Synthesis and Biological Characterization of Novel Inhibitors of Histone Deacetylases as Anticancer and Antiparasitic Agents

DISSERTATION

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Abstract

Epigenetics has emerged to become an important field of modern medicinal chemistry and drug discovery. Research in this field often combines different methods such as computational studies, chemical synthesis, biochemical and biophysical assays, and cellular testing. Combination of all these approaches together is essential to identify new probes and study their potential in different physiological and pathological systems. The publications presented in this thesis focus on identifying novel inhibitors against certain histone deacetylases (HDAC) isoforms and exploring their potential as anticancer and antiparasitic agents.

Firstly, a novel series of benzhydroxamates were identified as human HDAC inhibitors with decreased activity against other HDAC isoforms. These derivatives showed a strong HDAC8 inhibition phenotype in neuroblastoma cells. Secondly, the polypharmacology concept was applied to design dual-acting inhibitors of HDAC8 (as well as 6) and the bromodomain/PHD finger-containing protein (BRPF1). Despite their in vitro activity, the synthesized HDAC8/BRPF1 and HDAC6/BRPF1 dual inhibitors did not show cellular effects on acute myeloid leukemia cells (AML).

Benzhydroxamates were further explored against schistosomal histone deacetylases, with a focus on HDAC8 (smHDAC8). Several nanomolar inhibitors were identified and further explored. The structure-activity relationship (SAR) of the synthesized derivatives was determined, and some of the novel inhibitors also showed remarkable activity in vivo against the parasite. Based on these smHDAC8 inhibitors, a quantitative structure-activity relationship (QSAR) model was generated and refined to predict the biological activity of novel compounds. The developed smHDAC8 inhibitors were further explored and tested against other parasitic infections. Some of them showed antileishmanial and cestocidal activity, highlighting their potential as novel antiparasitic agents. Additionally, a continuous assay based on a thiotrifluoroacetyled substrate was successfully applied to measure HDAC8 catalytic activity. The assay is compatible with different microtiter plates and could facilitate enzymatic testing of more HDAC inhibitors.

Finally, the binding mode of balsalazide, a previously reported hit of the deacylase sirtuin 5 (Sirt5), was studied, and SAR analysis identified the structural parts essential for the inhibitory activity. Balsalazide was also found to be selective for Sirt5 over other sirtuins isoforms.

The results obtained in the current work could be potential starting points for future projects to optimize these novel chemotypes as HDAC inhibitors for anticancer and antiparasitic therapies.

Keywords: Epigenetics, HDAC, Anticancer, Antiparasitic, Benzhydroxamates, Neuroblastoma, Polypharmacology, Dual-acting inhibitors, BRPF1, Schistosomiasis, smHDAC8, QSAR, Antileishmanial, Cestocidal, Continuous HDAC assay, Balsalazide, Sirtuin 5.

Kurzfassung

Die Epigenetik spielt eine wichtige Rolle in der modernen Medizinischen Chemie und Wirkstoffforschung. Die Forschung auf diesem Gebiet kombiniert oft verschiedene Methoden wie Computergestützte Studien, chemische Synthese, biochemische und biophysikalische Assays, sowie zelluläre Tests. Das Zusammenspiel all dieser Methoden ist wesentlich, um neue epigenetische Modulatoren zu identifizieren und ihr Potenzial in verschiedenen physiologischen und pathologischen Systemen zu untersuchen. Die in dieser Dissertation präsentierten Veröffentlichungen konzentrieren sich auf die Identifizierung neuer Inhibitoren bestimmter Histon-Deacetylasen (HDAC)-Isoformen und die Untersuchung ihres Potenzials als Antikrebs- und Antiparasitenmittel.

Zunächst wurde eine neue Reihe von Benzhydroxamaten als humane HDAC-Inhibitoren mit verminderter Aktivität gegenüber anderen HDAC-Isoformen identifiziert. Diese Derivate zeigten in Neuroblastomzellen einen starken HDAC8-Hemmungsphänotypus. Desweiteren wurde ein polypharmakologisches Konzept angewendet, um dual wirkende Inhibitoren von HDAC8 (als auch 6) und der Bromodomäne und dem PHD-Finger-enthaltenden Protein (BRPF1) zu entwickeln. Trotz ihrer *in vitro*-Aktivität zeigten die synthetisierten HDAC8/BRPF1- und HDAC6/BRPF1-Dual-Inhibitoren keine zellulären Wirkungen auf Zellen der akuten myeloischen Leukämie (AML).

Benzhydroxamate wurden weiterhin gegen schistosomales HDAC8 (smHDAC8) untersucht und mehrere nanomolare Inhibitoren identifiziert. Untersuchungen zu Struktur-Aktivitäts-Beziehungen (SAR) der synthetisierten Derivate zeigten, dass einige von ihnen auch eine bemerkenswerte Aktivität gegen Parasiten aufweisen. Basierend auf diesen smHDAC8-Inhibitoren wurde ein Modell der quantitativen Struktur-Aktivitäts-Beziehung erstellt und verfeinert, um die biologische Aktivität zukünftiger Verbindungen vorherzusagen. In weiterführenden Untersuchungen wurden die entwickelten smHDAC8-Inhibitoren auch gegen andere parasitäre Infektionen getestet. Einige von ihnen zeigten antileishmanische und cestozide Aktivität, was ihr Potenzial als neuartige antiparasitäre Mittel unterstreicht. Darüber hinaus wurde ein kontinuierlicher Assay basierend auf einem thiotrifluoracetylierten Substrat erfolgreich angewendet, um die katalytische Aktivität von HDAC8 zu analysieren. Der Assay ist mit verschiedenen Mikrotiterplatten kompatibel und könnte die enzymatische Testung weiterer HDAC-Inhibitoren erleichtern.

Abschließend wurde der Bindungsmodus von Balsalazid, einem zuvor beschriebenen Sirtuin 5 (Sirt5)-Hit-Inhibitor, untersucht. Eine SAR-Analyse identifizierte die für die Aktivität wesentlichen Strukturteile. Somit wurde auch festgestellt, dass Balsalazid für Sirt5 gegenüber anderen Sirtuin-Isoformen selektiv ist.

Die in der aktuellen Arbeit erzielten Ergebnisse können potenzielle Ausgangspunkte für die weitere Optimierung der erhaltenen neuen Chemotypen als HDAC-Inhibitoren für Krebs- und antiparasitäre Therapien darstellen.

Schlagwörter: Epigenetik, HDAC, Antikrebs, Antiparasitika, Benzhydroxamate, Neuroblastom, Polypharmakologie, Dual-wirkende Inhibitoren, BRPF1, Schistosomiasis, smHDAC8, QSAR, Antileishmanial, Cestocidal, Continuous HDAC Assay, Balsalazid, Sirtuin 5.

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Dedication

To my mother and father, thanks for your prayers and for everything you went through so that I become the person I am now.

To my wife and children, thanks for being with me during this journey; the good and hard times. Thank you **Basma** for always supporting me, especially when I was down.

To my brother, thanks for always being there for me. I couldn't have accomplished that without you there in Egypt taking care of our parents.

To all my beloved ones who passed away during my journey here, may we see each other again in a better place!

Contents

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List of Abbreviations

Abbreviation	Full
Ac-CoA	Cofactor acetyl coenzyme A
ADP	Adenosine diphosphate
AML	Acute myeloid leukemia
BET	Bromodomain and Extra-Terminal
BETi	BET inhibitor
BRD	Bromodomain
BRPF	Bromodomain and PHD finger-containing protein
CBP/EP300	CREB binding protein/ E1A-associated protein p300
CD1 and CD2	Catalytic domains 1 and 2
CdLS	Cornelia de Lange syndrome
CFDA	China food and drug administration
CPS1	Carbamoyl phosphate synthetase 1
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
endo-siRNAs	Endogenous small interfering RNAs
ERRα	Estrogen Related Receptor α
FDA	U.S. Food and Drug Administration
HATs	Histone acetyl acetyltransferases
HCC	Hepatocellular carcinoma
HDACi	HDAC inhibitor
HDACs	Histone deacetylases
hEaf6	Human Esa1-associated factor 6 ortholog
hHDAC	Human HDAC
HMGCS2	3-Hydroxy-3-methylglutaryl-coA synthase 2
HSP90	Heat shock protein 90
HTS	High-throughput screening
ING5	Inhibitor of growth 5
KATs	Lysine acetyltransferases
KDACs	Lysine deacetylases
lncRNAs	Long non-coding rnas
miRNA	Micro RNAs

MOZ	Monocytic leukemic zinc finger
mRNA	Messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
ncRNAs	Non-coding rnas
PDB	Protein data bank
PHD	Plant Homeodomain
PROTACs	Proteolysis targeting chimeras
PTMs	Post-translational modifications
QASR	Quantitative structure-activity relationship
RMSD	Root-mean-square deviation
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SAHA	Suberanilohydroxamic acid
SAR	Structure–activity relationship
SDH	Succinate dehydrogenase
SIR2	Silent information regulator 2
Sirt	Sirtuin
SMC3	Subunit of the cohesin complex
smHDAC	Schistosomal HDAC
TRIM24	Tripartite motif containing 24
tRNA	Transfer RNA
TSA	Trichostatin A
UV	Ultraviolet

1. Introduction

1.1. Epigenetic mechanisms of gene control

The term epigenetics was first used to study gene interactions that control the phenotypic changes occurring during the development of an organism. This term was later extended to refer to inherited factors that control gene expression without altering the DNA sequence and consequently determine if and when certain genes are expressed or repressed. The different epigenetic mechanisms that regulate gene expression include DNA methylation, non-coding RNAs (ncRNAs), chromatin conformation, and histone modification [1, 2]. DNA methylation in mammalian cells is regulated through DNA methyltransferase enzymes (DNMTs) which add a methyl group at the C5 position of cytosine in CpG dinucleotides resulting in 5-methylcytosine. DNA methylation controls gene transcription by altering the binding affinity of different transcription and recognition factors to gene promoters [3]. Non-coding RNAs are transcribed from non-coding genome, but they are not translated to proteins. In addition to tRNA and rRNA and their distinct role in protein synthesis, several ncRNAs regulate gene expression at different levels. While long non-coding RNAs (lncRNAs) can alter the chromatin structure and the accessibility of transcriptional machinery to genes, endogenous small interfering RNAs (endo-siRNAs) and micro RNAs (miRNA) work at the post-transcriptional level through interference with mRNA function leading to gene silencing [1, 4]. Human genetic material is stored in the chromosomes, which consist of compacted chromatin fibers. The basic structural unit of chromatin is the nucleosome, which consists of DNA wrapped around histone proteins. Loose chromatin structure allows active transcription, while further compaction into 30 nm fibers prevents the accessibility of RNA polymerase and transcription factors to the DNA and therefore leads to gene silencing [5]. As histone modifications are within the main focus of this work, it will be discussed in more detail.

1.2. Histone post-translation modifications

Post-translational modifications (PTMs) of histones—along with DNA methylation—are the most extensively studied pathways of epigenetic control of gene expression [1]. Histones are essential components of the compact eukaryotic genetic material as around 147 base pairs of DNA are wrapped around eight units of histones (H3–H4 tetramer and two H2A–H2B dimers) to form the nucleosome [6, 7]. Histones are globular proteins rich in basic residues like lysine and arginine and are positively charged under physiological conditions. Therefore, they have strong contact with the negatively charged DNA and other nucleosomes leading to the compact chromatin structure [6, 7]. Histones are subject to many PTMs that include—but are not limited to—acetylation, methylation, phosphorylation, and ubiquitination (**Fig. 1**). These modifications constitute together what is called the histone code, which regulates several cellular functions under physiological conditions. However, deregulations in the histone code were related to

different pathological conditions [6, 7]. Since histone acetylation is within the main focus of this work, it will be discussed in more detail.

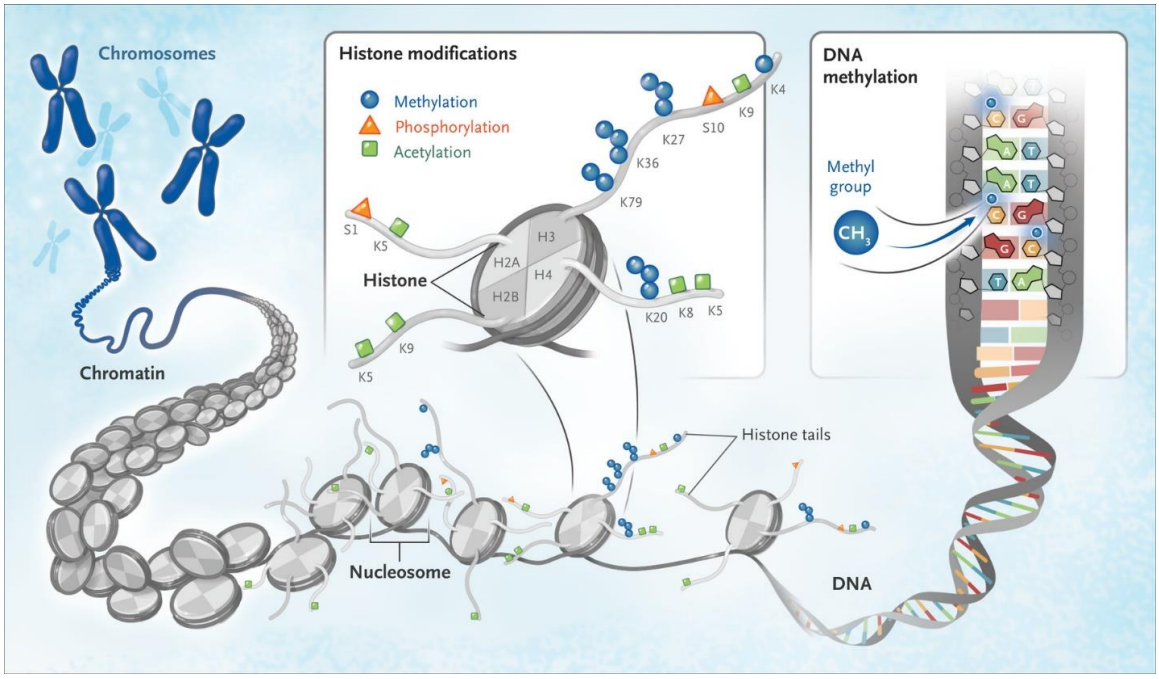


Figure 1: Chromatin structure, DNA methylation and histone post translational modifications. (Reproduced with permission from [8], Copyright Massachusetts Medical Society)

Acetylation of the lysine residues of the N-terminal tail of histones is a dynamic process that neutralizes the positive charge on the lysine, decreases the interaction between DNA and histones, and opens up the chromatin. This consequently increases the accessibility of the DNA by the transcription machinery leading to active transcription of the genes [6, 9]. In addition, several transcription activators or repressors can recognize acetylated residues, which then work as an anchor to recruit transcriptional complexes and that could be associated either with increased gene expression or gene silencing [6, 9-11]. Different regulators work in harmony to control the state of histone acetylation (**Fig. 2**). Lysine acetyltransferases (KATs) are the writers that add the acetyl group to lysine residues, while lysine deacetylases (KDACs) are the erasers that remove them. Bromodomains are the reader proteins that can recognize the acetylated residues [7, 9]. Failure to maintain the equilibrium between these key players—and consequently dysfunctional acetylation levels—is linked to dysregulated gene expression and several pathologies such as inflammatory and metabolic disorders, cardiovascular diseases, and cancer [7, 11-15].

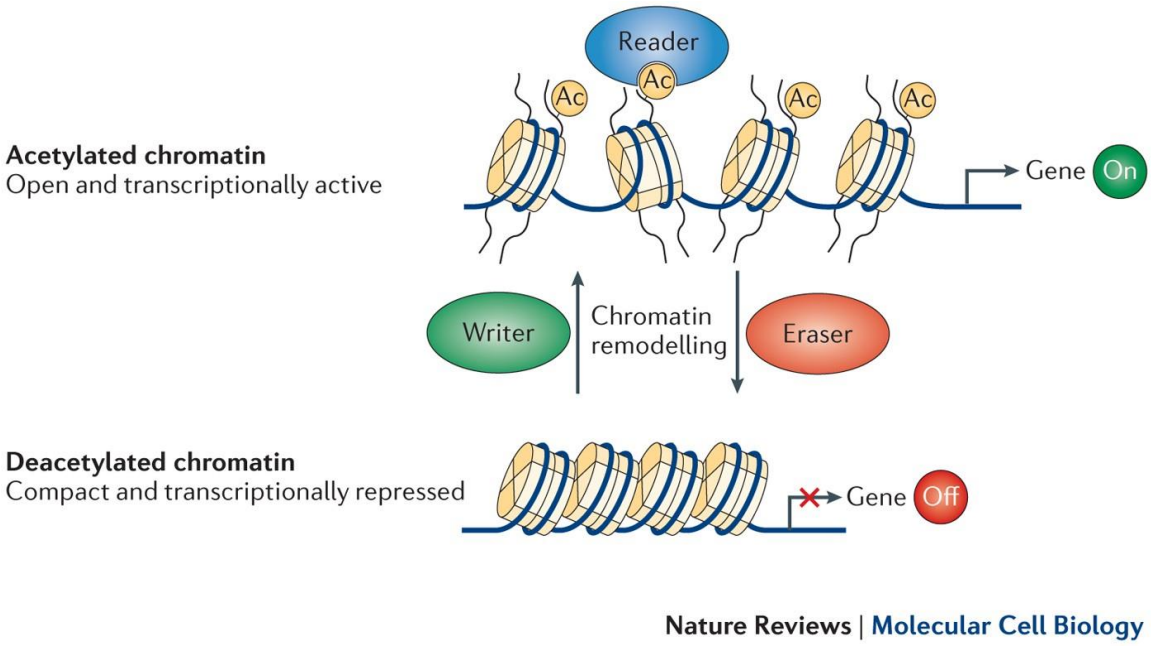


Figure 2: Main regulators of histone acetylation. (Reproduced with permission from [16])

It is worth mentioning that the abbreviations KATs and KDACs are more scientifically correct since these enzymes can acetylate and deacetylate lysine residues in histones and other non-histone proteins, as will be discussed later. However, as the terms histone acetyl acetyltransferases (HATs) and histone deacetylases (HDACs) are more common, they will be used to refer to these enzymes from now on. In addition to the acetyl group, these enzymes can also catalyze the addition and removal of other acyl groups on lysine residues creating distinct post-translational modifications [17].

