of BCP-ALL. *Blood advances* **8**, 4997-5011, doi:10.1182/bloodadvances.2023011145 (2024).
- 3 Stachura, P. *et al.* Unleashing T cell anti-tumor immunity: new potential for 5-Nonloxytryptamine as an agent mediating MHC-I upregulation in tumors. *Mol Cancer* **22**, 136, doi:10.1186/s12943-023-01833-8 (2023).

4 - Discussion

The overall treatment of leukemia has significantly advanced over the last decades. However, certain subtypes like AML continue to exhibit a poor prognosis [21]. In the case of AML, especially, HDAC inhibitors have gained substantial research interest. However, a single compound approach yielded only limited responses in patients. Concurrently, pan-HDAC inhibitors have been associated with severe hematologic toxicity, which restricts their therapeutic applicability. Therefore, identifying potent and specific synergistic drug combinations is essential for developing effective HDACi-based therapeutic strategies. In this thesis, several novel, potent, and selective HDACi have been characterized in regard to their anti-leukemic properties and ability to bind HDAC. Furthermore, potent drug combinations were identified and, in some cases, were directly associated with HDAC6.

4.1 - Structural modifications of HDAC inhibitors improved the protein binding properties and antileukemic effects

In all combined cumulative papers together more than 40 novel HDACi were synthesized and characterized in the context of leukemia. In the publication “Development of Fluorinated Peptoid-Based Histone Deacetylase (HDAC) Inhibitors for Therapy-Resistant Acute Leukemia” (Chapter 3.1 = C3.1), **10h** was found to be the most potent anti-leukemic compound. It displayed IC₅₀ of 0.1 μ M to 0.5 μ M but was simultaneously the least selective inhibitor with a selectivity index of 1.3 for HDAC6 vs HDAC1 (C3.1 - Table 1 & Figure 5a). It performed comparably well to the other pan inhibitor, vorinostat, while exceeding the cytotoxicity of ricolinostat (C3.1 - Table 2). This effect may be attributed to its increased binding affinity to HDAC1, validated via western blot analysis of histone H3 acetylation (C3.1 - Figure 5c). The connection between high cytotoxicity and the class I HDACs is well known, but it is furthermore possible that off-targets contribute to the cytotoxicity [282]. However, only limited conclusions can be drawn regarding its effects on healthy tissues due to limitations in healthy controls. In this study, healthy fibroblasts were used, which presents a limitation, as HDAC inhibitors typically exhibit reduced efficacy in adherent cell lines [282]. Most often, therapy-related toxicity occurs in regard to the hematologic compartment, which is not accurately represented by fibroblasts. The inclusion of isolated peripheral blood mononuclear cells (PBMCs) or CD34⁺ stem cells would enhance the physiological relevance of the study. An in-depth analysis of their complete HDAC inhibition profile would have been useful since only HDAC1 and HDAC6 were investigated. Based on the homology, a reasonable assumption would be that HDAC2 and HDAC3 were targeted

similarly, but less could be said about the other family members. Therefore, a further classification as pan or class I/IIb specific cannot be attributed.

Other experimental inhibitors from this publication had small-nanomolar binding affinities for HDAC6, while the binding affinity for HDAC1 showed considerable variability. **10p** emerged as the inhibitor with the highest selectivity for HDAC6 with a selectivity index of 191 compared to HDAC1 (C3.1 - Table 1). While it induced hyperacetylation of α -tubulin in western blot analyses, none of the tested concentrations altered histone H3 acetylation levels (C3.1 - Figure 5c). In the drug screening, no cytotoxicity against the leukemic cell lines or healthy controls was observed (C3.1 - Figure 5a). Since the used concentrations were capped at 25 μ M, no valid IC₅₀ was gathered from the drug response curve. Given that 1 μ M is commonly used as a benchmark for assessing compound cytotoxicity, and no reduction in cell viability was observed even at 25 μ M, these inhibitors can be considered non-cytotoxic under the tested conditions. The observed α -tubulin hyperacetylation conclusively demonstrates that HDAC6 was blocked. These results suggest that the cytotoxicity that was previously attributed to HDAC6 was either circumstantial (e.g. cancer type) or that these inhibitors had uncharacterized off-target effects. A similar conclusion was reached in a study where a highly selective HDAC6 inhibitor could not induce cytotoxicity at high micromolar concentrations while HDAC6 was already fully inhibited at low micromolar doses [283]. These findings support the increasing body of evidence suggesting that selective HDAC inhibition alone does not inherently confer cytotoxicity and that such effects are highly dependent on cellular context.

The inhibitors from the publication “Preferential HDAC6 inhibitors derived from HPOB exhibit synergistic antileukemia activity in combination with decitabine” (Chapter 3.2 = C3.2) displayed similar selectivity and activity profiles. While **1g** was less selective and significantly more cytotoxic in leukemia cells, **2b** was markedly more selective but demonstrated less potency. Compound **2b** was of particular relevance as it represented an intermediate between cytotoxicity and selectivity. A selectivity index of 25 between HDAC6 and HDAC1 was determined (C3.2 - Table 1 & Table 2). Therefore, the original selectivity of HPOB was maintained while the IC₅₀ was reduced by one order of magnitude. Cytotoxicity correlated with increased histone H3 acetylation, whereas low compound concentrations induced α -tubulin acetylation without measurable cytotoxicity (C3.2 - Figure 4B). In the drug screening, **2b** exhibited comparable activity to ricolinostat and CI994 (C3.2 - Figure 4A). Notably, compound **1g** was less potent against HDAC6 in leukemic cells than anticipated. Although the enzyme assay suggested approximately twice the binding efficacy for HDAC6, western blot analysis indicated a stronger preference for HDAC1 (C3.2 - Table 1, Table 2 & Figure 4B). Similar methodological limitations, such as the absence of healthy

control samples for assessing hematopoietic toxicity and the restricted HDAC binding affinity assays, also applied to the inhibitors described in this study.

In the study “Deciphering the therapeutic potential of novel pentyloxy-amide-based class I, IIb HDAC inhibitors against therapy resistant leukemia” (Chapter 3.3 = C3.3), thirteen derivatives of an optimized vorinostat analogue were investigated. Among the novel inhibitors, compounds **4d** and **4m** demonstrated substantially enhanced binding affinities for HDAC1–HDAC3 while maintaining comparable affinities for HDAC6 and HDAC8 (C3.3 - Table 1 & Table 2). Vorinostat exhibited relatively low affinity for HDAC4 and HDAC11, suggesting that its classification as a pan-HDAC inhibitor may be less accurate, particularly when compared to panobinostat. A marked enhancement in binding affinity was observed when larger substituents containing electron-donating heteroatoms were introduced at the para position. This effect may be attributed to stabilization of the enzyme–ligand interaction by the electron-donating heteroatom, either within the catalytic channel or, more plausibly, at the rim of the active site. Computational predictions and crystallographic analyses were consistent with this interpretation (C3.3 - Figure 3-5).

Both **4d** and **4m** outperformed not only several pan-HDACi like panobinostat, but also the conventional AML therapeutics daunorubicin or cytarabine in a differential drug sensitivity score (dDSS) based approach, incorporating pediatric leukemia PDX samples (C3.3 - Figure 7). As CD34⁺ progenitor cells and healthy T cells were included as controls, the data suggest that the novel inhibitors exhibited greater selectivity toward leukemic cells while exerting reduced effects on healthy immune cells. Several other next-generation HDACi, such as pracinostat, abexinostat, and givinostat, displayed a similar effect. Structural optimization that enhances class I HDAC binding may increase cytotoxicity toward healthy immune cells, given their functional dependence on these enzymes [218-228]. However, compounds **4d** and **4m** demonstrated more favorable dDSS profiles than vorinostat while maintaining comparable selectivity ratios, thereby challenging the previously proposed relationship between class I affinity and cytotoxicity. Even the more selective inhibitors ricolinostat and CI-994 failed to exhibit favorable dDSS profiles. This observation may be explained by off-target effects that reduce the viability of healthy control cells but are not shared by compounds **4d** or **4m**. It should be taken into account that the dataset was skewed toward ALL-derived PDX samples, as AML PDX models are more difficult to establish and are less common in pediatric oncology. Nonetheless, in all five AML PDX cases, compounds **4d** and **4m** achieved equal or higher dDSS values compared to cytarabine and daunorubicin/idarubicin.