1.3. Modulators of histone acetylation

1.3.1. Histone acetyltransferases

The "writers" histone acetyltransferases are a group of enzymes capable of transferring an acetyl group from cofactor acetyl coenzyme A (Ac-CoA) to lysine. They all have an acetyltransferase domain but are further classified into families and subtypes depending on sequence similarity, presence of other domains, and their cellular locations [18]. They can acetylate histone and other non-histone proteins such as p53 and assemble with other proteins and transcription factors into large multi-subunit protein complexes modulating their substrate specificity. Dysregulated HAT activity has been linked to inflammatory diseases, neurological disorders, and malignancies [11, 18].

1.3.2. Histone deacetylases

On the contrary, histone deacetylases are responsible for the removal of acyl (mostly acetyl) groups from lysine residues. Their substrates include histones and many non-histone proteins, such as p53, cytoskeleton proteins, RNA processing enzymes, and proteins involved in cell signaling and apoptosis [17]. Given the wide variety of their substrates, HDACs are involved in the regulation of multiple cellular functions, and like HATs, some HDACs work through the formation of large multiprotein complexes [17]. The human genome encodes 18 HDACs differing in size, cellular distribution, substrate, acyl group removed, and mechanism of catalytic activity. The two major categories of HDACs are the classical isoforms and sirtuins [17]. Classical histone deacetylases—for which the abbreviation HDACs will refer from now on—are 11 enzymes that have zinc ion in the active site responsible for their catalytic activity (**Fig. 3A**). These metalloenzymes differ in size, cellular localization, and substrates; however, they all have a conserved deacetylase domain. They are further classified—based on their similarity to yeast analogs—to classes I (HDACs 1-3, 8), IIa (HDACs 4, 5, 7, 9), IIb (HDACs 6, 10), and IV (HDAC11) [19-21]. Non-classical HDACs (class III) got the sirtuin nomenclature due to their homology to the yeast silent information regulator 2 (SIR2). These seven isoforms depend on nicotinamide adenine dinucleotide (NAD^+) for their deacetylase activity (**Fig. 3B**), as the nicotinamide moiety is cleaved and the acyl group is transferred from the lysine to ADP-ribose. Sirtuins share a conserved catalytic core formed of a Rossmann fold domain and a zinc-binding domain, with the active site lying at the interface between them [22, 23]. HDACs and sirtuins are involved in the regulation of different physiological functions, and their uncontrolled activity is linked to many pathological disorders. As a result, their structure, substrates, biological roles, and relation to disease were extensively studied, and many informative reviews on HDACs (such as [16, 17, 24-28]) and sirtuins (such as [22, 23, 29-31]) are available. Of particular interest for this work are class I **HDAC8**, class IIb **HDAC6** and **Sirt5**; therefore, they will be discussed in more detail.

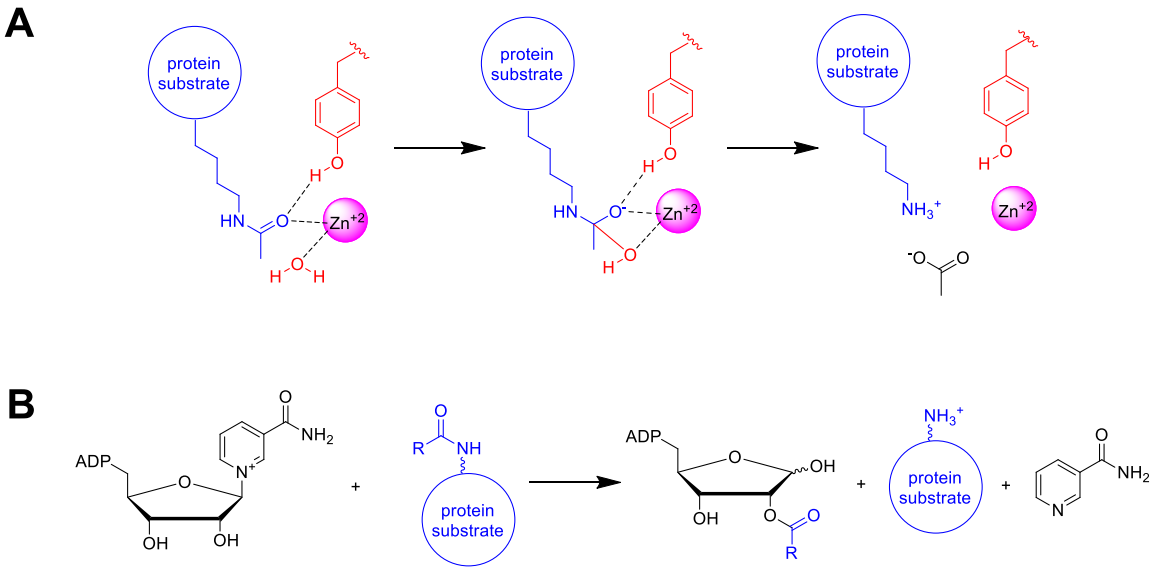


Figure 3: A. Mechanism of zinc ion mediated deacetylation by HDACs. B. Mechanism of NAD+ mediated deacylation by sirtuins.

1.3.2.1. HDAC6

HDAC6 is the largest member of classical HDACs as it has 1215 amino acids. This isoform also has a unique structure as it contains two distinct catalytic domains; CD1 and CD2. CD2 is responsible for histone and tubulin deacetylase activity and has specificity for different substrates, whereas CD1 is more specific for peptide substrates having a C-terminal acetyllysine residue [32]. HDAC6 interacts with ubiquitin through its zinc-finger ubiquitin-binding domain at its C-terminal, mediating interaction with the ubiquitin-proteasome and aggresome pathways to clear misfolded proteins [33]. HDAC6 is mainly found in the cytoplasm interacting with cytosolic proteins and substrates but can also shuttle between the cytoplasm and the nucleus in response to cellular signaling. Its main substrate is α-tubulin; however, it deacetylates other non-histone substrates such as cortactin, β-catenin, and Hsp90 and interacts with several proteins regulating its deacetylase activity [33]. As a result, HDAC6 plays specific physiological roles in different cellular pathways and is linked to a variety of human diseases such as cancer, neurodegenerative diseases, immunological disorders, and human rare diseases [32-34].

1.3.2.2. HDAC8

HDAC8 is perhaps one of the most studied HDAC isoforms. Although it belongs to class I classical HDACs, this 377 amino acids long isoform has unique structural and functional features that distinguish it from HDACs 1-3. Firstly it lacks the C-terminal protein-binding domain of other HDACs, and it is X-linked in humans [35]. Unlike HDACs1-3, which are exclusively located in the nucleus, HDAC8 could also be found in the cytoplasm, where it deacetylates many non-histone proteins such as cortactin, estrogen related receptor alpha (ERRα), SMC3 (subunit of the cohesin complex), and p53

at specific lysine residues. However, it is still a discussion if histones are bona fide HDAC8 substrates in vivo [35]. HDAC8 controls diverse processes such as sister chromatid separation, energy homeostasis, microtubule integrity, and muscle contraction. In addition, mutations in HDAC8 were linked to Cornelia de Lange syndrome (CdLS). Dysregulated HDAC8 is correlated with childhood neuroblastoma and T-cell lymphoma [35, 36].

1.3.2.3. Sirtuin 5

Sirt5 is one of the mitochondrial sirtuins isoforms but could also be found in the cytoplasm. Although it shares the main structural features of the catalytic active site with other sirtuins, it has unique arginine and tyrosine residues in the deep end of the substrate-binding pocket; therefore, it can specifically recognize negatively charged acyl-lysine groups (**Fig. 4**) [37]. Sirt5 was found to have strong deacylase activity in vitro and in vivo against malonyl, succinyl, and glutaryl modifications of lysine residues but much weaker deacetylase activity compared with other sirtuins [37]. Sirt5 regulates several metabolic enzymes such as carbamoyl phosphate synthetase 1 (CPS1), succinate dehydrogenase (SDH), and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), and consequently plays a role in the regulation of several cellular processes like ammonia detoxification, ketone body formation, reactive oxygen species (ROS) management and fatty acid oxidation. Dysregulated Sirt5 was linked to metabolic disorders, cancer, Alzheimer's disease, and Parkinson's disease [37-39].

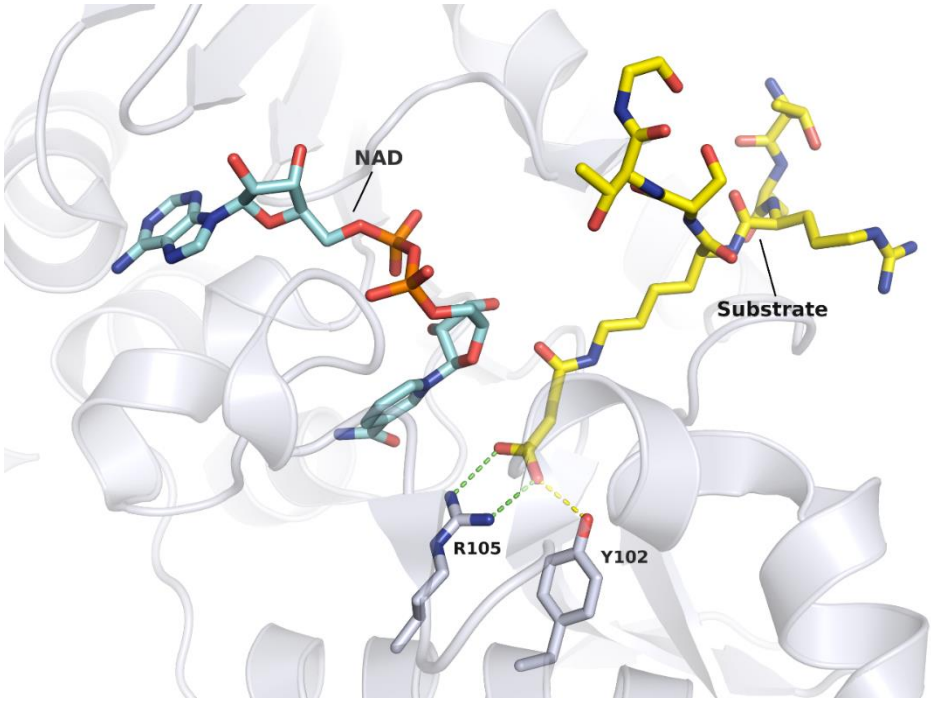


Figure 4: Crystal structure (PDB code: 3RIY) of Sirt5 with a succinyl-lysine peptide (yellow sticks) and NAD+ (cyan sticks) showing the interaction of Sirt5 unique Arg105 and Tyr102 residues with the negatively charged carboxylate of the succinyl group.

1.3.3. Bromodomains

Besides writers and erasers, the reader bromodomains also contribute to acetylation equilibrium through recognizing ε-*N*-acetylated lysine residues, and—together with other epigenetic readers—they are responsible for the recruitment of transcription factors to chromatin [40]. Bromodomains are proteins that have around 110 amino acids and usually occur as an integral part of larger protein complexes. The human proteome contains 61 bromodomains which are present within 46 different proteins and are classified into eight distinct families, with the Bromodomain and Extra-Terminal (BET, family II) and CREB binding protein/ E1A-associated protein p300 (CBP/EP300, family III) are the most extensively studied and characterized bromodomains [40, 41]. The majority of bromodomains have conserved asparagine and tyrosine residues responsible for the recognition of the acetyllysine; however, due to other structural differences, they vary in their substrate specificity. While some can recognize many acetylated substrates, others are specific to a certain acetylated lysine residue in a specific protein [41]. As for HATs and HDACs, bromodomains can read acetyl modifications also in non-histone proteins such as CREB binding protein (CREBBP) and recognize the acetylated lysine on p53 in response to cellular stress. Bromodomains are involved in the regulation of different cellular processes and are linked to different human pathologies such as autoimmune diseases and cancer [40-43]. The bromodomain and PHD finger-containing protein (BRPF) family is within the focus of this work and will be discussed in more detail.

1.3.3.1 Bromodomain and PHD finger-containing protein (BRPF)

The human BRPF family has a high degree of sequence homology within their bromodomains and shares a conserved domain structure of two N-terminal PHD domains linked with a zinc finger (recognize unmodified histone H3), a bromodomain (recognize acetylated lysine in H2A, H3, and H4 histones) and PWWP domain (recognize trimethylated 36th lysine residue of the histone H3) [44]. Three members of this family have been identified, namely BRPF1, 2, and 3, and they act as a scaffold for the recruitment and assembly of the histone acetyltransferases of the MYST family. Once the MYST HATs are recruited to histone through the bromodomain, they start to acetylate more residues creating a positive feedback loop and resulting in recruitment and stabilization of more HATs on the chromatin (**Fig. 5**) [44, 45]. This, in turn, locally increases the acetylation resulting in the up-regulation of gene expression. One clear example is BRPF1, which links the monocytic leukemic zinc finger (MOZ) catalytic HAT subunit to the inhibitor of growth 5 (ING5) and the human Esa1-associated factor 6 ortholog (hEaf6) subunits, forming quaternary complex, thereby promoting its HAT activity [45]. The activity of BRPF-containing HAT complexes was related to the regulation of hematopoiesis, and dysregulation was linked to acute myeloid leukemia (AML) [44-47].

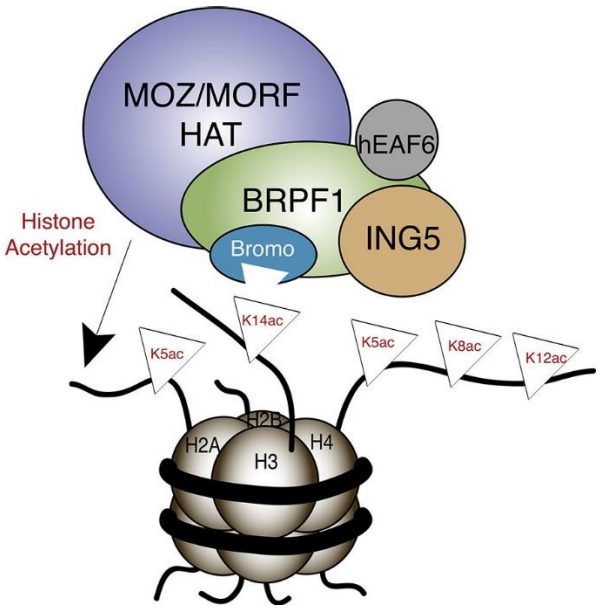


Figure 5: BRPF1 recruits the MOZ HAT complex to acetylated histone allowing further acetylation. (Reproduced with permission from [45])

1.4. HDACs and their inhibition in human pathologies

As previously mentioned, HDACs are major epigenetic players, and their dysregulation could increase the acetylation state and alter the expression of different genes leading to different pathological conditions. Their direct substrates also include many cellular non-histone proteins, meaning that they control many metabolic and physiological processes whose disturbance could cause various pathologies. Indeed, aberrant HDACs activity was linked to neurodegenerative [48, 49] and metabolic diseases [50], cardiovascular [51, 52] and inflammatory [53, 54] disorders, immune response to viral infection [55, 56], rare diseases [33] and most notably cancer [57, 58]. In addition, major human parasites also depend on HDACs and other epigenetic modulators for their survival and growth [59, 60]. Within the main focus of this work are HDAC inhibitors (HDACi) as anticancer and antiparasitic agents; therefore, they will be discussed in more detail.

1.4.1. HDAC inhibitors as anticancer agents

Although examined for many other purposes, HDAC inhibitors were most extensively investigated as anticancer agents. HDAC inhibitors were found to cause cancer cell death through different mechanisms such as interference in cell cycle and mitosis, altering chromatin structure, increasing the expression of tumor suppressor genes, reactive oxygen species (ROS) production, cell cycle arrest, angiogenesis inhibition, and apoptosis induction [17]. These effects were mainly observed for hematological malignancies where HDACi found their greatest therapeutic success as antiproliferative agents leading to five regulatory approved HDAC inhibitors for cancer chemotherapy. Moreover, many others are in preclinical and clinical development—either as single agents or in combination therapy—for oncology [17, 61].

HDAC inhibitors generally have three main structural components; zinc-binding group, linker, and capping group **(Fig. 6A)** and are traditionally classified according to their zinc-binding group to hydroxamic acid derivatives, benzamides (better described as *ortho*-aminoanilides), cyclic peptides, and carboxylic acid derivatives [61]. Also, some "unusual" HDACi containing other zinc binders such as thiols [62, 63], amides [64], acid hydrazides [65, 66], and ketones [67] have also been reported. FDA-approved drugs SAHA (Suberanilohydroxamic acid), belinostat, pracinostat, and panobinostat are all hydroxamates, while the depsipeptide romidepsin is reduced to the active thiol, and the five of them are approved for blood malignancies (**Fig. 6B**) [61]. Additionally, the *ortho*-aminoanilide tucidinostat **(Fig. 6B)** was approved by the china food and drug administration (CFDA) for treating peripheral T-cell lymphoma [17]. However, these inhibitors showed some serious dose-limiting adverse effects such as bone marrow toxicity, thrombocytopenia, and cardiac abnormalities, which were attributed to systemic HDAC inhibition of multiple isoforms [27, 68].