Based on these favorable results, an in vitro pharmacokinetic screening was conducted, which demonstrated suitable absorption, distribution, metabolism, and elimination

properties (C3.3 - Figure 12). These findings were consolidated by *in vivo* experiments, which showed that **4d** had a higher systemic exposure while having a similar elimination half-life in mice (C3.3 - Table 4 & Table 5). In an *in vivo* AML mouse model using immunocompetent animals, both **4d** and vorinostat significantly reduced leukemic growth following two cycles of daily treatment for five days each at a dose of 20 mg/kg (C3.3 - Figure 13C & Figure 13D). Although statistically significant, the reduction was modest and could potentially be improved by dose escalation of the HDAC inhibitor. Other studies have employed the more toxic inhibitor panobinostat at equivalent doses (20 mg/kg), potentially indicating a broader therapeutic window for **4d** or vorinostat [284]. Increasing the dosage could further emphasize the differences between **4d** and vorinostat, clarifying the superior *in vivo* efficacy of **4d**. Notably, no significant weight loss was observed in mice treated with **4d**, indicating appropriate tolerability at this dosage (C3.3 - Figure 13E). In conclusion, compound **4d** demonstrated *in vivo* activity but its true therapeutic potential requires further elucidation in an optimized experimental setting.

All of the described inhibitors show potential for clinical application. **10h**, **1g**, **4d**, and **4m** are less selective and exhibit a higher binding affinity towards class I HDACs. This may increase the risk of adverse effects due to the functional HDAC dependency of healthy immune cells. However, the dDSS *in vitro* assay for **4d** and **4m** and *in vivo* results of **4d** demonstrated a targeted leukemia cytotoxicity and suggest potential patient tolerability. **2b** represents an intermediate alternative that is more specific towards HDAC6 while maintaining a similar cytotoxicity profile. Particularly in combination with suitable synergy agents, it could serve as a valuable therapeutic component in clinical settings. The highly specific inhibitor **10p** exhibited selectivity comparable to that of tubastatin A and may be useful in HDAC6-targeted therapies. Although leukemia cells appear insensitive to HDAC6 inhibition alone, such inhibition may reveal potential therapeutic opportunities when combined with other agents. Additionally, HDAC6 is a protein of interest in numerous other clinical contexts, such as neurodegenerative diseases, neuroblastoma, gastrointestinal cancer, inflammatory diseases, and heart failure [285-289]. Beyond their therapeutic potential, these inhibitors provide valuable insights into drug-HDAC isoform interactions. The crystal structures elucidate the binding mode, while the enzyme/cell data allow a structure-activity relationship analysis. These insights may guide the design and synthesis of next-generation HDAC inhibitors.

4.2 - HDAC inhibitors synergize with commonly used AML chemotherapeutics

Since HDACi have shown limited efficacy as monotherapies, it is widely assumed that their therapeutic potential may be enhanced through synergistic drug combinations. Across the analyzed publications, three synergistic interaction partners were identified. In clinical trials, combinations of vorinostat or panobinostat with daunorubicin/idarubicin, cytarabine, azacytidine, decitabine, and bortezomib have already been evaluated [275-279]. While decitabine is known to act synergistically with pan-HDAC inhibitors, the observed synergy between HDAC6 inhibition and nucleoside analogs such as clofarabine and cytarabine remains less well characterized.

4.2.1 – Decitabine demonstrates high synergistic potential when combined with unselective inhibitors

Decitabine is a synthetic nucleoside analog that becomes incorporated into the DNA during replication [290]. Within the DNA, it inhibits DNA methyltransferase, which subsequently leads to DNA hypomethylation. Similar to HDAC inhibition, this process results in the reactivation of silenced genomic regions. A recent large-scale meta-analysis from 2022 concluded that decitabine monotherapy demonstrated clinical efficacy in AML [291]. However, both intrinsic and acquired resistance remain major clinical challenges, positioning decitabine in a similar therapeutic context as HDAC inhibitors. Previous studies established that panobinostat combined with decitabine produced a pronounced synergistic effect in diffuse large B-cell lymphoma [292]. In AML clinical trials, decitabine has been evaluated in combination with valproic acid and vorinostat [293, 294]. While valproic acid did not alter the therapeutic response to decitabine, the combination of vorinostat and decitabine was well tolerated and demonstrated encouraging therapeutic activity.

All tested experimental inhibitors exhibited varying degrees of synergy with decitabine in AML cell lines. Compounds **10h**, **2b**, **4d**, and **4m** exhibited notably high zero interaction potency (ZIP) scores (C3.1 - Figure 6a upper panels, C3.2 - Figure 5A-5B, C3.3 - Figure 10A-5B). The most synergistic regions exhibited ZIP scores exceeding 60, indicating strong synergistic interactions. **10p** and **1g** were also synergistic, albeit to a much lesser extent. **1g** was not able to go beyond a ZIP score of 20 (C3.2 - Figure 5E-5F), while **10p**'s most synergistic area was located at 20 μ M of **10p** (C3.1 - Figure 7). Based on these findings, it is unlikely that the synergistic mechanism is dependent on the inhibition of HDAC6. **10p** at these high concentrations should be able to completely inhibit HDAC6, according to the western blot data (C3.1 - Figure 5c). As HDAC inhibitors typically lose specificity at higher

concentrations, off-target effects may underlie the observed synergy. On the other hand, no H3 acetylation was observed even at 36 μ M, suggesting that the effect is independent of HDAC1 and HDAC3 inhibition. However, **10h**, **2b**, **4d**, and **4m** all showed significantly high ZIP scores, and especially **10h**, **4d**, and **4m** are unselective and potent HDAC class I inhibitors. **2b**, on the other hand, is HDAC6 preferential, but shows a similar behavior. Through the matrix screening data, it seems that the synergy is, to some extent, more dependent on the inhibition of class I HDACs than HDAC6. **1g**, despite being a potent class I HDAC inhibitor, produced markedly lower synergistic effects (ZIP area scores around 10–15) compared to other class I inhibitors (ZIP area scores up to 65), which calls this concept into question. Therefore, it is plausible that the observed synergy arises from a specific balance of HDAC isoform inhibition or from additional off-target effects.

Kalac et al. demonstrated that the acetylation of histone H3 increased when panobinostat was combined with decitabine [292]. Comparable effects were observed by western blot analysis for compounds **10h**, **4d**, and **4m** (C3.1 - Figure 6b, C3.3 - Figure 10C). These findings suggest that decitabine, although not affecting H3 acetylation alone, may potentiate the acetylation induced by HDAC inhibition. Indeed, previous studies have demonstrated that combining HDAC inhibitors with other epigenetic drugs substantially alters both the acetylome and methylome relative to single-agent treatment [295]. That study reported a pronounced downregulation of oncogenes such as MYC, KDM2B, and SUV39H1, which were repressed following combination treatment. These proteins are often associated with epigenetic alterations, which would suggest an indirect effect of the combination through driver gene repression. Moreover, DNA methylation is known to promote the recruitment of HDACs, thereby reinforcing transcriptional silencing at methylated loci [296]. A detailed analysis involving compound **4d** revealed a positive correlation between increased H3 acetylation and PARP cleavage when a fixed concentration of decitabine was combined with escalating concentrations of **4d** (C3.3 - Figure 10D-F). This finding indicates a potential mechanistic link between synergistic cytotoxicity and epigenetic modification. Vorinostat failed to elicit the same effect at the same concentrations, which underscores the potential of **4d** in combinational therapy.

Another important aspect is that the synergy appears to be specific for AML. Both AML cell lines HL60 and MOLM13 showed a exhibited comparable potent synergistic responses when treated. However, the B-ALL cell line HAL01 and the T-ALL cell line Jurkat displayed minimal synergy when treated with decitabine and **10h** or **2b** (C3.1 - Figure 6a lower panels, C3.2 - Figure 5C-D). This specificity may involve activation of AML-relevant tumor suppressor genes such as CDKN2B upon combined treatment [297]. Definite conclusions on the specificity of this combination are limited due to the low number of cell models. To improve this aspect, an in-depth investigation of HDACi combined with decitabine against

cell lines and PDX are warranted. Furthermore, healthy controls of crucial cells such as HSC would be informative for assessing patient tolerability. Nonetheless, the previous clinical trial with vorinostat and decitabine demonstrated the safety and clinical activity in AML.