It was then suggested that isoform-selective HDAC inhibitors could have a better safety profile than pan HDAC inhibitors through inhibition of specific isoforms associated with certain malignancies [27]. Moreover, due to different expression levels of HDAC subtypes in various tumors, developing inhibitors to target certain HDAC isoforms is of particular interest as these "probes" could identify the specific functions of these isoforms and the potential therapeutic benefits of their inhibition. Advances in structural biology and the increasing availability of crystal structures of different HDAC isoforms identified specific structural features that could be—and have already been—used to achieve isoform selectivity, at least in vitro [27]. Accordingly, many studies reported modifications in the cap group size and nature, linker length and type, and zinc-binding group to address the specific structural features of many HDAC isoforms [17, 27, 61, 68]. It is also worth mentioning that some of the developed "isoform-selective" inhibitors did not show the expected cellular antiproliferative effect, raising a debate whether the pan HDAC inhibitors—with their previously mentioned side effects—are still therapeutically more valuable [69, 70]. The quest for selective HDAC6 and 8 inhibitors—in particular—is highly active given their established role in different disorders, limited side effects that result from their inhibition or knockdown, and their applicability for selective targeting. Due to their importance for the current work, selective HDAC6 and 8 inhibitors are going to be discussed in more detail.

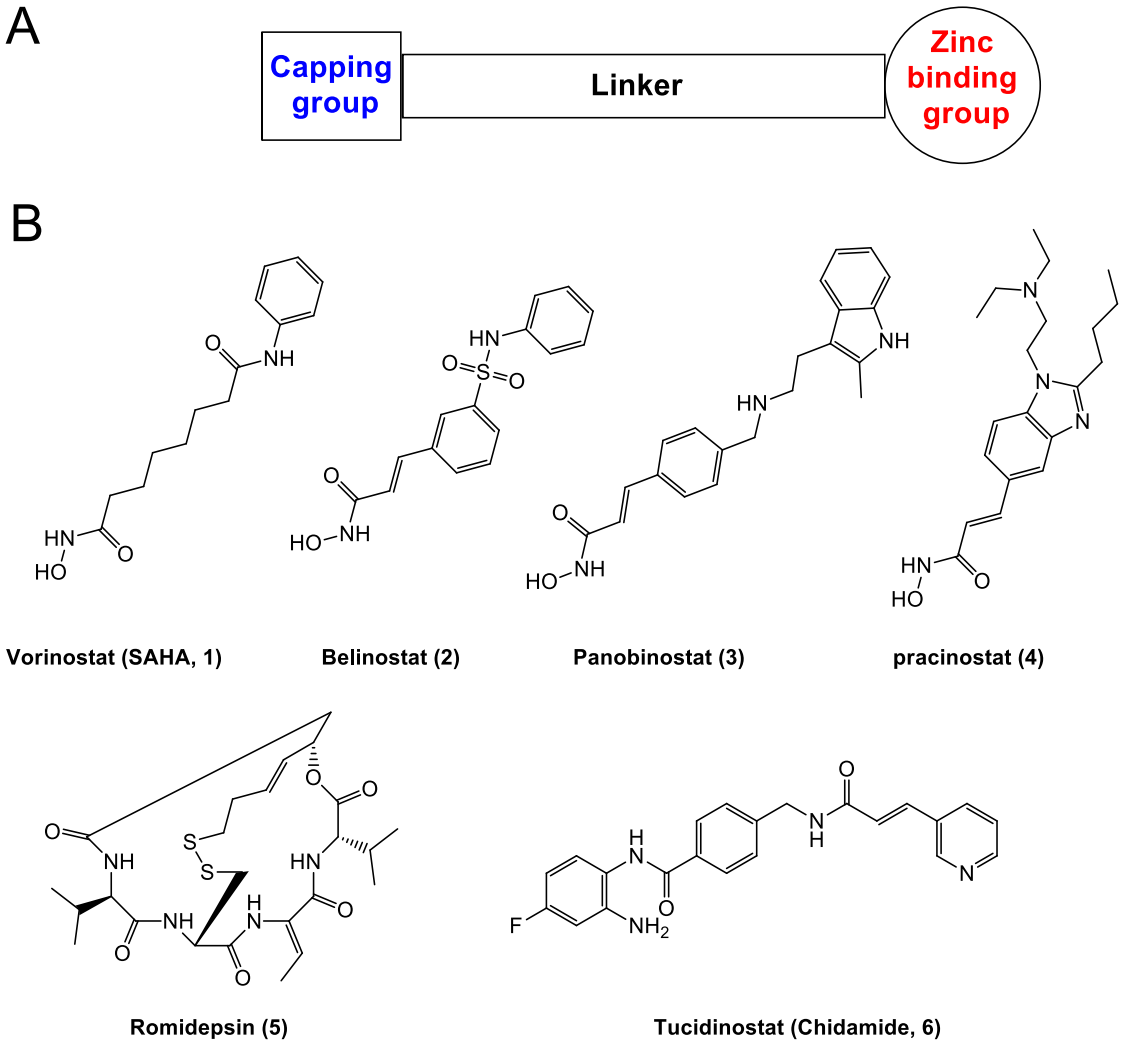


Figure 6: A. General structure features of HDAC inhibitors. B. HDAC inhibitors approved as anticancer agents.

1.4.1.1. Selective HDAC6 inhibitors and their potential as anticancer agents

Major HDAC6 substrates are various non-histone proteins involved in many cellular function ns, and dysregulation of this isoform was linked to many tumors. In addition, knockdown of this isoform did not have a toxic effect on mice [71] but showed antiproliferative effects against some cancer cell lines [72, 73], suggesting that HDAC6 selective inhibition could lead to anticancer agents with fewer side effects. Encouraged by the available HDAC6 crystal structures—mainly for the CD2 deacetylase domain—with different inhibitors, many efforts were devoted to developing inhibitors with preferential HDAC6 activity over other isoforms. Although the hydroxamic acid moiety was mainly used as a zinc-binding group, other non-hydroxamates were also reported [62]. To gain selectivity over other subtypes, HDAC6 selective inhibitors usually contain a relatively bulky—and in some examples a bifurcated cap group—to interact with the

large surface regulation domain of the enzyme. Additionally, HDAC6 has a wide hydrophobic channel that can accommodate either aromatic or longer aliphatic linkers [74]. Indeed, many HDAC6 selective inhibitors were reported showing nanomolar inhibitory activity, excellent selectivity over other isoforms in biochemical assays (**Fig. 7**), and some also exhibited promising in vitro activity against some cancer cell lines [62, 75-86]. Some recently published studies have however shown that selective HDAC6 inhibition in cells is not sufficient for an anticancer effect and that the observed antiproliferative effect of reported HDAC6 inhibitors might be the result of inhibiting other HDACs or other off-targets [70, 87]. Complying with that, it was shown that the selective HDAC6 inhibitor ricolinostat showed minimal clinical activity as a single agent, but this activity was enhanced—with an acceptable toxicity profile—by using it in combination therapies with other anticancer agents such as the proteasome inhibitor bortezomib or the alkylating agent bendamustine [72, 88, 89].

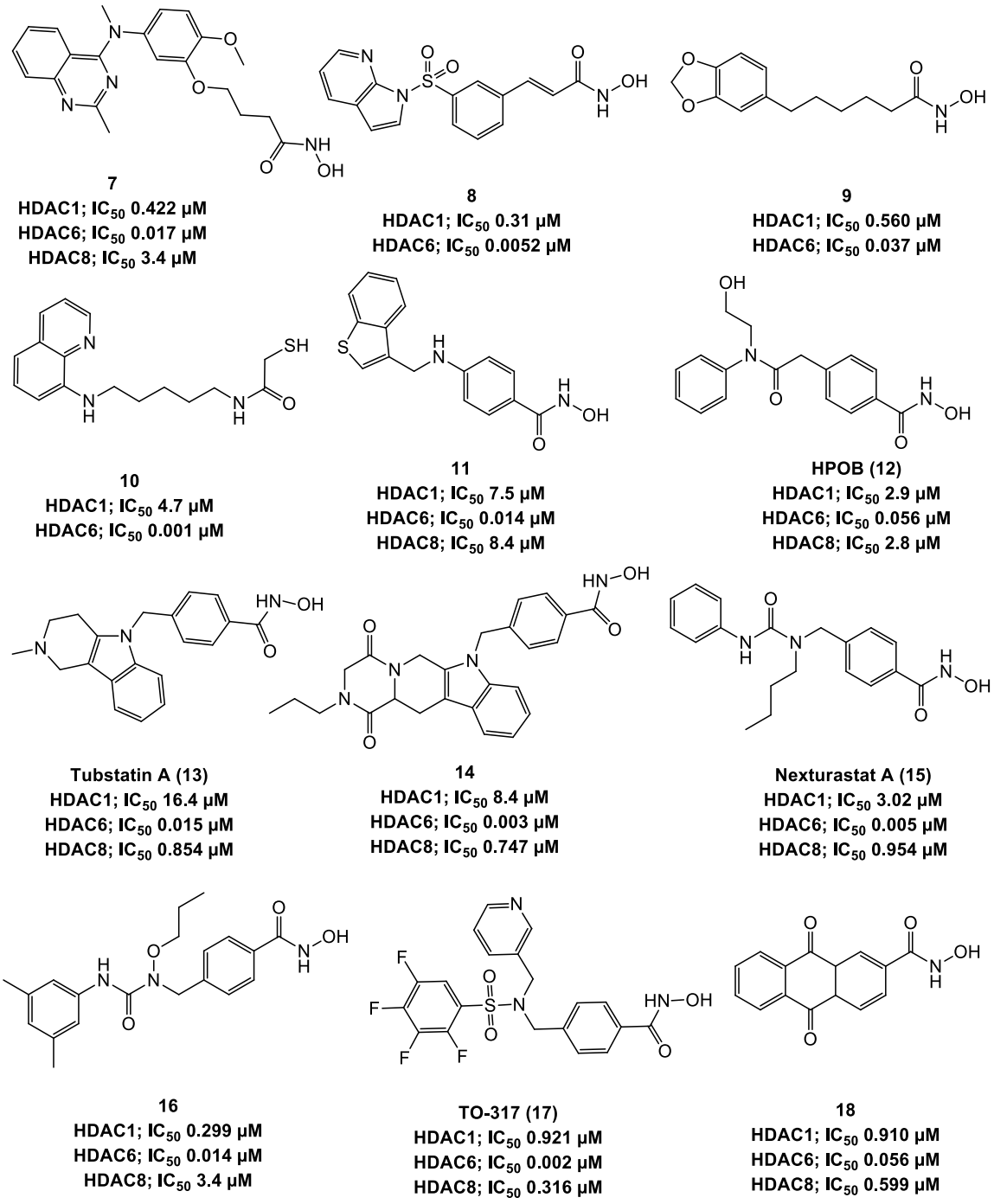


Figure 7: Examples of reported selective HDAC6 inhibitors.

1.4.1.2. Selective HDAC8 inhibitors as potential anticancer agents

HDAC8 was found to be overexpressed and upregulated in different human cancers such as colon cancer and neuroblastoma, whereas its knockdown with RNA interference (RNAi) decreased cellular proliferation and was effective against some cancer cell lines [36]. The majority of the reported HDAC8 inhibitors contain the hydroxamic acid group as the zinc binder, but other non-hydroxamates were also reported (reviewed in [36, 90]). Crystal structures of HDAC8 with different inhibitors revealed some unique structural determinants that could be utilized to design more selective inhibitors. The most important structural feature of HDAC8 is the "HDAC8 specific pocket" formed between L1 and L6 loops and the catalytic tyrosine [91]. Selective HDAC8 inhibitors could acquire an "L"-shaped confirmation so that the cap group can interact with this specific pocket. However, these L-shaped inhibitors cannot bind to other HDAC isoforms, as their L1 and L6 loops hinder the binding **(Fig. 8)** [36, 91]. In addition, the tunnel leading to the HDAC8 active site is shallow and hydrophobic. Therefore, inhibitors with shorter hydrophobic linkers and suitable cap groups—to address the selectivity pocket—are expected to be more active on HDAC8 [36]. Many virtual screening campaigns and computational approaches were initiated to find and design selective HDAC8 inhibitors [92-96], and numerous probes (**Fig. 9 and 10**) emerged that showed preferential in vitro inhibition of HDAC8 compared to other isoforms ([64, 96-105] and were reviewed in [36, 90]). Additionally, the selective HDAC8 inhibitor PCI-34051 was shown to induce apoptosis in cell lines derived from T-cell lymphomas or leukemias [101] and, selective HDAC8 inhibition was demonstrated to have antineuroblastoma activity [106].

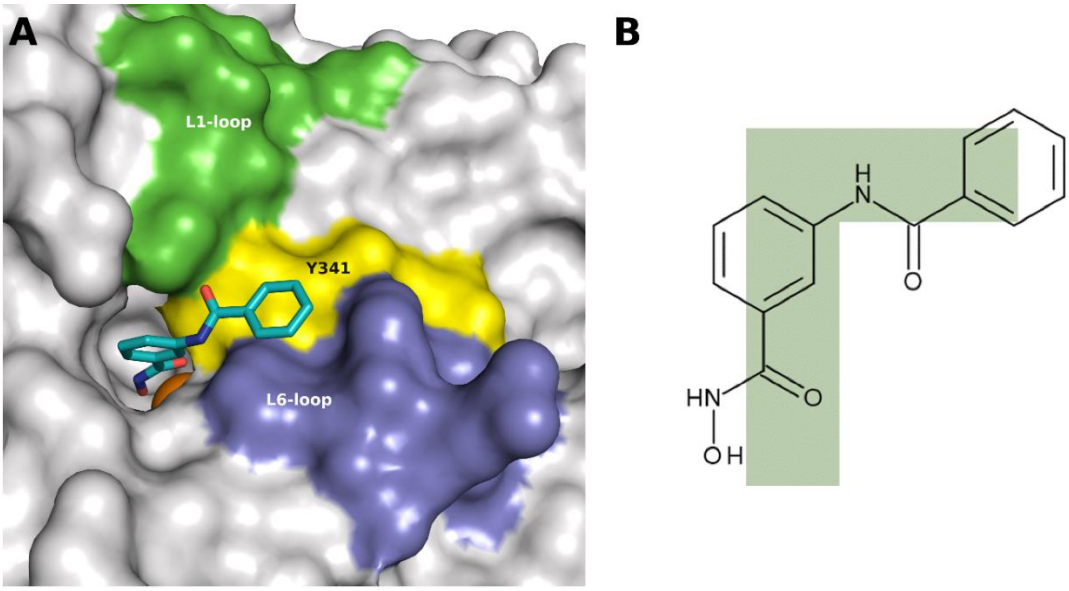


Figure 8: A. Surface depiction of smHDAC8 binding side in complex with a previously reported selective HDAC8 inhibitor [91, 107] (PDB ID 5FUE). The capping group (teal sticks) occupies the HDAC8-specific pocket formed between L1 (green), L6 (blue) and Tyr341 (yellow). B. L-shaped conformation of this selective HDAC8 inhibitor.

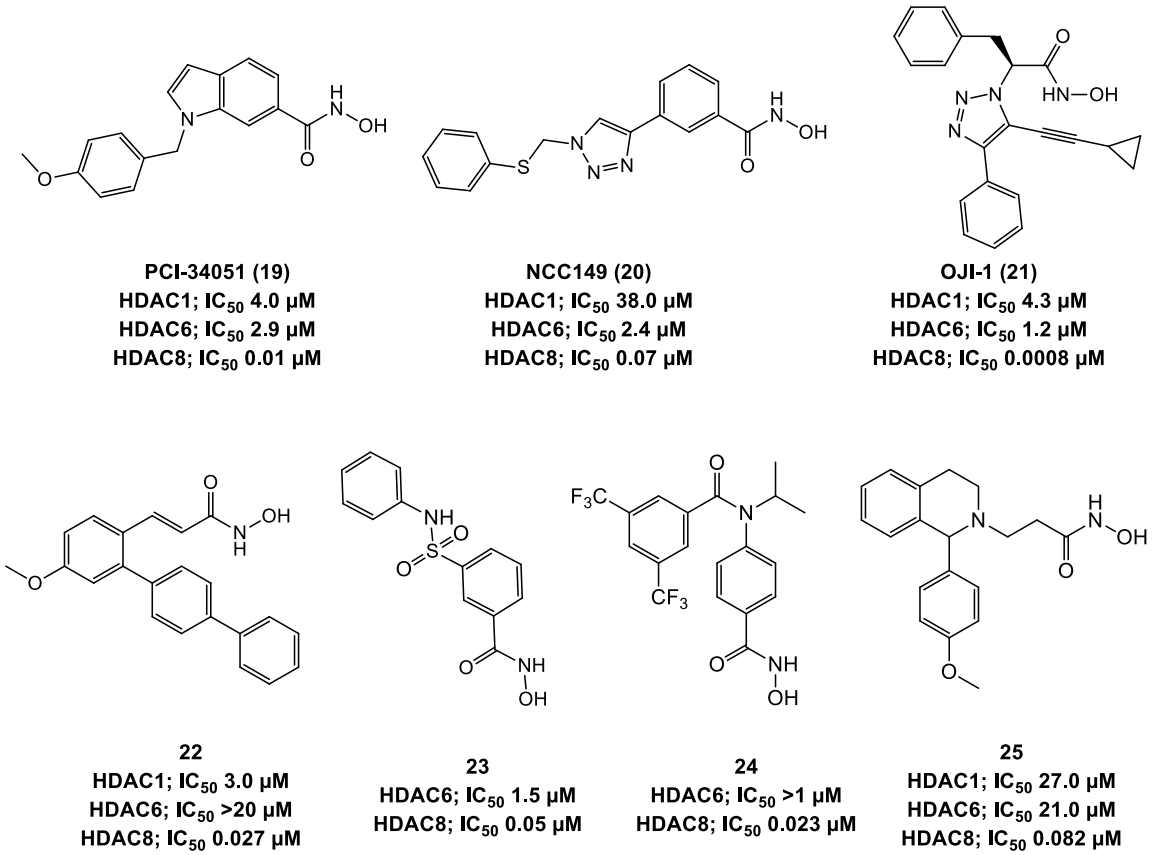


Figure 9: Examples of hydroxamic acid derivatives reported as selective HDAC8 inhibitors.