Beyond the synergistic cytotoxic interactions, it has been reported that the combination induced the expression of immunogenic neoantigens in AML [298]. Furthermore, it was found that DNA methyltransferase inhibitors like decitabine enhance antitumor immune signaling in ovarian cancer [299]. Therefore, this combination may enhance immunotherapy approaches by priming the immunogenicity of the AML cells. Compound **2b** may be of particular clinical interest due to its high synergistic potential combined with reduced toxicity toward healthy immune cells, consistent with its lower affinity for class I HDACs. For elderly patients who are ineligible for the conventional “3 + 7” regimen, this therapeutic approach may represent a viable alternative. In conclusion, these findings support the clinical feasibility of combining HDAC inhibitors with decitabine and justify further clinical evaluation.

4.2.2 – HDAC6 loss increases the susceptibility of leukemic cells towards cytarabine and clofarabine

Both cytarabine and clofarabine are nucleoside analogs that can incorporate into DNA during synthesis and inhibit replication during the S-phase, ultimately inducing apoptosis [300]. Due to the inhibition of DNA polymerases, the repair mechanism fails, leading to DNA damage accumulation. Cytarabine has long been a cornerstone of AML treatment, forming the basis of the standard “3 + 7” induction regimen, whereas clofarabine has been explored as a potentially improved alternative. In preclinical experiments with vorinostat and cytarabine, it has been shown that the simultaneous treatment of AML cells with both drugs resulted in antagonism [301]. This was reversed when the cells were pretreated with vorinostat and afterward treated with cytarabine, which led to combination indexes below 1, indicating synergy. A study found a direct link between the levels of functional HDAC1 or HDAC6 and cytarabine-induced apoptosis in pediatric AML [302]. Clinical trials of combining cytarabine with valproic acid failed due to the limited clinical activity of valproic acid. Other studies combined panobinostat with both cytarabine and daunorubicin or idarubicin [276, 303]. In both studies, the combination was well tolerated but occasionally resulted in thrombocytopenia. Notably, panobinostat treatment increased histone H3 acetylation in patient-derived samples, which correlated with improved overall survival. For clofarabine, a phase I clinical trial combining it with the HDAC inhibitor entinostat in patients with biphenotypic ALL/AML achieved an overall response rate of 50%, warranting further investigations [304].

In the study “Epigenetic remodeling via HDAC6 inhibition amplifies anti-tumoral immune responses in myeloid leukemia cells” (Chapter 3.4 = C3.4), a complete loss of HDAC6 in the CML cell line K562 significantly reduced the IC50 values of both cytarabine and clofarabine (C3.4 - Figure 6A). Validation across multiple assays confirmed that HDAC6 KO K562 cells exhibited reduced proliferation and increased apoptosis upon treatment with either agent (C3.4 - Figure 6B-6E, C3.4 Supplement Figure 6A-6C). These findings indicate that HDAC6 confers intrinsic resistance to cytarabine and clofarabine. Ricolinostat was chosen to validate these results pharmacologically since it is the furthest advanced HDAC6 preferential inhibitor in phase 2 clinical trials in multiple myeloma [305]. In wild-type K562 cells, ricolinostat produced a modest synergistic effect with both cytarabine and clofarabine, reaching ZIP synergy scores of approximately 30 (C3.4 – Figure 6F), thereby supporting the genetic findings from HDAC6-deficient cells. Similar results were obtained for MV4-11 (C3.4 - Figure 6G). Consequently, an extensive matrix synergy approach with lymphoblastic and myeloid cell lines or PDX was launched to systematically investigate the synergistic potential between HDAC6 preferential inhibitors and cytarabine or clofarabine (C3.4 - Figure 6H-I).

Samples from the myeloid lineage were particularly responsive to synergistic combinations, whereas synergy scores in B-ALL and T-ALL were generally lower. Among the two agents, clofarabine produced slightly higher synergy scores than cytarabine and performed more consistently in PDX models. It should be noted that the overall degree of synergy was comparably low compared to the findings with decitabine. Many samples exhibited responses near the additive range, indicating limited interaction strength. Notably, compounds **4d** and **4m** displayed substantially higher synergy scores than ricolinostat in HL-60 cells (C3.3 - Figure 11). This implies that the synergistic mechanism correlates with the co-inhibition of both HDAC6 and HDAC1, since ricolinostat is a significantly better HDAC6 than HDAC1 inhibitor. It has been shown that only the simultaneous targeting of both proteins has a significant effect on survival [302]. However, this study was done with knockdown models, which were not inhibitor-based, and lacked further robust synergy models like ZIP scoring. A logical next step would be to look at pan HDACi like panobinostat or preferential HDACi like the experimental inhibitor **2b** to investigate this phenomenon. As synergy with vorinostat was observed only under pretreatment conditions, sequential treatment strategies could be explored to optimize the observed effects [301].

The mechanism of synergy remains incompletely understood. One potential explanation lies in the interaction of HDAC6 with p53. Not only does HDAC6 modulate p53's stability and transcriptional activity, but it is also recruited by RUNX2 to silence p53-regulated genes [202-204]. This mechanism counteracts apoptosis induction and aligns with the observation that HDAC6-deficient K562 cells exhibit an increased tendency to undergo apoptosis upon

treatment with cytarabine or clofarabine. Concurrently, the proteomics data revealed that many of the DNA damage response proteins were downregulated when HDAC6 was knocked out in K562. Among these is CHEK1 a key enzyme which mediates cell-cycle arrest and the DNA damage response mechanisms (C3.4 - Supplemental 2A). Miyake et al. demonstrated that ricolinostat represses the function of CHEK1, which leads to synergy with adavosertib in p53-mutated squamous cell carcinoma cells [306]. Similar reports were shown in glioblastoma cells where HDAC6 inhibitors ablated the expression of CHEK1 and consequently reduced cell survival [307]. These observations highlight the need for further investigation into the functional relationship between CHEK1 and HDAC6 to elucidate the underlying mechanism of action.

A correlation analysis of cell line ZIP most synergistic area scores and inferred transcription factor or pathway activity RNA-seq data from the DepMap portal provided additional mechanistic insights (C3.4 - Figure 6J & Supplement 6F). The scores positively correlated with the negative regulation of DNA double strand break repair and the detoxification of reactive oxygen species (ROS). Both pathways are directly linked to the mechanism of action of cytarabine and clofarabine [308-310]. This suggests that the combination may be particularly effective against cells tending to have lower DNA repair mechanisms while tolerating reactive oxygen species. In this context, TP73 emerged as a key regulatory factor since synergy scores correlated negatively with its activity and it governs both pathways [308, 311]. Rehberger et. al. determined that TP73 confers resistance against cytarabine induced DNA damage [308]. Clinically, elevated TP73 expression even correlates with worse survival outcome and cytarabine resistance in AML [312]. A further observation was that upon Cytarabine treatment the expression of TP73 significantly increased in the KO (C3.4 - Supplement 6G). A sensible interpretation would be that the cells try to counteract the damage via TP73 induction however due to the low base line this compensatory upregulation might be too slow. Based on these findings incorporating HDACi with cytarabine in TP73 low expressing cases might be a suitable addition in to clinical therapy protocols. Nevertheless, further studies are required to investigate this relationship further and clarify the core component of this mechanism.