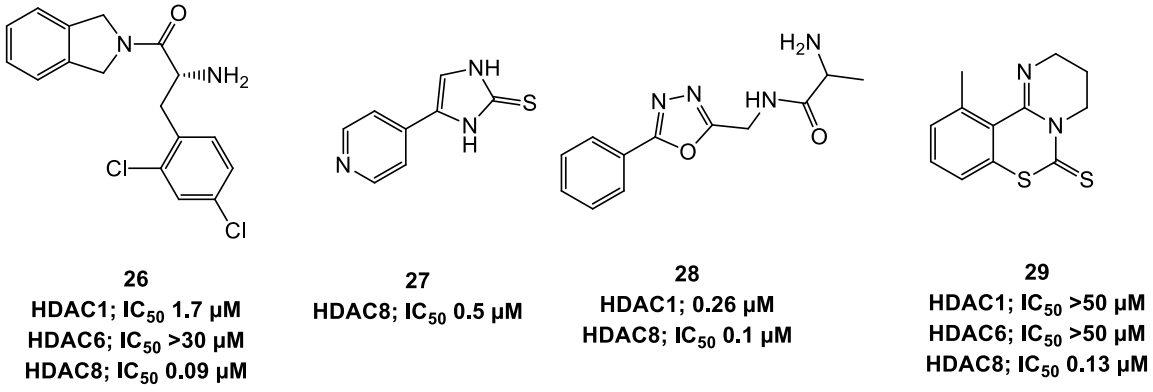


Figure 10: Examples of non-hydroxamates reported as selective HDAC8 inhibitors.

Some benzohydroxamates were also reported to possess promising activity toward both HDAC6 and HDAC8 isoforms, with good selectivity against HDAC1-3 (**Fig. 11**) [74, 108-110]. Furthermore, it was shown that modifications at C2, C4, or C5 of the alkyl linker of the pan inhibitor vorinostat could confer in vitro HDAC6/8 selectivity over other isoforms [111-113].

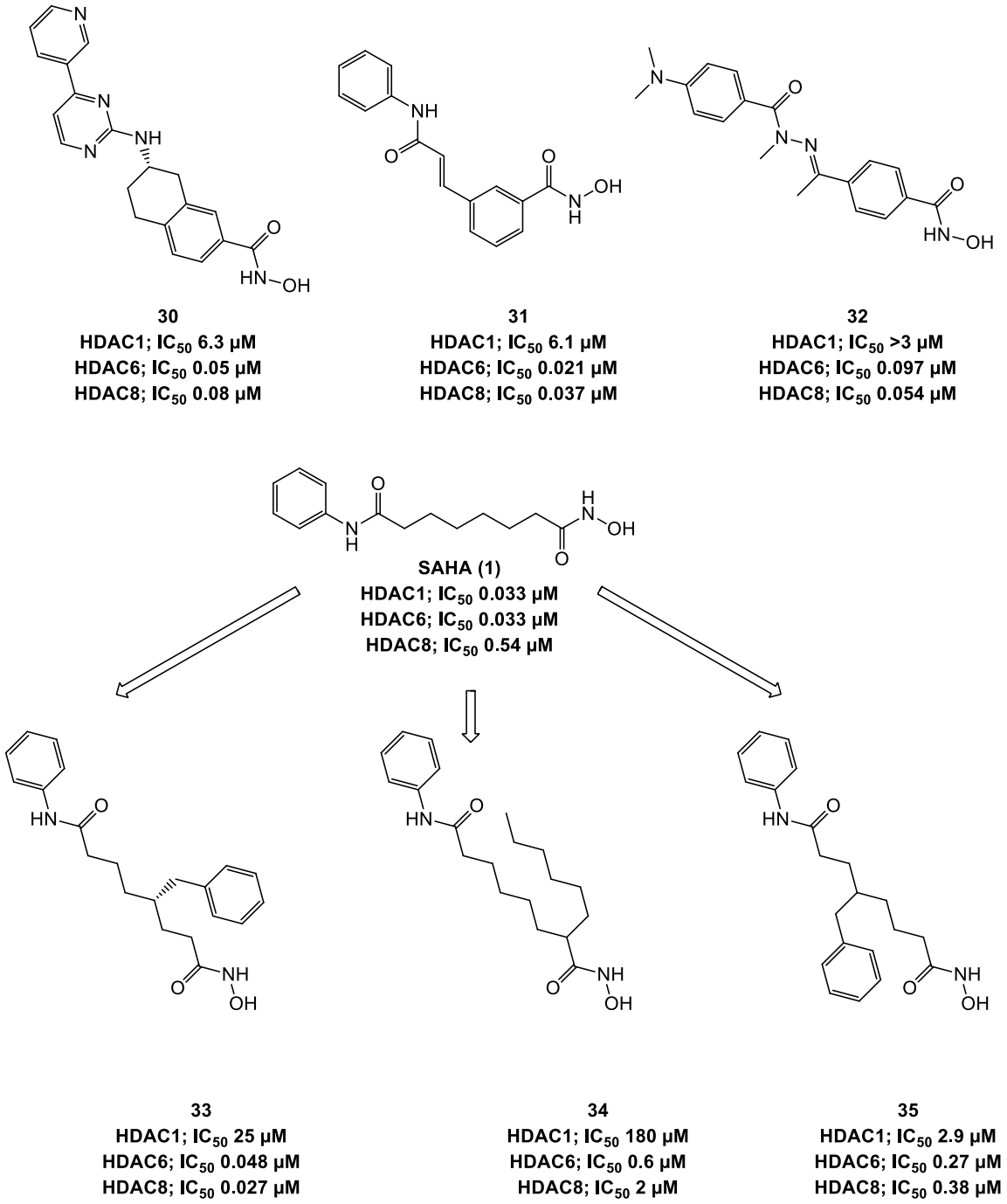


Figure 11: Examples of reported selective HDAC6/8 inhibitors.

1.4.2. HDAC inhibitors as antiparasitic agents

Neglected parasitic diseases affect hundreds of millions of people and cause high morbidity and mortality. Therefore they are considered a serious health concern, especially in poor and developing countries. Lack of vaccines and the limited number of available drugs led to extensive use of the exciting therapies raising the problem of drug resistance and consequently therapy failure. Available drugs also need long regimes, are usually active against only specific live stages of the parasites, and sometimes have severe side effects [59]. As a result, there is an urgent need to identify new antiparasitic agents with novel mechanisms of action. In this regard, the "piggyback" approach or repurposing of already approved drugs—that could have an antiparasitic effect—would be very useful as it could decrease the time and costs to develop novel antiparasitic agents [114]. Anticancer agents are attractive for this approach since cancer cells and parasites have many similarities including high metabolic and reproductive activity and the ability to survive within the host immune system [115]. In addition, parasites usually have a complex lifecycle characterized by several morphologically distinct forms which imply a tight control of gene expression. As a result, epigenetic modulators—especially histone deacetylases—were suggested as potential targets for antiparasitic therapy [59, 60]. Indeed, homologs of human HDACs were identified and characterized in major human parasites, and their inhibition with HDACi resulted—in some cases—in antiparasitic effects. While the current work focuses on HDAC inhibitors for schistosomiases, leishmaniasis, and cestode infections, HDACi for other parasitic infections such as malaria [116-121], trypanosomiasis [114, 122-124], and toxoplasmosis [125, 126] were also reported and reviewed [59, 60, 127].

1.4.2.1. HDAC inhibitors as antischistosomal agents

Schistosomiasis affects about 240 million people worldwide and is prevalent in poor tropical and subtropical areas [128]. The infection is caused by blood flukes from the genus *Schistosoma*, mainly *S. mansoni*, *S. haematobium*, and *S. japonicum*. Efforts to control schistosomiasis rely almost exclusively on preventive/curative chemotherapy through mass drug administration of praziquantel [129], which raises the concern of the development of drug resistance [130].

In schistosomes, several orthologs of the human enzymes have been characterized, belonging to classes I, II, and III [131, 132]. Class I smHDACs are expressed in all life cycles of the parasite, with the smHDAC8 isoform being the most abundant, whereas its human counterpart (hHDAC8) usually shows a lower level of expression in human cells compared to HDAC,1 and 3 [133]. This isoform has then a specific function for the parasite and could, therefore, be the perfect target for novel antischistosomal therapy [133, 134]. Indeed, many smHDAC8 inhibitors (**Fig. 12**) were reported showing in vitro inhibition of enzymatic activity accompanied by phenotypic effects such as impaired viability of different life stages of the parasite, decreased egg production, and morphological alterations in the adult worms [59, 135-138].

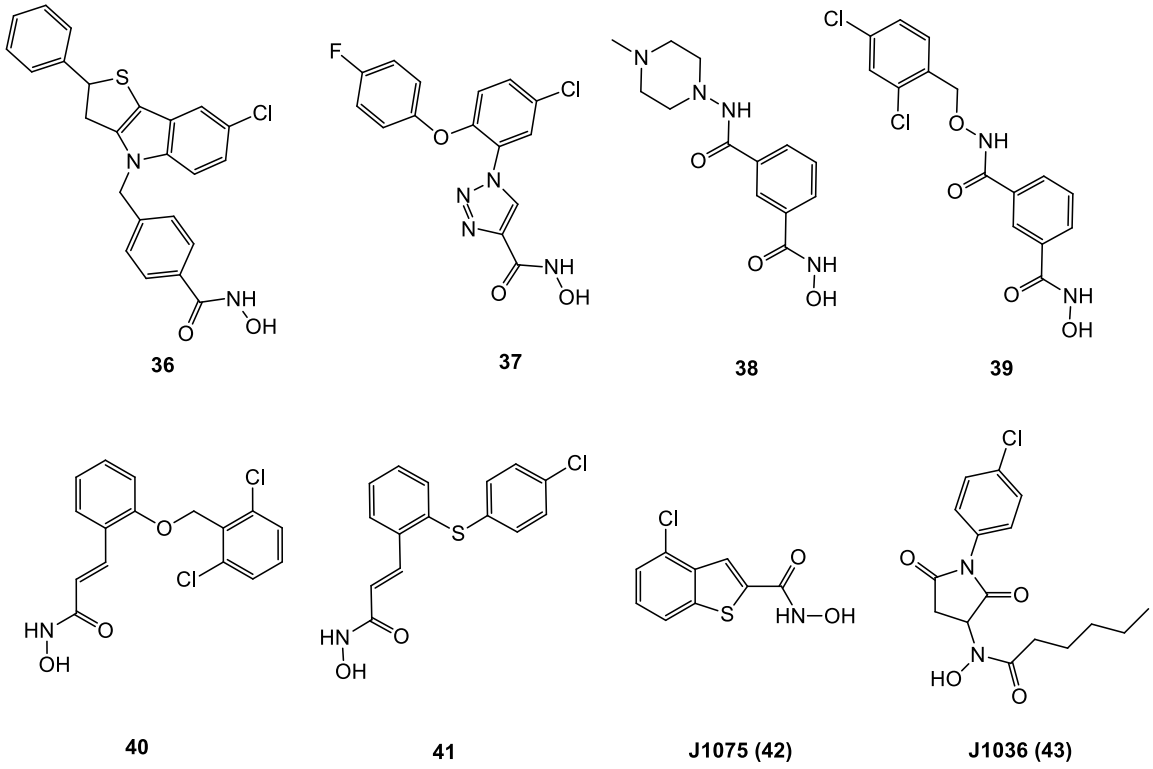
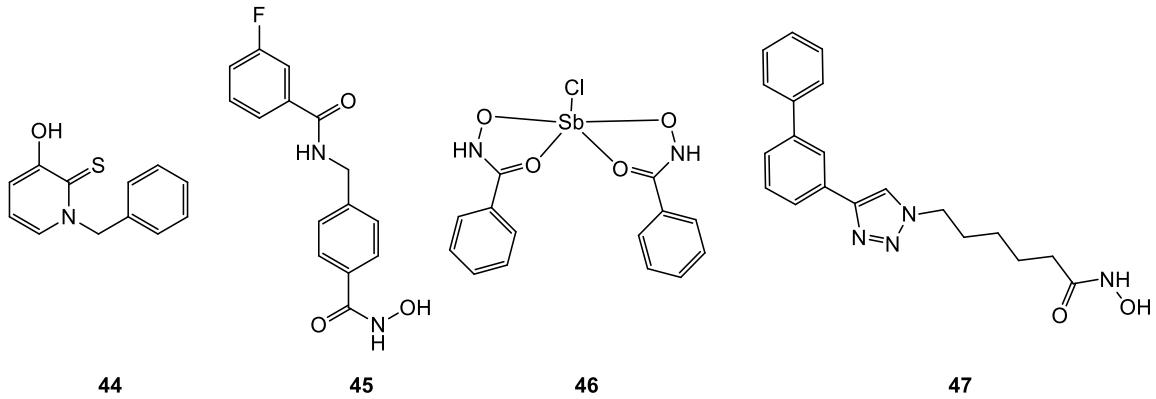


Figure 12: Examples of reported smHDAC8 inhibitors.

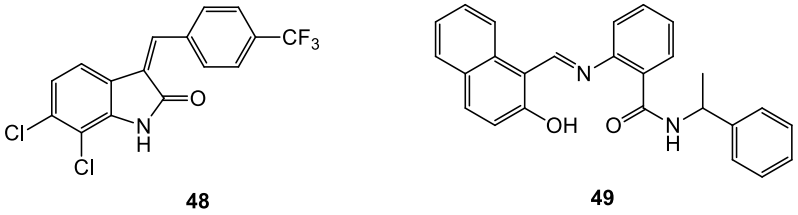
1.4.2.2. HDAC inhibitors as antileishmanial agents

Leishmaniasis affects around one million people per year and is caused by parasites from the genus *Leishmania*; such as *L. donovani, L. braziliensis, and L. amazonensis*. There are three forms of leishmaniasis; visceral, cutaneous, and mucocutaneous. Visceral leishmaniasis or kala-azar is the most severe form, while cutaneous leishmaniasis is considered the most common [139]. Unfortunately, there is a limited number of drugs for the treatment of leishmaniasis such as amphotericin B, miltefosine, paromomycin, and antimony agents. These drugs require a long period of treatment and have a severe toxicity profile [60]. Epigenetic targets, and especially HDACs, could represent a novel therapeutic target in leishmaniasis as three genes in the *Leishmania* genome were found to encode NAD^+-dependent deacetylases, while four genes encode class I and II zinc-dependent deacetylases [127]. Previous studies showed that *Leishmania* are susceptible to HDAC inhibition, and some HDAC and sirtuins inhibitors have antileishmanial action on specific life forms of the parasites (**Fig. 13**) [59, 60, 140-142]. However, it was also shown that the clinically approved HDAC inhibitors vorinostat, belinostat, panobinostat and romidepsin are ineffective against *Leishmania* [143].



44 **45** **46** **47**

reported HDAC inhibitors showing antileishmanial activity



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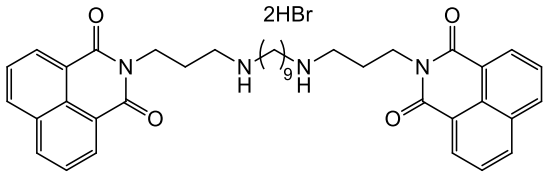

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reported sirtuins inhibitors showing antileishmanial activity

Figure 13: Examples of reported HDAC and sirtuins inhibitors showing antileishmanial activity.

1.4.2.3. HDAC inhibitors against cestode infections

Echinococcosis is a zoonotic disease caused by parasite tapeworms of the genus *Echinococcus* and is estimated to affect around one million people globally. It occurs in two forms; either cystic or alveolar. Likewise, Taeniasis (cysticercosis) is an intestinal infection with cestodes from the genus *Taenia* affecting 2.5-8 million people worldwide. If the larvae are transmitted to the brain, they cause neurocysticercosis, characterized by convulsions and epilepsy [144, 145]. These parasitic infections are classified as neglected tropical diseases and are prevalent in underdeveloped countries where hygienic standards are relatively lower. Treatment options are limited and include praziquantel, niclosamide, and albendazole [144, 145]. However, these antiparasitic agents are not well-tolerated by some patients and require prolonged treatment regimens. Additionally, they are not

highly effective, which necessitates the search for alternative therapies [146]. Encouraged by establishing HDACs as potential targets for trematodes infections (schistosomiasis), various genes encoding class I and II HDACs in some cestodes were identified and characterized, and their transcriptional expression levels throughout several developmental stages of *Echinococcus spp.* were analyzed. Moreover, the pan-HDAC inhibitor trichostatin A (TSA) was found to decrease parasite viability and induce morphological alterations on the parasites. These results suggested that HDACs represent potential drug targets in cestodes [146].

1.5. Bromodomains and their inhibition in human pathologies

Although the physiological and pathological functions of many bromodomains are yet to be fully identified and understood, the role of certain bromodomain families in some human pathologies was revealed. Being the most druggable, BET bromodomains were the most extensively studied acetyl readers, and their roles—not only in cancer [147-149] but also in renal [150], neurological [151], and other disorders [152]—were discussed. Lots of small molecules were identified as BET inhibitory probes with various selectivity profiles against different subfamily members, and some of them are now evaluated in preclinical and clinical phases against various malignancies (reviewed in [153-155]). Additionally, other bromodomains outside the BET family also received some interest, and selective inhibitors were successfully developed for some of them and showed potential therapeutic effects, mainly against tumors (reviewed in [156, 157]). As previously mentioned, the BRPF family is within the focus of this work, and their inhibitors for anticancer therapy will be discussed in more detail.

1.5.1. BRPF bromodomain inhibitors and their potential as anticancer agents

Over the last decade, many groups identified and optimized different chemical scaffolds as BRPF bromodomain inhibitors (**Fig. 14**). Given the highly conserved acetyllysine binding pocket, selectivity against other bromodomains, especially BET, has to be carefully considered. BRPF inhibitors containing 1,3-dimethyl benzimidazolone scaffold were reported showing not only high potency and selectivity for the BRPF family but in some cases also subtype selectivity for BRPF1. Optimization of these inhibitors afforded compound **(51, Fig. 14)** with improved potency, selectivity, and pharmacokinetic profile [158, 159]. Very recently, another derivative **(52, Fig. 14)** was reported to inhibit hepatocellular carcinoma (HCC) development in vitro and in vivo [160]. Moreover, two groups independently utilized this scaffold to design dual targeting inhibitors of the bromodomains of both BRPF1/2 and the transcription factor TRIM24 (**53** and **54, Fig. 14**). The optimized inhibitors were selective against these bromodomains and showed cellular activity. Compound **(54)** also showed modest activity against some cancer cell lines but was ineffective against breast cancer cell line MCF-7 [161-164].

Another group reported the discovery and optimization of pan BRPF inhibitors containing 1,3-dimethylquinolin-2-one scaffold, showing low nanomolar potency against BRPFs and excellent selectivity. The optimized inhibitor **(55, Fig. 14)** had good biopharmaceutical properties and displayed functional activity in cellular assays against cancer cell lines [47, 165]. Moreover, a hit-to-lead campaign identified 1,4-dimethyl-2,3-dioxo-quinoxaline **(56, Fig. 14)** and 2,4-dimethyloxazole derivatives as low micromolar probes for BRPF1 with good selectivity [166, 167].

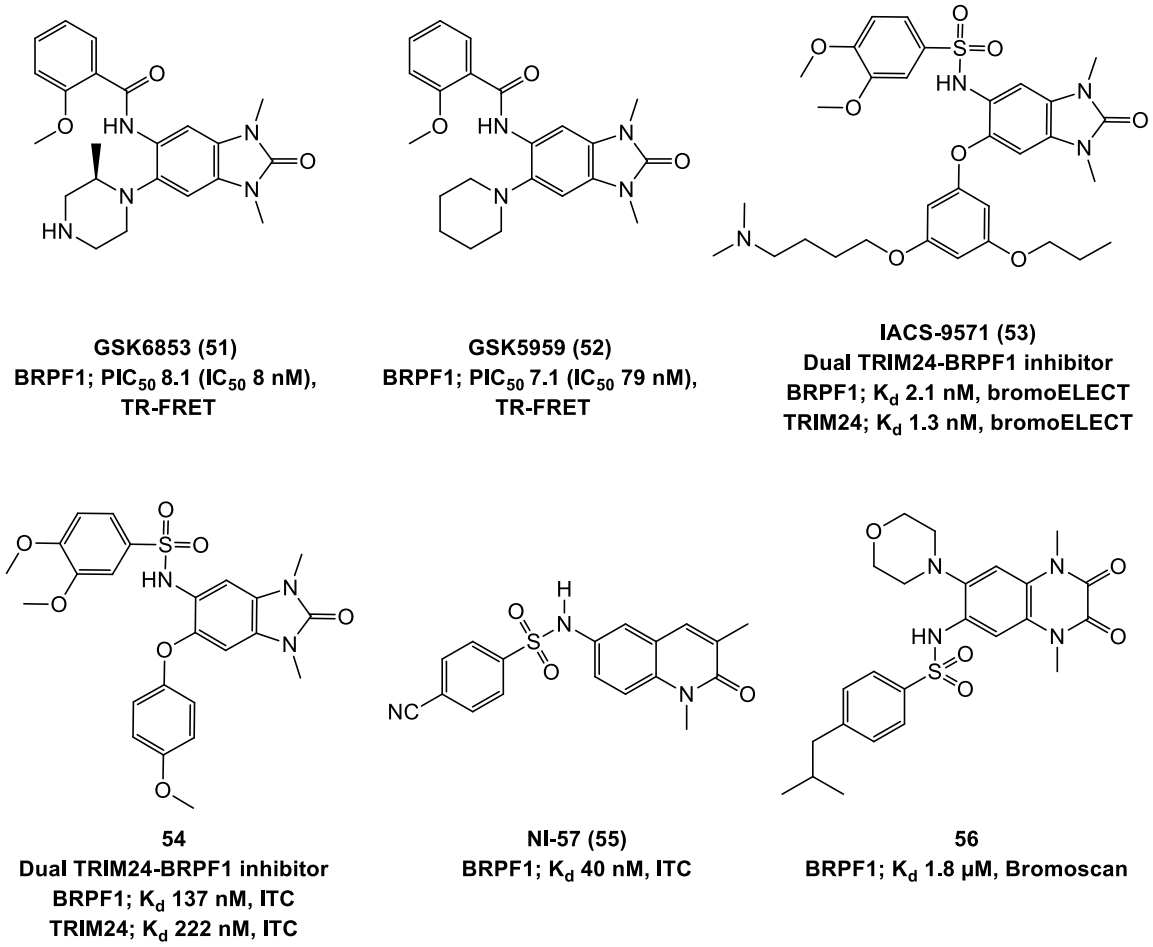

Figure 14: Examples of reported BRPF bromodomain inhibitors.

1.6. Polypharmacology approach in drug design

Multifactorial diseases such as inflammation, cancer, neurodegenerative, and metabolic disorders have a complex nature; therefore, it is challenging to completely cure such diseases through specific modulation of a single target. Indeed, many single agents for these diseases suffered from clinical failure and therapeutic resistance [168]. Other alternatives could be combination therapy by using different drugs to modulate different targets or designing therapeutic multitarget agents capable of simultaneously inhibiting multiple targets; an approach called polypharmacology. Multitarget drugs could have some advantages over drug cocktails, such as fewer side effects and drug-drug interactions, better patient compliance, and lower pharmacokinetic complexity. Accordingly, many reviews discussed the strategies to design multitarget agents as well as their potential in various multifactorial diseases [168, 169]. However, while designing dual-acting probes is a promising approach, achieving clinical effectiveness is still challenging, and many aspects have to be considered. Merging or fusing two scaffolds usually results in a bigger and more complex structure and could be associated with decreased drug-like properties. Another critical issue is balancing the activities on both targets since incorporating a second pharmacophore could interfere with the binding of the original one. Other challenges also include non-specific binding and different expression levels of targeted proteins [170-172].

Since dual-target epigenetic agents—and more specifically dual HDAC/BRD inhibitors—are within the main focus of this work, they will be discussed in more detail.

1.6.1. Dual HDAC/BRD inhibitors

Several genetic, epigenetic, and metabolic pathways could be dysregulated leading to cancer formation. For such a multifactorial disease, combination therapies of different modulators could achieve better clinical results than monotherapy, especially against solid and resistant tumors [168]. Over the past decade, lots of efforts were dedicated to designing dual-acting epigenetic inhibitors, targeting either two epigenetic modulators or epigenetic and metabolic pathways (reviewed in [173-175]). Being already approved for cancer treatment, no wonder that HDACi have received a great interest in this regard. Several dual-acting HDACi were designed to interact with a second target such as kinases, metalloproteinases, and topoisomerases, and some of them have already progressed to clinical trials (reviewed in detail in [176-179]). Similarly, bromodomains also got some interest, and some dual bromodomain/kinase inhibitors were investigated [180-183].

Of particular relevance to the current work are the attempts to design dual HDAC/BRD epigenetic inhibitors. Many groups rationalized the design of dual HDAC/BET inhibitors by changing the cap group of HDACi, mostly SAHA, to a reported BRD4 inhibiting

pharmacophore [184-189]. Indeed, the authors were not only able to achieve dual inhibitory activity for some compounds **(57-60**, **Fig. 15)** but also promising in vitro activity against some cancer cell lines. However, the results failed to show superior or synergistic activity over the original HDAC or BRD inhibitors. Also, the selectivity profile of these dual inhibitors on different HDAC isoforms was not optimal [184-189]. Recently, two groups have reported dual HDAC/BRD4 based on the scaffold of the BETi (+)-JQ1. Both compounds **(61** and **62, Fig. 15)** showed not only a balanced activity on BRD4 and class I HDACs but also a superior anticancer activity in pancreatic cancer cells compared to the parent compounds alone [190, 191]. Very recently, three dual HDAC/BRD4 inhibitors were reported based on the mode of binding of the BET inhibitor ABBV-744. The compounds were designed either as a pan HDAC, selective HDAC1, or selective HDAC6 inhibitor (**63**, **64,** and **65** respectively, **Fig. 15**), and they showed a submicromolar antiproliferative activity against AML cell line MV-4-11 [192].

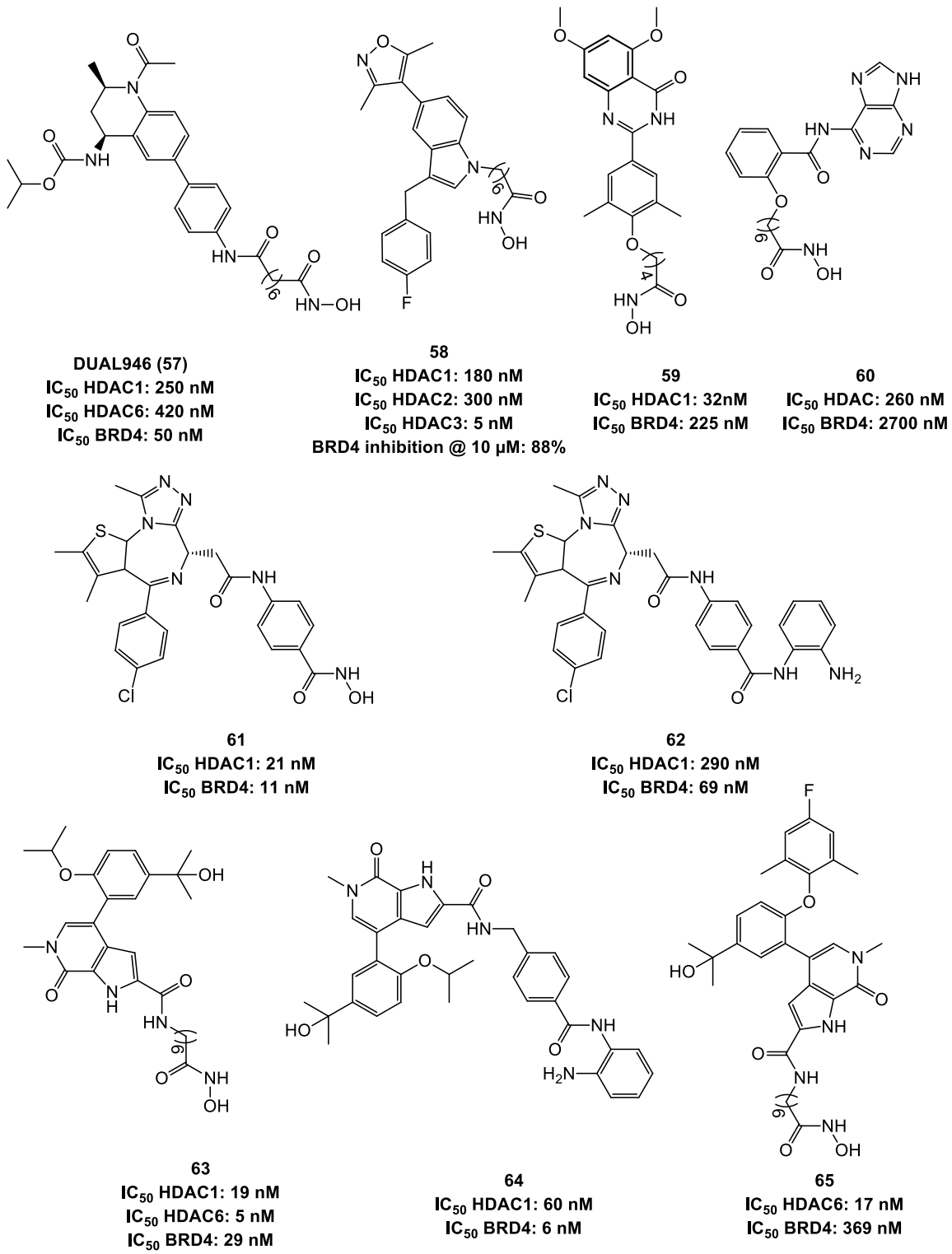

Figure 15: Some previously reported dual HDAC/BRD inhibitors.

2. Objectives of the work

As previously mentioned and discussed in the introductory part, epigenetics emerged as a promising field of modern medicinal chemistry research. On the one hand, some epigenetic anticancer agents—mainly histone deacetylases inhibitors—have already been approved for the treatment of various cancer types, encouraging researchers to deeply investigate epigenetic pathways, fully reveal their mechanisms and develop more therapies. On the other, the side effects and limitations associated with the current epigenetic agents also stimulated further research to overcome them and develop novel therapies with improved safety and effectiveness.

Therapeutic applications of epigenetic modulators are not limited to the field of oncology, as there is increasing evidence of their significance in different human pathologies. One example is human parasitic infections, widely considered as a promising field for the therapeutic potential of epigenetic modulators, especially HDAC inhibitors. Following that, the main objective of the current work is to design and synthesize novel inhibitors of histone deacetylases and evaluate their biological activity as anticancer and antiparasitic agents. To achieve that, the following specific objectives are applied:

Since dysregulated HDAC8 is correlated with childhood neuroblastoma, we firstly set to investigate the potential of HDAC8 inhibitors against this malignancy. Novel hHDAC8 inhibitors will be designed based on the available hHDAC8 structures while taking advantage of the structural differences between the different HDAC isoforms to achieve an acceptable selectivity profile. The synthesized compounds will be tested for their in vitro activity against the different HDAC isoforms to determine their inhibitory potency as well as their selectivity profile. Additionally, phenotypic screening in neuroblastoma cell lines will be carried out to assess the anticancer activity of the new compounds.

Another idea is to apply the polypharmacology concept to design and synthesize novel dual inhibitors of HDACs and BRPF bromodomains. Here, the design of the dual inhibitors is based on previously reported inhibitors of both targets as well as their crystal structures. Modeling studies will guide the optimization process to achieve a balanced activity on both targets. The synthesized dual inhibitors will first be tested in biochemical and biophysical assays for their activity against HDACs and BRPF bromodomains. Phenotypic screening in acute myeloid leukemia cell lines will be additionally performed to test the antileukemic activity of the dual agents.

Another focus will be on the development of HDAC inhibitors for an antiparasitic therapy. Previously reported smHDAC8 inhibitors will be optimized in this project. Different computational approaches such as binding free energy calculations, QSAR analysis, and docking studies will be implemented to explain the activity of the reported inhibitors and to suggest novel derivatives, whereas the mode of binding will be confirmed using cocrystallization studies. The newly synthesized derivatives will be

evaluated for their inhibitory activity against both parasitic and human HDAC isoforms, in addition to their cellular activity against the *Schistosoma* parasite.

Since HDACi have been shown to hold potential for the treatment of major parasitic diseases, other than schistosomiasis, the synthesized HDAC inhibitors will be further tested for their antileishmanial and cestocidal activity.

Since many compounds will be developed in the different projects by applying modern medicinal chemistry methods, it is also necessary to use in vitro screening assays that are able to handle such number of compounds. To achieve this the idea was also to develop a continuous assay to allow for rapid determination of histone deacylase activity.

In the last project of the current work a series of novel derivatives of the Sirt5 hit balsalazide are planned to be synthesized in collaboration with the group of Prof. Bracher from LMU Munich. The obtained results should be used to set up protein-ligand models able to rationalize the biological activities.

3. Results and Discussions

The results of this thesis include the following scientific manuscripts:

3.1. Structure-Based Design and Biological Characterization of Selective Histone Deacetylase 8 (HDAC8) Inhibitors with Anti-Neuroblastoma Activity

Tino Heimburg, Fiona R. Kolbinger, Patrik Zeyen, **Ehab Ghazy**, Daniel Herp, Karin Schmidtkunz, Jelena Melesina, Tajith Baba Shaik, Frank Erdmann, Matthias Schmidt, Christophe Romier, Dina Robaa, Olaf Witt, Ina Oehme, Manfred Jung, and Wolfgang Sippl

J. Med. Chem. **2017,** *60* (24), 10188-10204

https://pubs.acs.org/doi/10.1021/acs.jmedchem.7b01447

Abstract

Histone deacetylases (HDACs) are important modulators of epigenetic gene regulation and additionally control the activity of non-histone protein substrates. While for HDACs 1−3 and 6 many potent selective inhibitors have been obtained, for other subtypes much less is known on selective inhibitors and the consequences of their inhibition. The present report describes the development of substituted benzhydroxamic acids as potent and selective HDAC8 inhibitors. Docking studies using available crystal structures have been used for structure-based optimization of this series of compounds. Within this study, we have investigated the role ofHDAC8 in the proliferation of cancer cells and optimized hits for potency and selectivity, both in vitro and in cell culture. The combination of structure-based design, synthesis, and in vitro screening to cellular testing resulted in potent and selective HDAC8 inhibitors that showed anti-neuroblastoma activity in cellular testing.

3.2. Design, Synthesis, and Biological Evaluation of Dual Targeting Inhibitors of Histone Deacetylase 6/8 and Bromodomain BRPF1

Ehab Ghazy, Patrik Zeyen, Daniel Herp, Martin Hügle, Karin Schmidtkunz, Frank Erdmann, Dina Robaa, Matthias Schmidt, Elizabeth R. Morales, Christophe Romier, Stefan Günther, Manfred Jung and Wolfgang Sippl

Eur J Med Chem **2020,** *200*, 112338

https://www.sciencedirect.com/science/article/pii/S022352342030307X

Abstract

Histone modifying proteins, specifically histone deacetylases (HDACs) and bromodomains, have emerged as novel promising targets for anticancer therapy. In the current work, based on available crystal structures and docking studies, we designed dual inhibitors of both HDAC6/8 and the bromodomain and PHD finger containing protein 1 (BRPF1). Biochemical and biophysical tests showed that compounds **23a,b** and **37** are nanomolar inhibitors of both target proteins. Detailed structure-activity relationships were deduced for the synthesized inhibitors which were supported by extensive docking and molecular dynamics studies. Cellular testing in acute myeloid leukemia (AML) cells showed only a weak effect, most probably because of the poor permeability of the inhibitors. We also aimed to analyse the target engagement and the cellular activity of the novel inhibitors by determining the protein acetylation levels in cells by western blotting (tubulin vs histone acetylation), and by assessing their effects on various cancer cell lines.