4.3 - Exploring non-cytotoxic effects of HDAC6 inhibition in myeloid leukemia

Although HDAC6 was initially considered a promising therapeutic target, its actual impact on cancer cell viability has since been questioned. While some studies have reported cytotoxic or cytostatic effects of HDAC6 inhibition, these outcomes appear to be highly

context-dependent [237, 263-265, 283]. As previously discussed, the experimental HDAC6 inhibitor **10p** (C3.1) failed to induce cytotoxicity *in vitro* across major leukemia subtypes, despite clear evidence of functional HDAC6 inhibition. According to the DepMap database, none of the hundreds of different cell lines deriving from different cancer origins showed significant dependency (score >0.5) on HDAC6 [313]. Most cases, particularly among leukemia subtypes, clustered near the minimum threshold of dependency prediction scores. Moreover, HDAC6 KO in mice did not affect viability or development, although it resulted in pronounced α -tubulin hyperacetylation [314]. Collectively, these evidences suggest that HDAC6 does not play a crucial role in regard to cell survival. Nevertheless, HDAC6 inhibition may prove valuable by creating therapeutic opportunities that enhance existing treatment strategies.

Accordingly, in the study “Epigenetic remodeling via HDAC6 inhibition amplifies anti-tumoral immune responses in myeloid leukemia cells” we investigated the cytotoxic and non-cytotoxic properties of HDAC6 inhibition in myeloid leukemia. Using CRISPR/Cas9-generated HDAC6 knockout models, it was demonstrated that the proliferation of CML and AML cells was unaffected, although functional markers such as α -tubulin hyperacetylation confirmed loss of HDAC6 activity (C3.4 - Figure 1A-H). While these *in vitro* experiments lacked significant changes, *in vivo* leukemia progression was significantly impaired upon HDAC6 loss (C3.4 - Figure 1I-L). These findings suggest that the functional relevance of HDAC6 inhibition may primarily manifest within the *in vivo* context. Indeed, a survival analysis of patients participating in the TARGET study revealed that high expression of HDAC6 is connected to a poorer prognosis in AML (C3.4 - Supplement 1A-D). Data from the TCGA LAML cohort further indicated that AML subtypes harboring FLT3, IDH1, or NRAS mutations exhibited worse prognosis when HDAC6 expression was elevated (C3.4 - Supplement 1E-H). These observations further support the notion that the anti-leukemic effects of HDAC6 inhibition depend on a *in vivo* context. Based on established evidence, HDAC6 inhibition can impair metastasis formation, attenuate the heat shock response, and enhance immune activation - effects that inherently require an *in vivo* setting [181, 182, 208, 209, 215]. In the study “Epigenetic remodeling via HDAC6 inhibition amplifies anti-tumoral immune responses in myeloid leukemia cells”, several novel effects of HDAC6 loss/inhibition were identified, including upregulation of lysosomal proteins such as LAMP1 and RNase T2, increased TNF- α secretion by CD8⁺ T cells, and enhanced sensitivity to standard AML chemotherapeutics, namely cytarabine and clofarabine.

These findings were corroborated pharmacologically using the HDAC6-preferential inhibitor ricolinostat. It was selected because, along with citarinostat, it represents one of the most HDAC6-selective inhibitors to have advanced into clinical evaluation [281, 315]. However,

ricolinostat presents several limitations as a model compared to the complete KO of HDAC6. It retains an binding affinity towards class I HDAC, but at the same time is unable to bind to CD2 or the zinc finger domain of HDAC6 [316]. Consequently, HDAC6 may remain partially functional, as only the CD1 domain is inhibited. A potential solution involves the use of proteolysis-targeting chimeras (PROTACs), which not only inhibit their targets but also induce their proteasomal degradation [317]. HDAC6-selective PROTACs have been shown to be functionally active and capable of inducing selective degradation of HDAC6 [318, 319]. However, PROTACs also present several challenges for in vivo application, including low aqueous solubility, poor oral bioavailability, and limited cell permeability owing to their high molecular weight and structural complexity [320]. Therefore, ricolinostat seemed preferable despite the previously mentioned drawback. The following overlapping effects from KO and ricolinostat could be directly attributed to the primary deacetylase function of HDAC6 CD1.

4.3.1 – HDAC6 loss induces an accumulation of lysosomes

In the study “Epigenetic remodeling via HDAC6 inhibition amplifies anti-tumoral immune responses in myeloid leukemia cells”, loss of HDAC6 in K562 cells led to a significant increase in lysosomal proteins within both the proteome and secretome (C3.4 - Figure 2A & C, Supplement 2A). These findings were validated via immunofluorescence detection of the lysosomal membrane protein LAMP1 (C3.4 - Figure 2E-F). LAMP1 staining formed compact cytosolic puncta, confirming the proteomic findings of lysosomal enrichment. Lysosomes are vital organelles with multiple roles in maintaining cellular metabolism [321]. They degrade defective or foreign macromolecules via proteolytic enzymes, thereby maintaining protein homeostasis and contributing to cellular defense against pathogens. Consequently, lysosomes represent a central component of the autophagy machinery. Lysosomes further amplify programmed cell death pathways, such as apoptosis, through the release of lytic enzymes. In response to plasma membrane injury, lysosomes are recruited by extracellular Ca^{2+} influx to facilitate membrane repair and maintain cellular integrity.

In cancer, lysosomes can promote tumor growth and survival through functional exploitation by malignant cells [322]. To sustain their elevated metabolic demands, cancer cells exploit lysosomal degradation of non-essential proteins to overcome nutrient deprivation. Lysosomes can also sequester chemotherapeutic agents, neutralizing or expelling them via exocytosis to promote cancer cell survival [323]. Additionally, lysosomal degradation of cell-surface cancer antigens may contribute to immune evasion. During metastasis establishment, cancer cells form lysosome-rich invadopodia, which release cathepsins to

break down the extracellular matrix [324]. In several cancers, LAMP1 and LAMP2 are recognized as prognostic biomarkers associated with aggressive tumor progression [325-327]. Consequently, elevated lysosomal abundance is generally considered indicative of adverse prognosis.

However, the observed lysosomal accumulation (LAMP1 staining, C3.4 – Figure 2E-F) can be mechanistically explained by the biological function of HDAC6. HDAC6 coordinates and transports aggresomes for autophagosome formation and facilitates their fusion with lysosomes through interactions with cortactin and the cytoskeleton. Consequently, loss of HDAC6 results in lysosomal accumulation and impaired autophagic function [187]. Consistent with these findings, Zhan et al. reported that HDAC6 knockdown increased LAMP2 levels and reduced autophagic activity in odontoblasts [328]. Furthermore, HDAC6 mediates lysosomal transport along microtubules via dynein, promoting co-localization and fusion with autophagosomes [329, 330]. Another potential mechanism depends on LC3 deacetylation via HDAC6 to counter starvation with autophagic nutrient provisioning [331]. Interestingly, other studies have reported that tubulin acetylation induced by HDAC6 loss can positively influence lysosomal fusion, as observed in models of Duchenne muscular dystrophy [332]. Still, most studies found that HDAC6 functionally supports lysosomal fusion and autophagy. The observed lysosomal accumulation is therefore consistent with functional impairment and may be further enhanced by increased gene expression driven by chromatin relaxation (C3.4 - Figure 3D).

Under the assumption that the accumulation is not based on the impaired function but rather a reaction to cope with the HDAC6 loss, several therapeutically interesting options open up. Lysosomal dependency is a double-edged sword, which can be exploited to target the dependent cells specifically. ROS or lysosomotropic compounds can compromise lysosomal membrane integrity, potentially leading to the release of lytic enzymes and induction of apoptosis [333-335]. Ferroptosis-inducing agents like disulfiram, could display amplified cytotoxicity when lysosomes accumulate [336]. Other agents, such as chloroquine, inhibit autophagic flux by increasing the lysosomal pH and inactivating acid hydrolases, ultimately leading to autophagic cell death [337]. This mechanism could counteract potential chemoresistance associated with lysosome accumulation. However, during the drug screening, the K562 KO cells did not acquire resistance but rather exhibited increased vulnerability to a broad range of tested compounds (C3.4 - Figure 6A). Notably, cytarabine and clofarabine are of particular interest in this context, as both induce ROS as part of their cytotoxic mechanism. These findings support the conclusion that lysosomal accumulation does not confer drug resistance but rather reflects functional impairment.