3.3. Synthesis, Structure-Activity Relationships, Cocrystallization and Cellular Characterization of Novel Smhdac8 Inhibitors for the Treatment of Schistosomiasis

Ehab Ghazy, Tino Heimburg, Julien Lancelot, Patrik Zeyen, Karin Schmidtkunz, Anne Truhn, Salma Darwish, Conrad V. Simoben, Tajith B. Shaik, Frank Erdmann, Matthias Schmidt, Dina Robaa, Christophe Romier, Manfred Jung, Raymond Pierce and Wolfgang Sippl

Eur J Med Chem **2021,** *225*, 113745

https://www.sciencedirect.com/science/article/pii/S0223523421005948

Abstract

Schistosomiasis is a major neglected parasitic disease that affects more than 265 million people worldwide and for which the control strategy consists of mass treatment with the only available drug, praziquantel. In this study, we chemically optimized our previously reported benzhydroxamate-based inhibitors of Schistosoma mansoni histone deacetylase 8 (smHDAC8). Crystallographic analysis provided insights into the inhibition mode ofsmHDAC8 activity by the highly potent inhibitor 5o. Structurebased optimization of the novel inhibitors was carried out using the available crystal structures as well as docking studies on smHDAC8. The compounds were evaluated in screens for inhibitory activity against schistosome and human HDACs (hHDAC). The in vitro and docking results were used for detailed structure activity relationships. The synthesized compounds were further investigated for their lethality against the schistosome larval stage using a fluorescence-based assay. The most promising inhibitor 5o showed significant dose-dependent killing of the schistosome larvae and markedly impaired egg laying of adult worm pairs maintained in culture.

3.4. Binding Free Energy (BFE) Calculations and Quantitative Structure–Activity Relationship (QSAR) Analysis of Schistosoma Mansoni Histone Deacetylase 8 (Smhdac8) Inhibitors

Conrad V. Simoben, **Ehab Ghazy**, Patrik Zeyen, Salma Darwish, Matthias Schmidt, Christophe Romier, Dina Robaa and Wolfgang Sippl

Molecules **2021,** *26* (9), 2584

https://www.mdpi.com/1420-3049/26/9/2584

Abstract

Histone-modifying proteins have been identified as promising targets to treat several diseases including cancer and parasitic ailments. In silico methods have been incorporated within a variety of drug discovery programs to facilitate the identification and development of novel lead compounds. In this study, we explore the binding modes of a series of benzhydroxamates derivatives developed as histone deacetylase inhibitors of Schistosoma mansoni histone deacetylase (smHDAC) using molecular docking and binding free energy (BFE) calculations. The developed docking protocol was able to correctly reproduce the experimentally established binding modes of resolved smHDAC8–inhibitor complexes. However, as has been reported in former studies, the obtained docking scores weakly correlate with the experimentally determined activity of the studied inhibitors. Thus, the obtained docking poses were refined and rescored using the Amber software. From the computed protein–inhibitor BFE, different quantitative structure–activity relationship (QSAR) models could be developed and validated using several cross-validation techniques. Some of the generated QSAR models with good correlation could explain up to ~73% variance in activity within the studied training set molecules. The best performing models were subsequently tested on an external test set of newly designed and synthesized analogs. In vitro testing showed a good correlation between the predicted and experimentally observed IC50 values. Thus, the generated models can be considered as interesting tools for the identification of novel smHDAC8 inhibitors.

3.5. Histone Deacetylases Inhibitors as New Potential Drugs against Leishmania Braziliensis, the Main Causative Agent of New World Tegumentary Leishmaniasis

Luciana Ângelo de Souzaa, Matheus Silva e Bastosb, Joice de Melo Agripino, Thiago Souza Onofre, Lourdes Fanny Apaza Callaa, Tino Heimburg, **Ehab Ghazy**, Theresa Bayer, Victor Hugo Ferraz da Silva, Paula Dutra Ribeiro, Leandro Licursi de Oliveira, Gustavo Costa Bressan, Márcia Rogéria de Almeida Lamêgo, Abelardo Silva-Júnior, Raphael de Souza Vasconcellos, Ana Márcia Suarez-Fontes, Juliana Almeida-Silva, Marcos André Vannier-Santos, Raymond Pierce, Wolfgang Sippl and Juliana Lopes Rangel Fietto

Biochem. Pharmacol. **2020,** *180*, 114191

https://www.sciencedirect.com/science/article/pii/S0006295220304275?via%3Dihub

Abstract

The protozoan parasite Leishmania braziliensis is a major causative agent of the neglected tropical diseases Cutaneous and Mucocutaneous Leishmaniases in the New World. There are no vaccines to prevent the infection and the treatment relies on few drugs that often display high toxicity and costs. Thus, chemotherapeutic alternatives are required. Histone Deacetylases (HDACs) are epigenetic enzymes involved in the control of chromatin structure. In this work, we tested an in-house library of 78 hydroxamic acid derivatives as putative inhibitors of L. braziliensis HDACs (HDACi). The compounds were evaluated in relation to the toxicity to the host cell macrophage and to the leishmanicidal effect against L. braziliensis during in vitro infection. Eight HDACi showed significant leishmanicidal effects and the top 5 compounds showed effective concentrations (EC50) in the range of 4.38 to 10.21 μM and selectivity indexes (SI) from of 6 to 21.7. Analyses by Transmission Electron Microscopy (TEM) indicated induction of apoptotic cell death of L. braziliensis amastigotes with a necrotic phenotype. An altered chromatin condensation pattern and cellular disorganization of intracellular amastigotes was also observed. A tight connection between the mitochondrion and nuclear protrusions, presumably of endoplasmic reticulum origin, was found in parasites but not in the host cell. In flow cytometry (FC) analyses, HDACi promoted parasite cell cycle arrest in the G2-M phase and no changes were found in macrophages. In addition, the direct effect of HDACi against the promastigotes showed apoptosis as the main mechanism of cell death. The FC results corroborate the TEM analyses indicating that the HDACi lead to changes in the cell cycle and induction of apoptosis of L. braziliensis. The production of nitric oxide by the infected macrophages was not altered after treatment with the top 5 compounds. Taken together, our results evidenced new HDACi as promising agents for the development of new treatments for American Tegumentary Leishmaniasis caused by L. braziliensis.

3.6. The Potential for Histone Deacetylase (HDAC) Inhibitors as Cestocidal Drugs

Hugo Rolando Vaca, Ana María Celentano, María Agustina Toscanini, Tino Heimburg, **Ehab Ghazy**, Patrik Zeyen, Alexander-Thomas Hauser, Guilherme Oliveira, María Celina Elissondo, Manfred Jung, Wolfgang Sippl, Federico Camicia and Mara Cecilia Rosenzvit

PLoS neglected tropical diseases **2021,** *15* (3), e0009226

https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0009226

Abstract

Background

Echinococcosis and cysticercosis are neglected tropical diseases caused by cestode parasites (family Taeniidae). Not only there is a small number of approved anthelmintics for the treatment of these cestodiases, but also some of them are not highly effective against larval stages, such that identifying novel drug targets and their associated compounds is critical. Histone deacetylase (HDAC) enzymes are validated drug targets in cancers and other diseases, and have been gaining relevance for developing new potential anti-parasitic treatments in the last years. Here, we present the anthelmintic profile for a panel of recently developed HDAC inhibitors against the model cestode Mesocestoides vogae (syn. M. corti).

Methodology/Principal findings

Phenotypic screening was performed onM. vogae by motility measurements and optical microscopic observations. Some HDAC inhibitors showed potent anthelmintic activities; three of them -entinostat, TH65, and TH92- had pronounced anthelmintic effects, reducing parasite viability by ~100% at concentrations of ≤ 20 μM. These compounds were selected for further characterization and showed anthelmintic effects in the micromolar range and in a time- and dose-dependent manner. Moreover, these compounds induced major alterations on the morphology and ultrastructural features of M. vogae. The potencies of these compounds were higher than albendazole and the anthelmintic effects were irreversible. Additionally, we evaluated pairwise drug combinations of these HDAC inhibitors and albendazole. The results suggested a positive interaction in the anthelmintic effect for individual pairs of compounds. Due to the maximum dose approved for entinostat, adjustments in the dose regime and/or combinations with currently-used anthelmintic drugs are needed, and the selectivity of TH65 and TH92 towards parasite targets should be assessed.

Conclusion, significance

The results presented here suggest that HDAC inhibitors represent novel and potent drug candidates against cestodes and pave the way to understanding the roles of HDACs in these parasites.

3.7. One-Atom Substitution Enables Direct and Continuous Monitoring Of Histone Deacylase Activity

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Biochemistry **2019,** *58* (48), 4777-4789

https://pubs.acs.org/doi/abs/10.1021/acs.biochem.9b00786

Abstract

We developed a one-step direct assay for the determination of histone deacylase (HDAC) activity by substituting the carbonyl oxygen of the acyl moiety with sulfur, resulting in thioacylated lysine side chains. This modification is recognized by class I HDACs with different efficiencies ranging from not accepted for HDAC1 to kinetic constants similar to that of the parent oxo substrate for HDAC8. Class II HDACs can hydrolyze thioacylated substrates with approximately 5−10-fold reduced kcat values, which resembles the effect of thioamide substitution in metallo-protease substrates. Class IV HDAC11 accepts thiomyristoyl modification less efficiently with an ∼5-fold reduced specificity constant. On the basis of the unique spectroscopic properties of thioamide bonds (strong absorption in spectral range of 260−280 nm and efficient fluorescence quenching), HDAC-mediated cleavage of thioamides could be followed by ultraviolet−visible and fluorescence spectroscopy in a continuous manner. The HDAC activity assay is compatible with microtiter plate-based screening formats up to 1536-well plates with Z' factors of >0.75 and signal-to-noise ratios of >50. Using thioacylated lysine residues in p53-derived peptides, we optimized substrates for HDAC8 with a catalytic efficiency of >250000 M^{-1} s^{-1}, which are more than 100-fold more effective than most of the known substrates. We determined inhibition constants of several inhibitors for human HDACs using thioacylated peptidic substrates and found good correlation with the values from the literature. On the other hand, we could introduce N-methylated, N-acylated lysine residues as inhibitors for HDACs with an IC50 value of 1 μM for an N-methylated, N-myristoylated peptide derivative and human HDAC11.

3.8. Identification of the Subtype-Selective Sirt5 Inhibitor Balsalazide through Systematic SAR Analysis and Rationalization via Theoretical Investigations

Carina Glas, Johannes C.B. Dietschreit, Nathalie W€ossner, Lars Urban, **Ehab Ghazy**, Wolfgang Sippl, Manfred Jung, Christian Ochsenfeld and Franz Bracher

Eur J Med Chem **2020,** *206*, 112676.

https://www.sciencedirect.com/science/article/pii/S0223523420306486

Abstract

We report here an extensive structure-activity relationship study of balsalazide, which was previously identified in a high-throughput screening as an inhibitor of Sirt5. To get a closer understanding why this compound is able to inhibit Sirt5, we initially performed docking experiments comparing the binding mode of a succinylated peptide as the natural substrate and balsalazide with Sirt5 in the presence of NADþ. Based on the evidence gathered here, we designed and synthesized 13 analogues of balsalazide, in which single functional groups were either deleted or slightly altered to investigate which of them are mandatory for high inhibitory activity. Our study confirms that balsalazide with all its given functional groups is an inhibitor of Sirt5 in the low micromolar concentration range and structural modifications presented in this study did not increase potency. While changes on the N-aroyl-b-alanine side chain eliminated potency, the introduction of a truncated salicylic acid part minimally altered potency. Calculations of the associated reaction paths showed that the inhibition potency is very likely dominated by the stability of the inhibitor-enzyme complex and not the type of inhibition (covalent vs. non-covalent). Further in-vitro characterization in a trypsin coupled assay determined that the tested inhibitors showed no competition towards NADþ or the synthetic substrate analogue ZKsA. In addition, investigations for subtype selectivity revealed that balsalazide is a subtype-selective Sirt5 inhibitor, and our initial SAR and docking studies pave the way for further optimization

4. Summary of the results

The results obtained are briefly summarized in this part.

4.1. Identification of novel epigenetic modulators for the anticancer therapy

The results obtained in studies **3.1** and **3.2** are going to be briefly discussed in this part. The main focus of these studies was the design and synthesis of probes that could inhibit distinct HDAC isoforms and evaluating their biological activity in some cancer cell lines.

4.1.1. Identification of novel hHDAC8 inhibitors with potential anti-neuroblastoma activity

Based on our previously reported smHDAC8 inhibitors [107], we explored the potential of benzhydroxamates as inhibitors of the homologous human isoform. Docking studies of the initial hits highlighted that lipophilic interactions of the capping group with the residues lining the HDAC8-specific pocket might be exploited to design HDAC8-selective inhibitors. Docking studies revealed that meta-substituted benzhydroxamates could not fit in the narrow pocket of HDAC1, explaining the in vitro selectivity over HDAC1. To further explore the structure-activity relationship of HDAC8 inhibitors, we synthesized different series of inhibitors. The linker was changed from amide in the original hits to amine, inverse amide, and ether linkers in the newly synthesized derivatives (**Fig. 16**). Different substituents—at the 4-position of the benzhydroxamic acid core—and cap groups were also included to maximize the interactions in the hydrophobic side pocket of HDAC8.

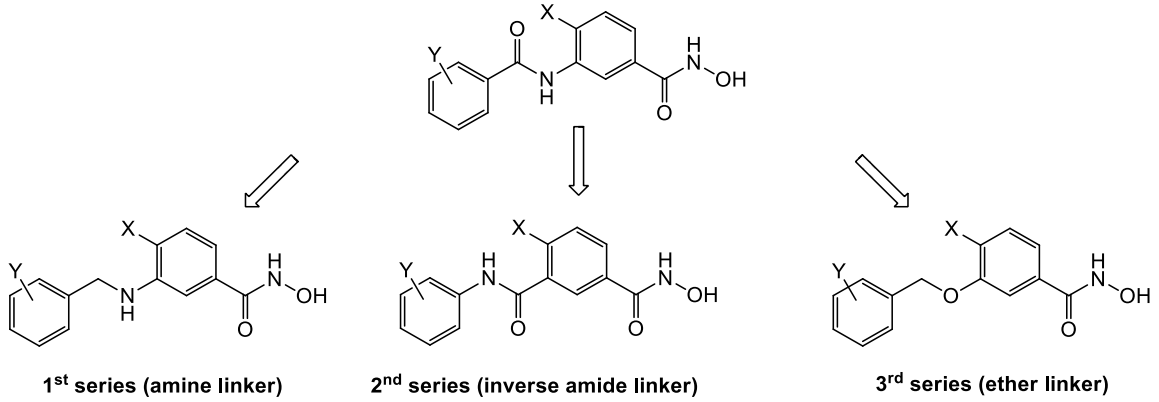


Figure 16: Design strategy of novel HDAC8 inhibitors.

Compared to the original hits, two compounds from the amine series **8b and 8f** showed increased in vitro HDAC8 activity and better selectivity against HDAC1 and 6. This improvement was also observed for one compound from the ether series **(20a)**, while for the inverse amide series, the increase in HDAC8 activity was accompanied by decreased selectivity against HDAC6 **12b**. The most promising compounds (**Fig. 17**) were then tested for their cellular effects, where they showed target engagement and hyperacetylation of HDAC8 and HDAC6 substrates but no effect on HDAC1 substrate.

Treating neuroblastoma cells with the compounds showed upregulation of specific differentiation marker genes and impaired colony formation ability, indicating a remarkable HDAC8 inhibition phenotype. The compounds also showed a relatively weak cytotoxic effect on normal human cell lines, highlighting the potential of selective HDAC inhibitors to target neuroblastoma with minimum side effects.

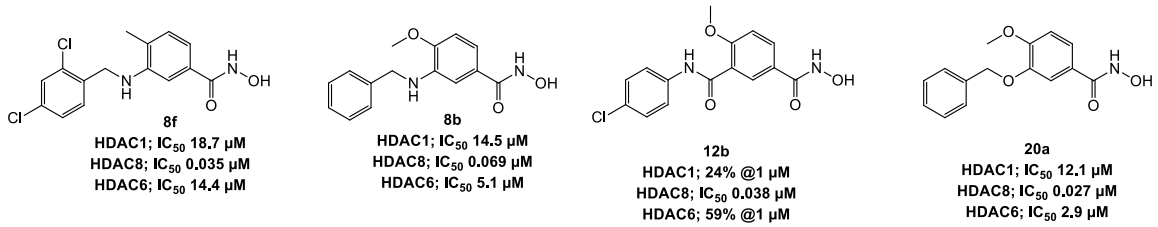


Figure 17: The most promising HDAC8 inhibitors for the treatment of Neuroblastoma.

4.1.2. Identification of dual targeting inhibitors of histone deacetylase 6/8 and bromodomain BRPF1, and their potential as antileukemic agents

Here, we build on the results obtained in the previous study and explore the polypharmacology concept as an interesting, but also challenging, approach to develop more effective epigenetic therapies. We aimed to design dual inhibitors of specific HDAC isoforms and the bromodomain and PHD finger-containing protein 1 (BRPF1). Therefore, we started by merging the essential structural features of our previously reported HDAC8 inhibitors with a BRPF1 binding scaffold to come up with dual inhibitors for both targets (**Fig. 18**) using different linkers to link both parts. The in vitro activity of the synthesized compounds on both targets revealed that linker nature is a limiting factor to get a balanced activity on both targets. While compounds containing amine and inverse amide linkers showed strong HDAC8 inhibition accompanied with weak BRPF1 activity, compounds bearing a sulfamoyl linker displayed a submicromolar inhibition of both targets. Docking studies in BRPF1 revealed that the bent conformation adopted by the sulfonamide derivatives resembles that of the cocrystallized ligand and is more favorable for BRPF1 binding. On the other side, the conformation adopted by amide derivatives leaves the benzhydroxamic acid moiety solvent exposed (**Fig. 19**). One of the best compounds was **23a** showing a submicromolar inhibition of HDAC8 and BRPF1 and a low in vitro activity against HDAC1 and 6 (**Fig. 18**).