Conversely, antibody-drug conjugates could benefit from lysosome accumulation if lysosomal function is retained. After target binding and internalization, these antibodies are trafficked through the endosomal pathway into the lysosome and release their activated payload [338]. A prominent example in this case of AML treatment is gemtuzumab ozogamicin, which targets CD33 to release calicheamicin [57]. An alternative which targets CD33 and demonstrated high activity in preclinical studies is vadastuximab talirine [339]. It releases a pyrrolobenzodiazepine dimer, which crosslinks DNA to induce cell death. In AML, the cell surface receptor CLL-1 can be targeted as an alternative to CD33 via the antibody DCLL9718A [340]. It carries and releases similar to vadastuximab talirine a pyrrolobenzodiazepine dimer. Several studies already found a link between antibody-drug conjugate potency/efficacy by modulating lysosomal processing [341, 342]. Inducing lysosomal accumulation selectively in leukemic cells could enhance ADC specificity, provided that this phenomenon is not universally present across cell types. This would potentiate the safety of the treatment by lowering off-toxicity against healthy cells. Therefore, HDAC6 inhibition-mediated lysosomal accumulation could represent a valuable adjunct to emerging antibody-drug conjugate therapies for AML.

4.3.2 – The ablation of HDAC6 potentially leads to lysosome secretion

Another notable finding was the increased abundance of lysosomal lumen proteins in the secretome of K562 HDAC6 KO cells (C3.4 - Figure 2B-C & 2D, Supplement 2B). The presence of lysosomal components in the extracellular space may indicate extracellular digestion, defense mechanisms normally deployed by neutrophils or a cell stress counter mechanism [343]. In regards to cell stress, a stress signal cascade will initiate extracellular exocytosis. This process traffics lysosomes to the cellular periphery, docks them to the plasma membrane and induces a Ca^{2+} dependent fusion of lysosome and cell membrane leading to an externalization of the lysosomal content [344]. This especially relates to either lysosomal or cell membrane damage. If the lysosomal membrane is compromised this could have dire consequences since the leaking enzymes would damage cellular components [345, 346]. Lee et al. demonstrated that HDAC6 loss results in the accumulation of immature autophagic vesicles [187]. Furthermore, it is known that lysosome dysfunction increases the risk of lysosome permeabilization and cell death [347]. Therefore, the secretion could act as a rescue mechanism, which could act as an exploitable weakness. Drugs like vacuolin-1 could inhibit Ca^{2+} dependent exocytosis, which could in turn result in cytotoxic stress [348]. Therefore, a reasonable next step would be to investigate the synergy mechanism.

HDAC6 is one of the best-established regulators of lysosome trafficking however direct connections the lysosome secretion process remains sparse. Nevertheless, an at least indirect regulatory involvement appears plausible. Mechanistically, this would suggest a negative regulation by HDAC6, since the extracellular lysosomal content increases upon HDAC6 loss. Reed et al. demonstrated in 2006 that the motor protein kinesin1 was more tightly bound and traveled longer distances on acetylated tubulin [349]. This observation is supported by evidence showing that acetylated microtubules possess higher bundling affinity, thereby enhancing kinesin-1 motility [350]. Organelle transport, including that of mitochondria, is enhanced along acetylated microtubules [351]. It has been shown that the deletion of α -tubulin acetylase S6K1 impairs the autophagic flux and lysosome-autophagosome-fusion through spatial separation [352]. This highlights the critical role of α -tubulin acetylation in regard to lysosome mobility and provides a sensible mechanistic rationale.

Another potential facet is the identification of neutrophil degranulation components in the secretomics analysis of the K562 HDAC6 KO models (C3.4 – Figures 2D). These granules typically fight pathogens, by releasing toxic agents, but in cancer their effect is more nuanced [353]. The released enzymes cause inflammation and produce reactive oxygen species, which can damage the tumor cells. In the K562 NOD SCID Gamma (NSG) mouse model, the prolonged survival observed in the HDAC6 KO group (C3.4 – Figures 1I–J) may be attributed to the KO cells themselves generating a cytotoxic or immunostimulatory microenvironment. Leukemic cells are known to be susceptible to cytotoxic components secreted by neutrophils [354, 355]. Currently, there is very little information about the connection of neutrophil degranulation induction and HDAC6 inhibition. Therefore, additional investigations of these processes are necessary and whether similar effects could occur in patient.

4.3.3 – The accumulation of tumor suppressor RNase T2 is induced by targeting HDAC6

Among the lysosome-associated proteins enriched following HDAC6 loss, RNase T2 was consistently upregulated across all tested cell lines (C3.4 - Figure 4A-C, Supplement 4A). Treatment with the HDAC6-preferential inhibitor ricolinostat similarly increased RNase T2 levels, confirming that this effect can be pharmacologically reproduced *in vitro* (C3.4 - Figure 4D-K, Supplement 4K). RNase T2 belongs to the highly conserved T2 family endoribonucleases and catalyzes the cleavage of single-strand (polyA) RNA molecules into mono- or oligonucleotides [356]. RNase T2 predominantly localizes to lysosomes but is also found in mitochondria and the endoplasmic reticulum, and can additionally be secreted into

the extracellular space [357]. In the innate immune system, RNase T2 plays an essential role by recognizing and degrading pathogenic RNA, thereby activating immune sensing pathways through TLR7 and TLR8 to initiate responses against intracellular pathogens [358, 359]. Secreted RNase T2 can also function as an alarmin to recruit immune cells in response to infections, pathogens, or tissue damage [360]. Furthermore, RNase T2 regulates the turnover of mitochondrial and ribosomal RNA, thereby influencing cellular energy metabolism and protein synthesis [361, 362].

Beyond its role in innate immunity, RNase T2 has also been recognized as a tumor suppressor. The RNASET2 gene, located on chromosome 6q27, is frequently deleted across multiple cancer types, resulting in reduced or absent transcript expression [363]. Loss of RNase T2 expression was shown to accelerate tumor growth in ovarian cancer xenografts. Additionally, RNase T2 downregulation was associated with drug resistance in ovarian cancer, highlighting that its overexpression could mitigate drug resistance and reduce relapse risk [364]. In human T-ALL caused by human T-cell leukemia virus type 1 it has been demonstrated that the viral Tax protein causes RNaseT2 downregulation [365]. An analysis of patient survival data from the TARGET AML cohort revealed that high RNASET2 expression in bone marrow samples correlates with significantly improved overall survival ($p = 5.48 \times 10^{-5}$; $n = 547-1166$; data not shown). In addition to its potential as a prognostic biomarker, recombinant RNase T2 protein may serve as a therapeutic agent, as suggested by studies in colon cancer models [366]. The subsequently induced ratio of low functional HDAC6 against high RNase T2 had significantly improved survival rates in the TARGET data, underscoring its relevance in this disease (C3.4 - Supplement 4N).

The tumor-suppressive function of RNase T2 appears largely independent of its catalytic activity and instead relies on immune-cell recruitment and interactions with interleukins [367]. Primary target of RNase T2 in its function as an immune modulator are macrophages. Essentially, it can recruit macrophages and turns the anti-inflammatory M2 state to the pro-inflammatory M1 phenotype macrophages within the tumor microenvironment [368, 369]. M1 macrophages exert anti-tumor activity by secretion of pro-inflammatory cytokines like TNF- α and IL12. This polarization may be clinically advantageous, as tumor-associated macrophages (TAMs) typically support tumor growth. In a mouse model, Acquati et al. (2010) demonstrated that depletion of macrophages using clodronate abolished the tumor-suppressive effect of RNase T2 [370]. Upon comparing the effect of high vs low HDAC/RNase T2 ratio, decreased levels of M2 macrophages were associated with improved survival chances in the TARGET study (C3.4 - Supplement 3P). Another immune cell type that gets stimulated by RNase T2 is dendritic cells [371, 372]. As dendritic cells are essential activators of cytotoxic CD8⁺ T cells through antigen presentation, their

activation substantially enhances anti-tumor immunity. In this context, dendritic cells were shown to contribute substantially to the *in vivo* anti-leukemic effects of panobinostat [267].

Although RNase T2 secretion following HDAC6 loss may influence the *in vivo* phenotype, this effect is likely limited in the NSG mouse model, which is severely immunocompromised (C3.4 - Figure 1I-J). In NSG mice, T cells, B cells, and NK cells are functionally deficient, whereas macrophages, dendritic cells, granulocytes, and mast cells retain partial activity [373]. Furthermore, the NOD background confers intrinsically reduced macrophage activation, impaired antigen presentation, and defective phagocytic function. Therefore, additional characterization of NSG macrophages with different RNase T2 expression might elucidate the mechanism. Similar considerations could be made for dendritic cells, but it seems reasonable to assume a much minor role due to the loss of T cells as their principal downstream effector. Beyond immunomodulatory activity, RNase T2 may also exert direct cytotoxic effects, for example by amplifying ROS production during oxidative stress [374]. This observation aligns with elevated neutrophil signatures in the K562, potentially corroborating higher ROS production in the HDAC6 KO (C3.4 - Figure 2C-D). RNase T2 overexpression can induce induced apoptosis through TRAF2 binding as a cell stress response in both melanocytes and keratinocytes [367]. Given the potential involvement of multiple pathways, further studies are required to clarify the precise mechanisms underlying RNase T2-mediated cytotoxicity.

Another notable observation of the RNase T2 overexpression is that it appears rather specific towards the myeloid lineage when compared to the lymphoid lineage (C3.4 - Supplement 4C-F). Since myeloid leukemia cells potentially differentiate into neutrophils with elevated lysosome levels, assuming a certain tendency towards RNase T2 expression is logical. However, the regulatory mechanisms by which HDAC6 controls RNase T2 expression remain poorly understood. In K562 cells, genes such as RNase T2 and LAMP1 displayed increased chromatin accessibility, accompanied by elevated RNA transcription and protein abundance (C3.4 - Figure 3C-D, Figure 4A-C). This indicates that HDAC6 (in)directly represses RNase T2 in myeloid lineage leukemia. In accordance, an analysis of the TARGET ALL cohort revealed that low RNASET2 expression correlated with improved survival, which was negatively associated with M1 macrophage abundance and positively associated with M2 macrophages ($p=0.037$, $n=92-291$, data not shown). While mostly seen as a tumor suppressor, in the case of clear cell renal cell carcinoma, high RNase T2 amounts are an indicator of poor survival [375]. This observation highlights the context-dependent and tumor-type-specific nature of RNase T2 function. In conclusion, these findings indicate that HDAC6 inhibition may activate components of the innate immune system through lysosomal proteins such as RNase T2, consistent with favorable patient survival data.

4.3.4 – HDAC6 inhibition primes myeloid leukemia cells for CD8⁺ T cells mediated cytotoxicity

Increasing evidence indicates that HDAC6 inhibition synergizes with CD8⁺ T-cell–based immunotherapies. In melanoma and myeloma, ricolinostat disrupted the PD-1/PD-L1 axis, leading to a significantly enhanced activity of CD8⁺ T cells [207]. In a CLL mouse model, the selective HDAC6 inhibitor ACY-738 reduced the level of exhausted effector CD8⁺ T cells [271]. The HDAC6 inhibitor AVS100 not only facilitated the tumor infiltration of M1 macrophages but also CD8⁺ T cells into colon cancer models [376]. This was accompanied by increased T-cell–mediated inflammatory signatures, including elevated TNF- α expression. Consequently, in our study, the impact of ricolinostat on T-cell–mediated cytotoxicity was investigated in a syngeneic, immunocompetent mouse model of AML. Both ricolinostat and the HDAC6 KO enhanced intracellular TNF- α levels in the bone marrow but also in an *in vitro* co-culture (C3.4 - Figure 5B & 5E-F). This was accompanied by increased *in vitro* cytotoxic activity, whereas *in vivo* effects were modest, underscoring the need for suitable combination partners (C3.4 - Figure 5J-K). A modest yet statistically significant downregulation of PD-L1 expression on macrophages was observed following ricolinostat treatment (data not shown). In K562 cells, ricolinostat induced the expression of MHC class I components, potentially enhancing T-cell recognition.

The effect of HDAC6 inhibition on T cell activation in myeloid and lymphoid leukemia has been poorly characterized to date. Generally, AML patients display patterns of CD8⁺ T cell exhaustion and senescence [377]. Overcoming this functional blockade often distinguishes chemotherapy responders from non-responders, directly linking patient survival to adaptive immune competence. In light of the previously observed synergistic cytotoxicity with cytarabine, ricolinostat may activate both innate and adaptive immune pathways. However, the precise mechanisms underlying increased susceptibility to T-cell–mediated cytotoxicity remain to be elucidated. Prolonged TNF- α exposure could harm the patient by increasing invasiveness, genomic instability, and immune suppressive cell recruitment [378, 379]. Enhancing cytotoxicity through rational combination with conventional chemotherapeutics could therefore offer therapeutic benefit. Combining DNMT inhibitors with HDAC6 inhibitors has been shown to synergistically activate CD8⁺ T cells and NK cells by reversing the immunosuppressive tumor microenvironment, as demonstrated in ovarian cancer models [299]. The well-established synergy between decitabine and broad HDAC inhibition could further enhance immune engagement. Such effects may be particularly advantageous for elderly AML patients, who often exhibit reduced immune responsiveness and poor prognosis. Overall, these findings support HDAC6 inhibition as a promising therapeutic strategy that may bridge chemotherapy with modern immune-based treatment approaches.

5 - Outlook

The findings presented in this work provide a foundation for advancing HDAC-targeted therapeutic strategies in acute leukemia. Insights gained from the characterization of novel inhibitors may inform the design of further optimized compounds. However, strategies aimed solely at reducing IC50 values through enhanced binding affinity toward class I HDACs may prove counterproductive, given the fundamental dependence of hematopoietic cells on these enzymes. A more rational approach would involve the development of compounds with defined isoenzyme selectivity, which would be particularly valuable once HDAC dependencies across distinct leukemia subtypes are systematically established. Nonetheless, the high degree of structural and functional homology among HDAC family members, combined with the limited understanding of class IIa HDAC mechanisms, highlights the need for further fundamental research in this area.

The identified synergistic drug combinations demonstrate translational potential in the clinical setting. Both cytarabine and decitabine are standard chemotherapeutic agents in AML treatment, and their efficacy may be enhanced by the addition of HDAC6 inhibitors. While proteomic and ATAC-seq analyses have provided preliminary mechanistic insights into the interactions with cytarabine and clofarabine, definitive mechanisms remain unresolved. Correlation analyses suggest an association between drug synergy and pathways related to DNA repair, oxidative stress detoxification, and TP73 activity. Future investigations should therefore focus on assessing the impact of HDAC6 inhibitors in combination with cytarabine or clofarabine on DNA damage responses. This could include evaluating general DNA damage via γ H2AX immunoblotting, detecting double-strand breaks using TUNEL assays, and quantifying reactive oxygen species (ROS) levels under combination versus single-agent treatment. Given the role of glutamate secretion as a resistance mechanism against ROS, extracellular glutamate concentrations should also be measured. We found that TP73 could serve as a predictive resistance marker and was induced more in a combinatory treatment, however its final functionality has to be determined. As a sensitivity marker a knockdown via siRNA in a high TP73 expression cell line would be an appropriate tool to investigate the protective function of the protein.

An additional critical step is the evaluation of these combinations *in vivo* to determine their therapeutic efficacy and immune-related effects. In particular, the observed CD8⁺ T-cell stimulation upon HDAC6 inhibitor treatment suggests that combinatorial approaches may leverage immune-mediated antileukemic responses. Since AML treatment outcomes are closely linked to the interaction between chemotherapy and the immune system, immunocompetent syngeneic mouse models are likely to provide more physiologically relevant insights than immunodeficient human xenograft models. With such a syngeneic *in*

vivo experiment not only synergistic reduction of the leukemic burden could be investigated but also changes in the immune composition in the bone marrow via FACS. To get a holistic representation of the impact through combinatory treatment, a single cell RNA-seq experiment from cells isolated from the bone marrow would be a powerful strategy. Especially CITE-seq, which conserves the structure of the bone marrow, while revealing the single cell RNA expression profile could elucidate this rather complex interplay. Since these methods remain rather expensive, an alternative would be the CODEX multiplexed tissue imaging technique. However, this method is only suitable to investigate a smaller predefined panel of proteins. A similar follow up for a synergistic combination of HDACi and decitabine seems reasonable due to known immunostimulatory effects from neoantigen expression induced by this treatment.