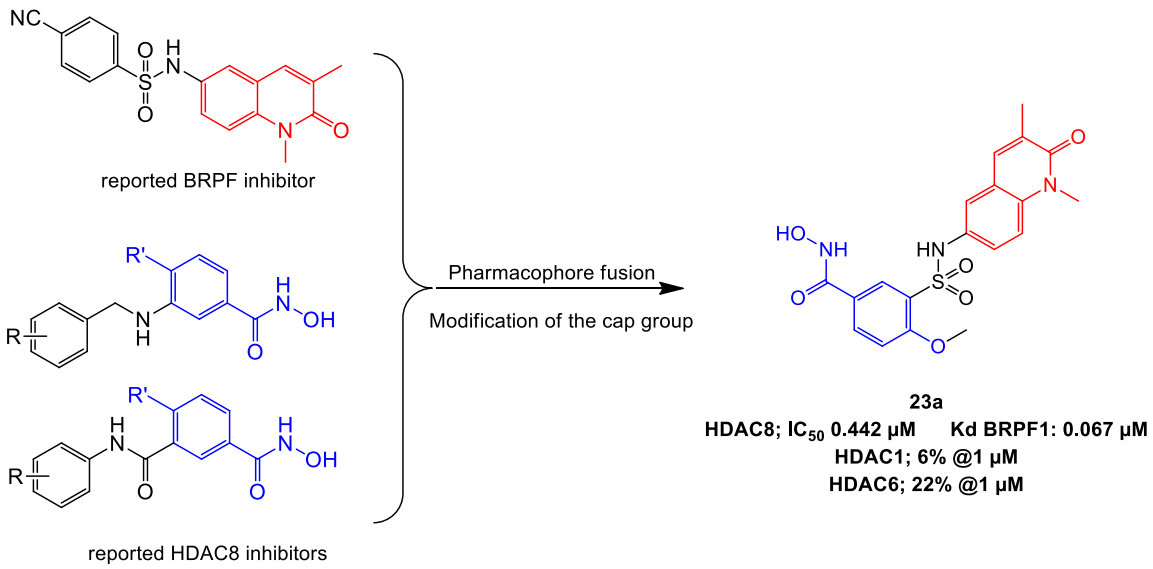


Figure 18: Pharmacophore merging to design a dual HDAC8/BRPF1 inhibitor.

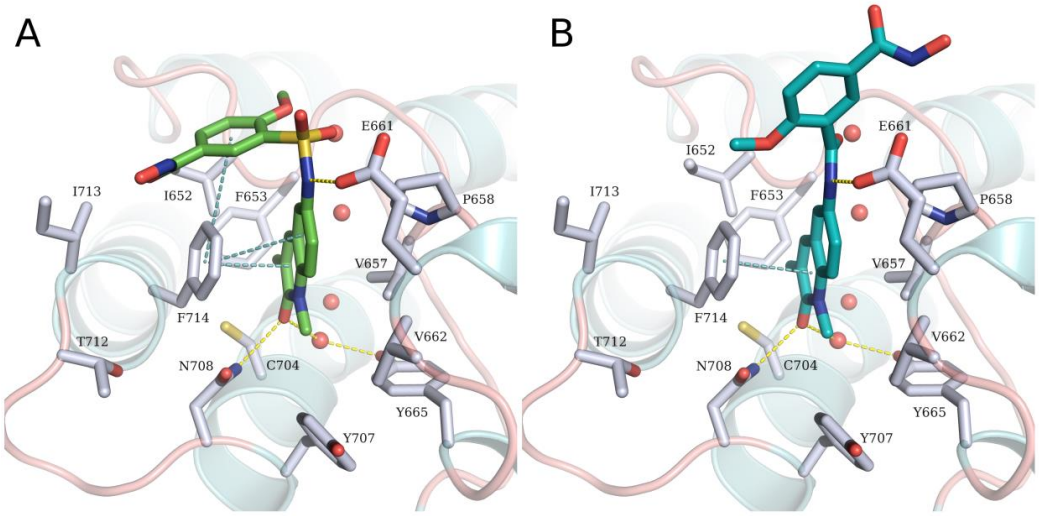


Figure 19: A. Predicted binding mode of derivative 23a (bearing sulfamoyl linker and showing the bent conformation, green sticks) in BRPF1 (PDB ID: 5MYG). B. Predicted binding mode of another derivative (having an amide linker, and showing the benzhydroxamic acid moiety solvent exposed, cyan sticks) in BRPF1. The ligands are shown as green sticks, side chains of binding site residues as white sticks, and water molecules as red spheres. Yellow-dashed lines indicate hydrogen bond interactions and cyan-dashed lines π-π stacking interactions.

We then extended the scope to include HDAC6 as a target for our inhibitors. The substitution pattern on the benzhydroxamic acid moiety was changed from meta- to para position to shift the HDAC activity more towards HDAC6 (**Fig. 20**). The sulfamoyl linker was either kept as such or increased in length to include an additional methylene group. The sulfonamide derivative **37** appeared to be the best HDAC6/BRPF1 dual inhibitor showing a submicromolar in vitro inhibitory activity against both targets (**Fig. 20**). Meanwhile, the derivative bearing an additional methylene group in the linker showed an enhanced in vitro HDAC6 activity, albeit a diminished binding to BRPF1. Docking studies and molecular dynamic simulations revealed that—owing to the flexibility of the additional methylene group in the linker—the preferred conformation could not be adopted, and the benzhydroxamic acid moiety is again solvent-exposed.

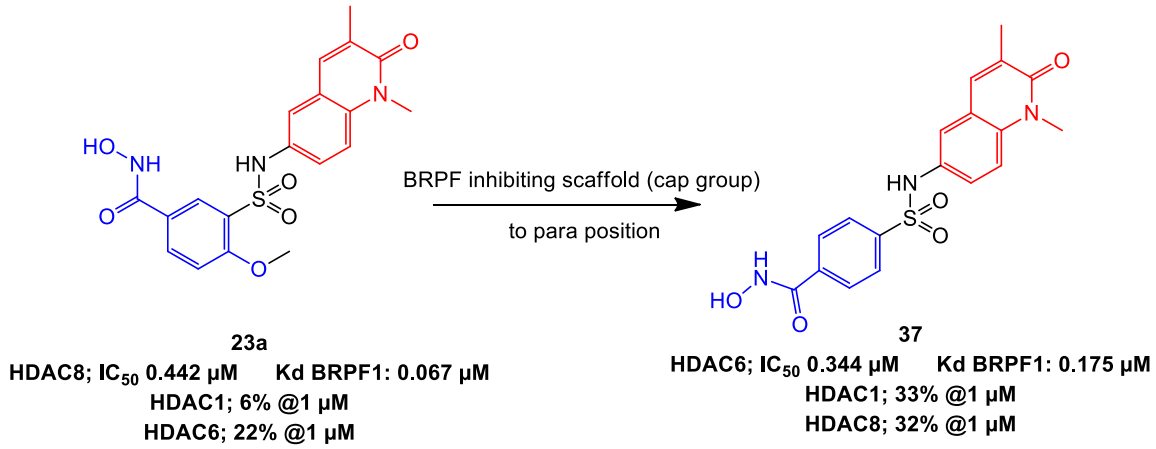


Figure 20: Changing the substitution pattern to design dual HDAC6/BRPF1 inhibitor.

The compounds were then tested for their cytotoxic effect in acute myeloid leukemia cell lines where they showed some activity only at high concentrations. However, one of the negative controls—lacking the hydroxamic acid moiety—showed a modest anticancer effect. Therefore, we hypothesized that the lack of cellular activity is perhaps a result of decreased permeability. This was further supported by target engagement studies which showed that the most in vitro active HDAC6 inhibitor (**44b**, HDAC6 IC_{50} value: 152 nM) could not induce tubulin hyperacetylation. Then, we tried to mask the hydroxamic acid moiety by synthesizing the corresponding ester prodrugs, a reported approach to overcome decreased cellular uptake and poor tissue penetration caused by the highly polar hydroxamic acid group. Unfortunately, these masked hydroxamates showed activity only at high concentrations. The results presented in this study showed how challenging the polypharmacology approach is and that merging two active pharmacophores does not guarantee a balanced and potent activity on both targets.

4.2. Identification of novel epigenetic modulators for human parasitic infections

The results obtained in studies **3.3-3.6** are going to be briefly discussed in this part. The main focus of these studies was to design novel more potent smHDAC8 inhibitors for schistosomiasis and explore their potential against other parasites.

4.2.1. Identification of novel smHDAC8 inhibitors and their antischistosomal activity

Here we follow up on our previous studies [107, 193], where we identified *Schistosoma mansoni* histone deacetylase 8 (smHDAC8) as a validated target for the antischistosomal therapy and identified some benzhydroxamates as smHDAC8 inhibitors such as the lead compound **TH65**. Structural studies showed that the hydrophobic biphenyl capping group of **TH65** could occupy an HDAC8-specific pocket and undergo extensive interactions with the hydrophobic amino acids lining the pocket. We then synthesized different derivatives where the capping group was varied to include different substituted phenyl, biphenyl, bicyclic and tricyclic rings (**Fig. 21**). Moreover, we synthesized some derivatives with an inverted amide linker to benefit from the reported HDAC8 inhibitory activity of our previous inverse amides. The chemical optimization was guided by docking studies, and the synthesized inhibitors were screened for their in vitro activity against smHDAC8 and other human HDACs. The new derivatives retained the nanomolar inhibition of both sm- and hHDAC8 and generally showed a decreased activity against HDAC1 and 6.

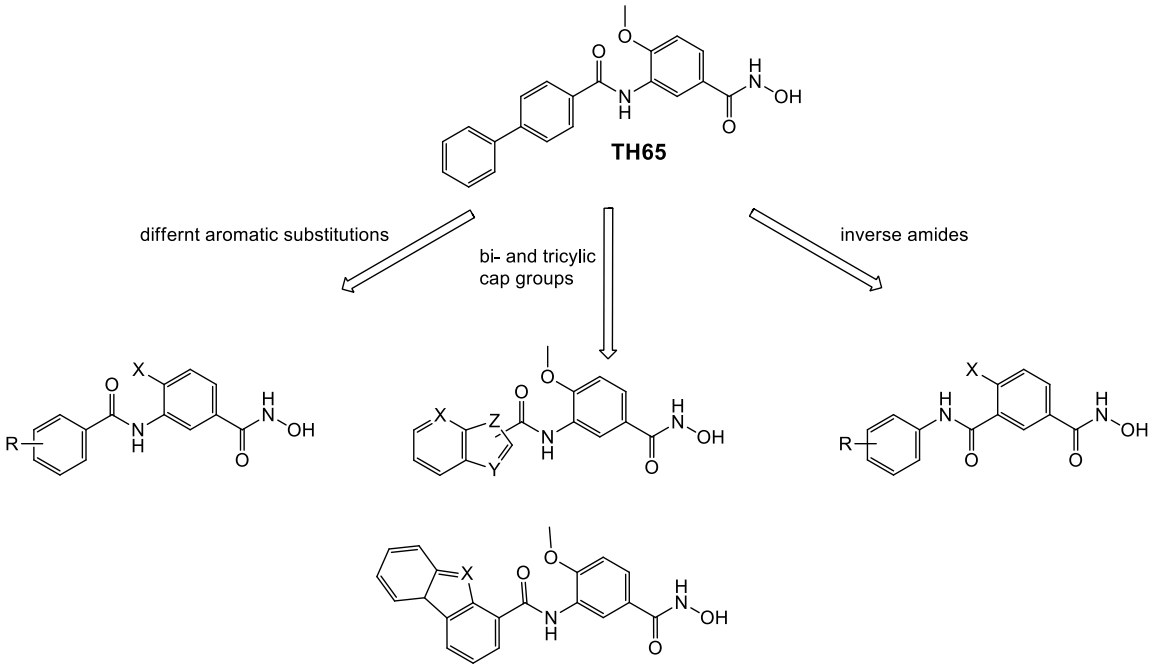
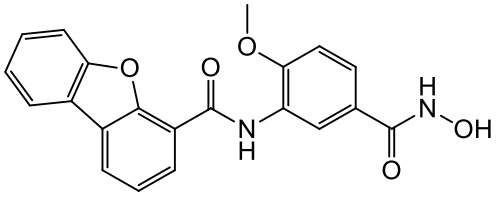


Figure 21: Design strategy to obtain novel smHDAC8 inhibitors.

We then evaluated the activity of the newly synthesized compounds on *S. mansoni* schistosomula using Alamar Blue-based assay, where some derivatives showed a more remarkable effect than the lead compounds. Compound **5o** (**Fig. 22**) with the dibenzofuran capping group was the most active with an EC_{50} of 3.5 µM on schistosomula. However, its lipophilicity and poor solubility hampered further in vivo studies, whereas the activity on schistosomula could not be retained using more soluble analogs. Crystal structure of smHDAC8 in complex with **5o** confirmed our docking results and emphasized the importance of the hydrophobic interactions between the capping group and HDAC8 side pocket.



5o

smHDAC8; IC_{50} 0.27 µM EC_{50} schistosomula *S. Mansoni*: 3.5 µM

hHDAC8; IC_{50} 0.32 µM

hHDAC1; IC_{50} 18.51 µM

hHDAC6; IC_{50} 0.29 µM

Figure 22: smHDAC8 inhibitor 5o showing the best activity on schistosomula *S. mansoni*.

4.2.2. Development and validation of QSAR models for smHDAC8 inhibitors

In study **3.4** different in silico methods were used to aid in designing new smHDAC8 inhibitors. Firstly, PDB entries of smHDAC8-ligand complexes were carefully examined, and the protein structure PDB ID 6HRQ was selected, as it showed the best cross-docking results (RMSD values ≤ 2 Å). Our docking protocol was then validated, as it could reproduce the experimentally determined binding mode of the already reported ligands (RMSD values ≤ 2 Å). Our previously reported hydroxamates [107] (training set) were then docked to 6HRQ, but we found a poor correlation between the docking scores (Glide-SP) and experimentally reported activities. As a result, the affinities of these ligands to smHDAC8 were further analyzed and different models were used to estimate the binding free energy of each docking pose.

The computationally estimated binding free energies were then used to generate QSAR models and—to improve the performance—the effect of considering further computed 2D descriptors was also investigated. Continuous improvement of the initial models through including 2D descriptors and removing some outliers led to the final satisfactory model with improved correlation with the biological data and good cross-validation results **(97, Fig. 23)**. To further evaluate the best QSAR models, some of the smHDAC8 inhibitors developed in study **3.3** were utilized as a test set. As in the training set, the same protocols for docking and binding free energy calculations were applied to the test set, and their predicted pIC_{50} values were calculated. The difference between experimentally determined and predicted pIC_{50} values was low (< 0.7 log unit) indicating that the models have a good predictive ability and could be further used to predict the activity of future smHDAC8 inhibitors.

$pIC_{50}\_pred = 3.45878 - 0.03231 * \Delta G + 11.84528 * PEOE\_VSA\_FPPOS$

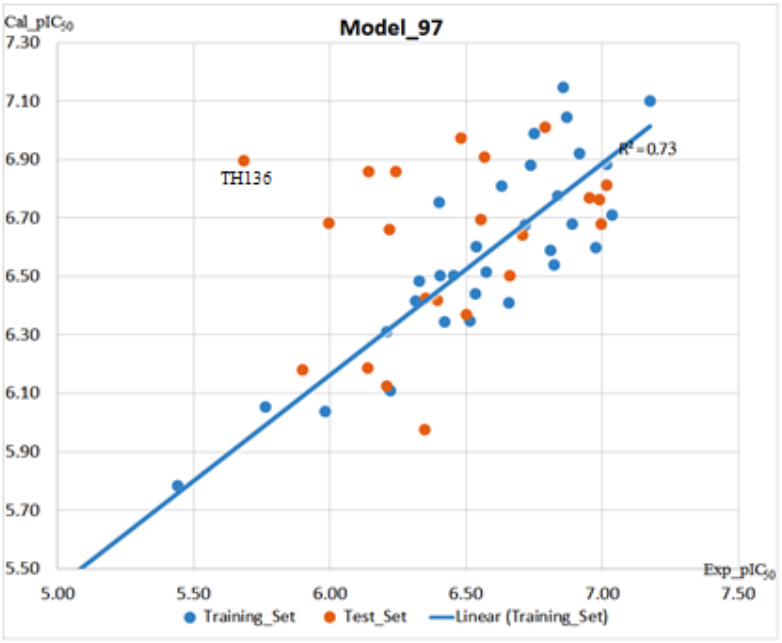


Figure 23: Equation of model 97 & correlation plot between the experimental pIC50 values (X-axis) and the calculated pIC50 values (Y-axis) for the training set molecules (blue points) using model 97 as well as the distribution of the newly designed set of molecules (orange points) along the linear regression line.

4.2.3. HDAC inhibitors are potential antileishmanial and cestocidal agents

In study **3.5** we aimed to determine the potential effects of HDAC inhibitors on *Leishmania braziliensis*. Firstly, the hydroxamates that we previously developed and optimized for HDAC8 ([107] and study **3.3**) were tested for their cytotoxic effects on the host cells macrophages. The least toxic compounds were then tested in an infection assay on the amastigotes, where eight compounds showed a significant leishmanicidal effect. Five of them showed EC_{50} values between 4 and 10 μM and selectivity index values between 6 and 22 over the host cells macrophages. The ultrastructural changes exerted by HDAC inhibitors on the amastigotes were then investigated and an altered chromatin condensation pattern and cellular disorganization were observed. Similar effects were not observed in the host cells. Our next step was to assess the direct effects of HDAC inhibitors on the extracellular promastigotes, where cell cycle arrest in the G2-M phase and apoptosis were observed as the main mechanisms of cell death. Also, nitric oxide production by the infected macrophages was not altered after treatment with the inhibitors, suggesting a possible direct leishmanicidal effect. Altogether, our results highlight the potential of HDACi as possible candidates for antileishmanial therapy.