To further investigate the observed alterations in lysosomes, a logical next step would be to elucidate the underlying biological mechanisms responsible for these changes. For specific visualization and quantification of lysosomes, the use of a LysoTracker probe would be an appropriate and effective approach. These fluorophores react to lower pH-values by changing their spectra, which can be used to infer lysosome functionality. Normally they operate from pH 4.5 to 5.0 and alterations going into the neutral area would indicate impaired functions. If lysosomes are found to accumulate and remain functionally active, a subsequent step could involve investigating their biophysical interactions with autophagosomes and endosomes to better understand the dynamics of intracellular trafficking and degradation pathways. However, the effects of HDAC6 on aggresome transport α -tubulin and cortactin acetylation are already well documented. Therefore, it might be more insightful to study how the lysosomal accumulation affects the cellular metabolism through autophagy restriction. A wider panel of HDAC6 KO models in combination of RNA-seq analysis with the META-Flux analysis R package could elucidate compensatory mechanisms. Using lysosome dependent drugs like gemtuzumab ozogamicin could be interesting if the lysosomes remain functional. A potential next step would be to treat HDAC6 KO cells with an antibody-drug conjugate or to perform a combinatorial matrix screening using HDAC6 inhibitor to identify synergistic therapeutic interactions. If the lysosome functionality is impaired it would be more feasible to look into drugs which get deactivated in the lysosome.

A major limitation of the present study is that RNase T2 upregulation was observed, but its functional role was not investigated. Although RNase T2 has been described as a tumor suppressor, there is no direct evidence linking it to reduced leukemic burden in AML, and its broader relevance in this disease context remains unexplored. Given that RNase T2 primarily exerts effects through macrophage activation, a logical next step would be to isolate macrophages from mouse models or healthy human donors for functional assays. If

primary samples are limited, an alternative approach would be to differentiate AML cell lines such as THP-1 into macrophage-like cells using PMA. These macrophages could then be co-cultured with HDAC6 KO or ricolinostat-treated leukemia cells, or exposed to conditioned media from these cultures, to assess indirect effects mediated by secreted factors. Cytokine profiling could subsequently be performed to determine macrophage polarization states (e.g., TNF- α or iNOS for M1, IL-10 or TGF- β for M2).

To directly assess tumor-suppressive function, RNase T2 KO or knockdown models should be established and evaluated in vivo. If RNase T2 functions as a tumor suppressor, loss of expression would be expected to accelerate leukemic progression and reduce survival in recipient mice. Following survival analysis, immune profiling of the bone marrow should be conducted, with a particular emphasis on macrophage abundance and proliferative state. While the referenced studies have advanced the understanding of HDAC6 inhibition in leukemia, these critical questions surrounding RNase T2 function remain unresolved and warrant further investigation.

6 - Conclusion

This dissertation investigated the role and therapeutic potential of HDAC6 inhibition in leukemia, with a particular emphasis on the clinically challenging subtype of AML. In collaboration with medicinal chemists, several novel HDAC6 inhibitors were synthesized and characterized for their antileukemic activity. Standard chemotherapeutics such as cytarabine and decitabine demonstrated strong synergistic effects with HDAC6 inhibition, particularly in AML models, with the most pronounced efficacy observed for selective HDAC6 inhibitors. Both genetic KO and pharmacological inhibition of HDAC6 using ricolinostat were examined across a diverse panel of leukemia cell lines and PDX samples. Distinct response patterns were observed between AML and ALL models. HDAC6 inhibition induced activation signatures within both the innate and adaptive immune systems, suggesting that this pathway may be leveraged to enhance immunotherapeutic strategies. Collectively, these findings advance the mechanistic understanding of HDAC6 biology in leukemia and provide a conceptual framework for integrating HDAC6-targeted approaches into clinical treatment paradigms. While this initial preclinical data seems promising, of course, much more effort is needed to broaden the understanding of the synergistic interaction and immune stimulatory effect *in vivo*. Future work should also clarify the extent to which ricolinostat's activity reflects true HDAC6 specificity versus off-target effects on other HDAC isoforms or unrelated proteins. Taken together, the studies included in this thesis underscore the therapeutic potential of HDAC(6) inhibition in leukemia, particularly when used in rational combination strategies, and demonstrate that selective HDAC6 inhibition possesses distinct and clinically relevant properties.

Further co-authorship publications

The following publications were co-authored during the PhD but not included as cumulative publications:

1. **Multicomponent Synthesis, Binding Mode, and Structure-Activity Relationship of Selective Histone Deacetylase 6 (HDAC6) Inhibitors with Bifurcated Capping Groups**

Nina Reißing, Melf Sönnichsen, Jeremy D. Osko, Andrea Schöler, **Julian Schliehe-Diecks**, Alexander Skerhut, Arndt Borkhardt, Julia Hauer, Matthias U. Kassack, David. W. Christianson, Sanil Bhatia, Finn K. Hansen

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DOI <https://doi.org/10.1021/acs.jmedchem.9b01888>

2. **Synergistic induction of apoptosis in resistant head and neck carcinoma and leukemia by alkoxyamide-based histone deacetylase inhibitors**

Leandro A. Alves Avelar, Christian Schrenk, Melf Sönnichsen, Alexandra Hamacher, Finn K. Hansen, **Julian Schliehe-Diecks**, Arndt Borkhardt, Sanil Bhatia, Matthias U. Kassack, Thomas Kurz

European Journal of Medicinal Chemistry – February 5, 2021

2-Year Impact Factor (2025): 6.0

DOI: <https://doi.org/10.1016/j.ejmech.2020.113095>

3. **Oxa Analogues of Nexturastat A Demonstrate Improved HDAC6 Selectivity and Superior Antileukaemia Activity**

Dr. Marc Pflieger, Melf Sönnichsen, Nadine Horstick-Muche, Jing Yang, **Julian Schliehe-Diecks**, Andrea Schöler, Prof. Dr. Arndt Borkhardt, Dr. Alexandra Hamacher, Prof. Dr. Matthias U. Kassack, Prof. Dr. Finn K. Hansen, Dr. Sanil Bhatia, Prof. Dr. Thomas Kurz

ChemMedChem – February 24, 2021

Impact Factor (2025): 3.5

DOI: <https://doi.org/10.1002/cmdc.202001011>

4. **Borinostats: Solid Phase Synthesis of Carborane-Capped Histone Deacetylase Inhibitors with a Tailor-Made Selectivity Profile**

Christoph Selg, Andrea Schöler, **Julian Schliehe-Diecks**, Maria Hanl, Laura Sinatra, Arndt Borkhardt, Menyhárt B. Sárosi, Bhatia, Evamarie Hey-Haw, Finn K. Hansen

Chemical Science – August 4, 2021

Impact Factor (2025): 8.1

DOI: <https://doi.org/10.1039/D1SC02268G>

5. **4-Acyl Pyrrole Capped HDAC Inhibitors: A New Scaffold for Hybrid Inhibitors of BET Proteins and Histone Deacetylases as Antileukemia Drug Leads**

Linda Schäker-Hübner, Robin Warstat, Heinz Ahlert, Pankaj Mishra, Fabian B. Kraft, **Julian Schliehe-Diecks**, Andrea Schöler, Arndt Borkhardt, Bernhard Breit, Sanil Bhatia, Martin Hügler, Stefan Günther, Finn K. Hansen

Journal of Medicinal Chemistry – September 28, 2021

Impact Factor (2025): 7.3

DOI: <https://doi.org/10.1021/acs.jmedchem.1c01119>

6. **Synthesis, Antiplasmodial, and Antileukemia Activity of Dihydroartemisinin–HDAC Inhibitor Hybrids as Multitarget Drugs**

Lukas von Bredow, Thomas Martin Schäfer, Julian Hogenkamp, Maik Tretbar, Daniel Stopper, Fabian B. Kraft, **Julian Schliehe-Diecks**, Andrea Schöler, Arndt Borkhardt, Sanil Bhatia, Jana Held, Finn K. Hansen

Pharmaceuticals – March 9, 2022

Impact Factor (2025): 4.99

DOI: <https://doi.org/10.3390/ph15030333>

7. **The Mycotoxin Beauvericin Exhibits Immunostimulatory Effects on Dendritic Cells via Activating the TLR4 Signaling Pathway**

Xiaoli Yang, Shafaqat Ali, Manman Zhao, Lisa Richter, Vanessa Schäfer, **Julian Schliehe-Diecks**, Marian Frank, Jing Qi, Pia-Katharina Larsen, Jennifer Skerra, Heba Islam, Thorsten Wachtmeister, Christina Alter, Anfei Huang, Sanil Bhatia, Karl Köhrer, Carsten Kirschning, Heike Weighardt, Ulrich Kalinke, Rainer Kalscheuer, Markus Uhrberg, Stefanie Scheu

Frontiers in Immunology – April 8, 2022

Impact Factor (2025): 7.0

DOI: <https://doi.org/10.3389/fimmu.2022.856230>

8. **Development of a First-in-Class Small-Molecule Inhibitor of the C-Terminal Hsp90 Dimerization**

Sanil Bhatia, Lukas Spanier, David Bickel, Niklas Dienstbier, Vitalij Woloschin, Melina Vogt, Henrik Pols, Beate Lungerich, Jens Reiners, Narges Aghaallaei, Daniela Diedrich, Benedikt Frieg, **Julian Schliehe-Diecks**, Bertan Bopp, Franziska Lang, Mohanraj Gopalswamy, Jennifer Loschwitz, Baubak Bajoghli, Julia Skokowa, Arndt Borkhardt, Julia Hauer, Finn K. Hansen, Sander H. J. Smits, Joachim Jose, Holger Gohlke, Thomas Kurz

ACS Central Science – April 27, 2022

Impact Factor (2026): 11.1

DOI: <https://doi.org/10.1021/acscentsci.2c00013>

9. The mycotoxin viriditoxin induces leukemia-and lymphoma- specific apoptosis by targeting mitochondrial metabolism

Fabian Stuhldreier, Laura Schmitt, Thomas Lenz, Ilka Hinxlage, Marcel Zimmermann, Philipp Wollnitzke, **Julian Schliehe-Diecks**, Yang Liu, Paul Jäger, Stefanie Geyh, Nicole Teusch, Christoph Peter, Sanil Bhatia, Rainer Haas, Bodo Levkau, Andreas S. Reichert, Kai Stühler, Peter Proksch, Björn Stork & Sebastian Wesselborg

Cell Death & Disease – November 8, 2022

Impact Factor (2026): 12.2

DOI: <https://doi.org/10.1038/s41419-022-05356-w>

10. Solid-Phase Synthesis of Cereblon-Recruiting Selective Histone Deacetylase 6 Degraders (HDAC6 PROTACs) with Antileukemic Activity

Laura Sinatra, Jing Yang, **Julian Schliehe-Diecks**, Niklas Dienstbier, Melina Vogt, Philip Gebing, Luisa M. Bachmann, Melf Sönnichsen, Thomas Lenz, Kai Stühler, Andrea Schöler, Arndt Borkhardt, Sanil Bhatia, Finn K. Hansen

Journal of Medicinal Chemistry – December 6, 2022

Impact Factor (2025): 7.3

DOI: <https://doi.org/10.1021/acs.jmedchem.2c01659>

11. Co-targeting HSP90 alpha and CDK7 overcomes resistance against HSP90 inhibitors in BCR-ABL1⁺ leukemia cells

Melina Vogt, Niklas Dienstbier, **Julian Schliehe-Diecks**, Katerina Scharov, Jia-Wey Tu, Philip Gebing, Julian Hogenkamp, Berna-Selin Bilen, Silke Furlan, Daniel Picard, Marc Remke, Layal Yasin, David Bickel, Munishikha Kalia, Alfredo Iacoangeli, Thomas Lenz, Kai Stühler, Aleksandra A. Pandyra, Julia Hauer, Ute Fischer, Rabea Wagener, Arndt Borkhardt & Sanil Bhatia

Cell Death & Disease – December 6, 2023

Impact Factor (2026): 12.2

DOI: <https://doi.org/10.1038/s41419-023-06337-3>

12. Proteogenomic profiling uncovers differential therapeutic vulnerabilities between TCF3::PBX1 and TCF3::HLF translocated B-cell acute lymphoblastic leukemia

Lena Blümel, Flavia Bernardi, Daniel Picard, Jacob Torrejon Diaz, Vera H. Jepsen, Rebecca Hasselmann, **Julian Schliehe-Diecks**, Jasmin Bartl, Nan Qin, Beat Bornhauser, Sanil Bhatia, Blerim Marovka, Veronique Marsaud, Florent Dingli, Damarys Loew, Martin Stanulla, Jean-Pierre Bourqin, Arndt Borkhardt, Marc Remke, Olivier Ayrault, Ute Fischer

Haematologica – July, 2024

Impact Factor (2025): 8.2

13. Synergistic drug interactions of the histone deacetylase inhibitor givinostat (ITF2357) in CRLF2-rearranged pediatric B-cell precursor Acute Lymphoblastic Leukemia identified by high-throughput drug screening

Athanasios Oikonomou, Titus Watrin, Luigia Valsecchi, Katerina Scharov, Angela Maria Savino, **Julian Schliehe-Diecks**, Michela Bardini, Grazia Fazio, Silvia Bresolin, Andrea Biondi, Arndt Borkhardt, Sanil Bhatia, Giovanni Cazzaniga, Chiara Palmia

Helyion – July, 2024

Impact Factor (2025): 3.6

DOI: <https://doi.org/10.1016/j.heliyon.2024.e34033>

14. A brain organoid/ALL co-culture model reveals the AP-1 pathway as critically associated with CNS involvement of BCP-ALL

Philip Gebing, Stefanos Loizou, Sebastian Hänsch, **Julian Schliehe-Diecks**, Lea Spory, Pawel Stachura, Vera Jespen, Melina Vogt, Aleksandra Pandyra, Herui Wang, Zhengping Zhuang, Johannes Zimmermann, Martin Schrappe, Gunnar Cario, Ameera Alsadeq, Denis M. Schewe, Arndt Borkhardt, Lennart Lenk, Ute Fischer, Sanil Bhatia

Blood advances – September 25, 2024

Impact Factor (2026): 7.7

DOI: <https://doi.org/10.1182/bloodadvances.2023011145>

15. H1-0 is a specific mediator of the repressive ETV6::RUNX1 transcriptional landscape in preleukemia and B cell acute lymphoblastic leukemia

Vera H. Jepsen, Andrea Hanel, Daniel Picard, Rigveda Bhawe, Rebecca Hasselmann, Juha Mehtonen, **Julian Schliehe-Diecks**, Carla-Johanna Kath, Vithusan Suppiyar, Yash Prasad, Katerina Schaal, Jia-Wey Tu, Nadine Rüchel, Ersen Kameri, Nan Qin, Herui Wang, Zhengping Zhuang, Rabea Wagener, Lena Blümel, Tobias Lautwein, Daniel Hein, David Koppstein, Gesine Kögler, Marc Remke, Sanil Bhatia, Merja Heinäniemi, Arndt Borkhardt, Ute Fischer

HemaSphere – April 2, 2025

Impact Factor (2024): 14.6

DOI: <https://doi.org/10.1002/hem3.70116>

16. Discovery of Histone Deacetylase 8-Specific Proteolysis-Targeting Chimeras with Anticancer Activity against Hematological Malignancies

Shiyang Zhai, Marie Kemkes, Cindy-Esther Kponomaizoun, Jan Gerhartz, Felix Feller, Jia-Wey Tu, Dominika Ewa Pieńkowska, Julian Schliehe-Diecks, Ina Dressel, Michael Gütschow, Radosław P. Nowak, Christian Steinebach, Sanil Bhatia, Finn K. Hansen

Journal of Medicinal Chemistry – January 2, 2026

Impact Factor (2025): 7.3

DOI: <https://doi.org/10.1021/acs.jmedchem.5c00939>

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