The pan HDAC inhibitor Trichostatin A was reported to decrease the viability of some cestode parasites, cause significant morphological alterations and increase acetylation of total proteins [146]. In study **3.6**, we followed up on these results and aimed to test the effect of class I and II selective HDAC inhibitors on cestode parasites represented by the larva stage of *Mesocestoides vogae*. While class II HDAC inhibitors (mainly designed as selective HDAC6 inhibitors) did not have a significant effect on parasite viability and morphology, some class I selective inhibitors—including the ones that we previously optimized as smHDAC8 inhibitors—showed a potent cestocidal activity. The most active compounds were TH65, TH92, and interestingly the HDAC1,2,3 inhibitor entinostat, showing anthelmintic effect in a time- and dose-dependent manner as well as extensive damage on the tegument and other morphological alterations. Their in vitro IC_{50} values on the larva were then determined and the anthelmintic agent albendazole were included as a reference. The three inhibitors showed low micromolar IC_{50} values and were more potent than albendazole (**Fig. 24**). Moreover, their anthelmintic effect was found to be irreversible. Finally, different combinations of these inhibitors with albendazole were tested and all the combinations were found to have potent, time-dependent anthelmintic effects. The results presented in this study help reveal the role of HDACs in cestodes and thus, aid in novel cestocidal agents development.

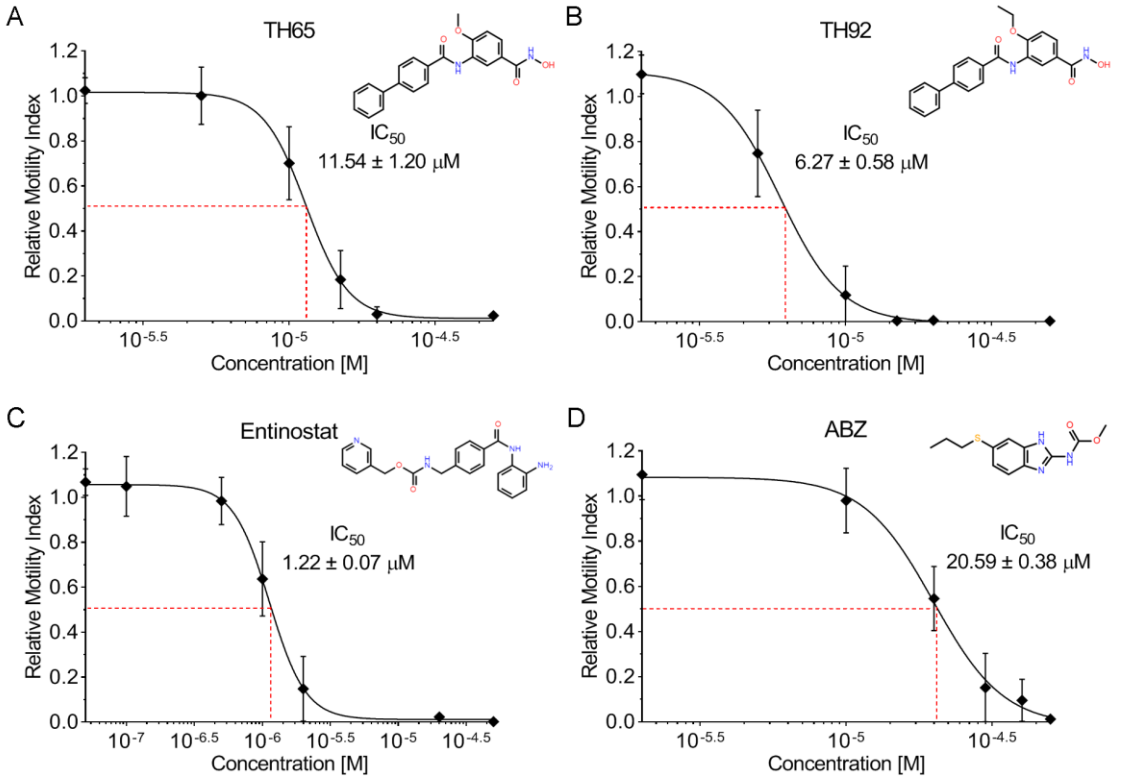


Figure 24: Anthelmintic dose-dependent effect of selected HDAC inhibitors and albendazole (ABZ). The anthelmintic dose-dependent effect was determined for the selected HDAC inhibitors (A) TH65, (B) TH92, and (C) entinostat, and (D) the current anthelmintic drug ABZ at 6 days of incubation.

4.3. Development of continuous assay to monitor HDAC activity

In almost all of the previous studies, we determined the activity of our HDAC8 inhibitors using a discontinuous assay in which the HDAC catalyzed reaction was coupled to a proteolytic reaction using the protease trypsin, specific for the free lysine side chain in the reaction product, followed by fluorescence-based readout for the proteolytic reaction. To enable us to screen more compounds, we aimed in study **3.7** to develop a continuous and direct assay for the determination of HDAC activity. Direct assays are easier because there is no coupling with another enzymatic reaction, and it is also possible to see kinetic effects in the progress curves. Therefore, substrates with lysine residues carrying different acyl groups including thioacetyl and thiotrifluoroacetyl moieties were synthesized (**peptides I and II**, **Fig. 25**). Thioamide bonds have unique spectroscopic properties since they have a UV absorption wavelength different from their reaction products, and they are also efficient fluorescence quenchers. Therefore, HDAC-mediated cleavage of thioamides could be followed by ultraviolet−visible and fluorescence spectroscopy continuously. Our results showed that substrates having a thiotrifluoroacetyl group were more widely accepted and hydrolyzed by different HDAC isoforms than their thioacetyl analogs. Even within the same class, while HDAC8 efficiently hydrolyzed both thioacyl groups, HDAC1 did not recognize these modifications. Interestingly, it was also found that HDAC11, a fatty acid deacylase, could accept a thiomyristoyl substrate. Surprisingly, introducing a methyl group on the nitrogen of the scissile amide bond resulted in peptides that are no longer HDAC substrates but could inhibit their enzymatic activity. HDAC11 was then shown to be efficiently inhibited by an *N*-methylated derivative of a myristoylated substrate with an IC_{50} value of almost 1 μM.

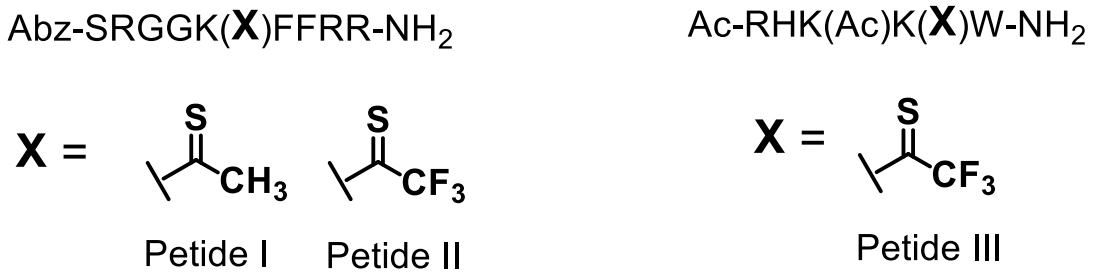


Figure 25: Structure of some thioacylated peptide derivatives used in this study (Abz = 2-aminobenzoyl, Ac = acetyl).

The newly developed HDAC8 assay is compatible with microtiter plate-based screening formats up to 1536-well plates, and the inhibition constants for several reported HDAC8 inhibitors could be reproduced. Some peptides derived from p53—one of the known in vivo HDAC8 substrates— were then modified by introducing thioacyl moieties and a thiotrifluoroacetylated substrate (**peptide III**, **Fig. 25**) was obtained that is more than 100-fold more effective than most of the known substrates reported to date.

4.4. Exploring structure activity relationship of a series of derivatives based on a sirt5 hit

The aim of study **3.8** was to get a deeper look at the previously reported Sirt5 hit balsalazide (**Fig. 26**) [194], explain its activity against the enzyme, and explore the possibility of further optimization to get more potent derivatives. Docking studies suggested that the carboxylate group on the β-alanine side chain of the compound interacts with the basic residue Arg105 (**Fig. 26**), also responsible for the binding of dicarboxylated substrates. A series of balsalazide derivatives was hence designed and synthesized in which single functional groups were either deleted or minimally altered in order to explore the structure-activity relationship. Although none of the new analogs showed a superior activity over the initial hit, it was noted that—in accordance with the docking studies—the amide and carboxylate groups on the β-alanine side chain are crucial for the activity, and derivatives lacking these functionalities showed a reduced Sirt5 inhibitory activity. Therefore, the carboxylic acid in the β-alanine-derived side chain has to be present and at an appropriate distance to the aromatic ring. Replacement of the amide bond with an ether or a secondary amine as well as inverting its orientation also led to a decreased activity. Interestingly, changes on the salicylic acid part were tolerated to some extent. Removing either the aromatic hydroxy or carboxylic, or both of them resulted in no major drop in inhibitory activity compared to balsalazide which also showed subtype selectivity for Sirt5 over Sirt1, 2, and 3 and no competition towards NAD^+. Our results suggest that balsalazide could represent a useful chemical tool for investigating the physiological roles of Sirt5.

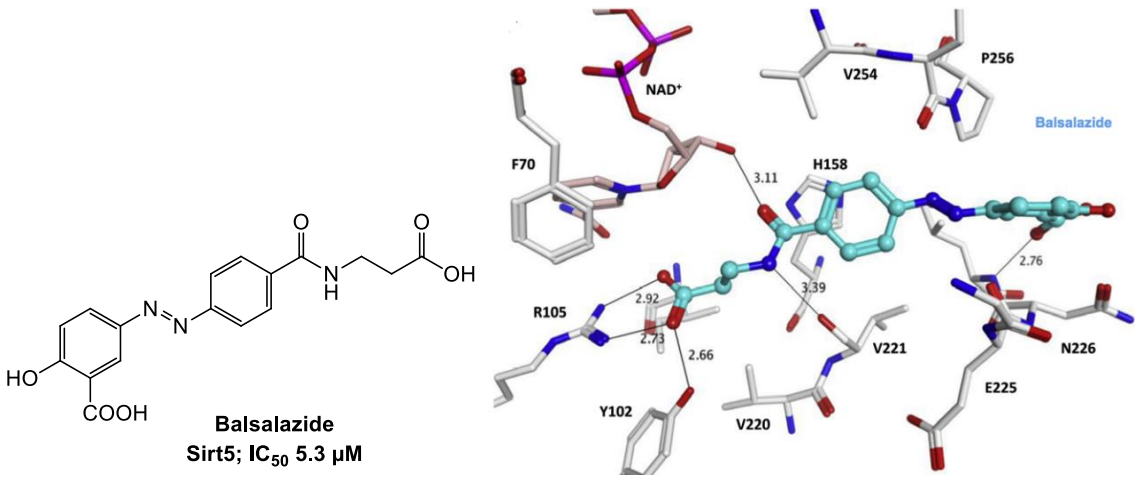


Figure 26: Structure of balsalazide and its docking pose in Sirt5 in the presence of NAD^+ (carbon atoms of NAD^+ are colored salmon, hydrogen bonds are shown as black lines and distances are given in Å).

5. General Conclusion and Perspectives

Advances in proteomics and molecular biology have revealed plenty of proteins that could represent potential drug targets for human diseases. However, the exact role of many of these proteins in different pathological conditions is yet to be revealed. Therefore, it is crucial to develop chemical probes that could potently and selectively inhibit a specific target. Such a challenging task requires a concerted effort that includes—but is not limited to—in silico approaches, drug design, chemical synthesis, in vitro biochemical and biophysical assays, target engagement studies, and phenotypic screening. The results presented in the current work highlight the combination of these approaches to identify and optimize novel modulators of some epigenetic key players, mainly histone deacetylases, and explore their potential as anticancer and antiparasitic agents.

1- We combined structure-based design, synthesis, and in vitro screening and identified several benzhydroxamates as low nanomolar inhibitors of human HDAC8. We could also show that some of them exhibited decreased activity against HDAC1 and 6, and our docking studies could explain this preferential in vitro activity of our inhibitors on HDAC8. Cellular activity of the new inhibitors was then confirmed using target engagement studies and phenotypic screening against neuroblastoma cells, where some derivatives showed a strong HDAC8 inhibition phenotype. One possible future extension of this work could be to evaluate these inhibitors in other cancer cell lines such as colon, lung, breast, pancreatic cancers where HDAC8 is reported to be overexpressed. Hydroxamates are indeed potent zinc chelators, but they also have many off-targets as well as suboptimal pharmacokinetic properties [20]. Since the HDAC8 inhibitors explored in this work are solely hydroxamates, it would be interesting to design inhibitors containing other zinc binders—some are already reported, see introduction part **1.4.1.2**—for HDAC8 activity and antineuroblastoma effect. One possible starting point could be further optimization of some previously reported amino acid-derived HDAC8 inhibitors [64]. Crystal structures of these inhibitors with HDAC8 are available, paving the way for a structure-guided optimization process. As a hot topic in drug discovery, targeted protein degradation could represent another promising application of our HDAC8 inhibitors. It is chemically feasible to functionalize our inhibitors and attach them to different E3 ligase warheads to synthesize HDAC8 PROTACs (proteolysis targeting chimeras). This strategy could then be used to evaluate the effect of HDAC8 degradation in various cellular systems including cancer cell lines.

2- We applied the polypharmacology concept to our HDAC8 inhibitors by merging their most essential structural features with a BRPF1 bromodomain-inhibiting scaffold to synthesize dual HDAC8/BRPF1 inhibitors. HDAC6 was also in our scope, as we were able—through simple structure modifications—to shift the activity towards this isoform and obtain dual HDAC6/BRPF1 inhibitors. In vitro assays identified some derivatives with submicromolar IC_{50} values against the targets, and modeling studies explained the results and guided the optimization process. Regarding cellular activity, neither our dual inhibitors nor their prodrug form showed effects in acute myeloid leukemia cells where

BRPF1 inhibitors are reportedly active. Possible reasons could be limited cellular permeability or the inability of the dual inhibitors—at the tested concentrations—to interact with their targets. Cellular permeability could be determined and perhaps enhanced by using some drug delivery formulations or other prodrug forms. However, the second reason illustrates one of the major challenges of designing dual-acting inhibitors. It is extremely challenging to balance the activity on two different targets. Simple structure modifications were found to enhance the activity on one but abolish the affinity to the other. In other words, the low nanomolar activity of the parent compounds could not—in our work—be achieved for the dual inhibitors. Although some recent reports showed dual HDAC/BET inhibitors with superior activity over the original inhibitors (see introduction part **1.6.1**), this was not the case for the majority of studies, highlighting a serious challenge for designing dual-acting agents. Also, one should not forget that some of the reported very potent "isoform-selective" HDAC inhibitors showed some cellular antiproliferative effects only at higher concentrations at which they act non-selectively on different HDAC isoforms in the cells [70]. For future dual-acting agents, it is then to be discussed if it is more therapeutically beneficial to use pan or selective HDAC inhibitors, with which targets they should be combined, and in which cell lines they could be tested.

3- We optimized our previously reported smHDAC8 inhibitors and studied their structure-activity relationship. A combination of docking and cocrystallization studies enabled further exploration of their binding mode, while the enzymatic assays showed their preferential activity for sm- and hHDAC8 over the human isoforms HDAC1 and 6. We identified one compound with an EC_{50} value in the low micromolar range against the parasitic larva and observed that lipophilicity is a determining factor for activity against the parasite. On the one hand, conferring lipophilicity to smHDAC8 inhibitors is feasible, as bulky hydrophobic capping groups could be accommodated in the active site. On the other, very high lipophilicity would decrease water solubility and hinder in vivo testing. Although our attempts to prepare a formulation for our most active compound were not successful, future collaboration with working groups in the pharmaceutical technology field could be beneficial in this regard.

4- In line with the structure-guided optimization and chemical synthesis, the developed smHDAC8 inhibitors were utilized to build a QSAR model to help us predict the activity of future planned derivatives. A poor correlation between the docking scores of the inhibitors and their biological activity was observed; therefore, binding free energy calculations were used to re-score the poses. Using the new scores—and through a continuous optimization process—an acceptable model with a reliable predictive ability was developed. This model presents another tool to aid in identifying novel smHDAC8 inhibitors. Although obtaining good smHDAC8 activity using hydroxamates-based inhibitors is a feasible target, selectivity against other human isoforms, especially hHDAC8, is challenging. It would be interesting to investigate if QSAR models could be generated to predict HDAC selectivity.

5- We again utilized our smHDAC8 inhibitors, but this time to investigate if they could be beneficial against other parasitic infections, namely leishmaniasis and cestode infections. Firstly some compounds showed a significant leishmanicidal effect against amastigotes with EC_{50} values in the low micromolar range. Moreover, the host cells did not show the phenotypic effects observed in the parasites, indicating a potential safety margin. Secondly, we moved to cestode parasites and observed an anthelmintic activity in a time- and dose-dependent manner. Some of our inhibitors have also better IC_{50} values on the larva stage of *Mesocestoides vogae* than the anthelmintic agent albendazole, and combinations of this drug with our inhibitors are even more potent. The results confirmed that the "Piggyback" approach (see introduction part **1.4.2**) could be applied to identify new antiparasitics for schistosomiasis, leishmaniasis, and cestode infections. It is also to be noted that many epigenetic targets, and especially histone deacetylases, were identified in other human parasites such as *Plasmodium sp.* and *Trypanosoma sp.* In some cases, their 3D structures were either determined through crystallographic studies or generated via homology modeling. This could pave the way for a structure-guided optimization process to develop potent HDAC inhibitors against these parasites. Since some antiparasitic therapies involve a combination of drugs to achieve better efficacy and overcome resistance, this could also be applied to HDAC inhibitors. They could be either tested in combination therapy or merged with other antiparasitic scaffolds to generate a potential dual-acting antiparasitic agent.

6- To facilitate enzymatic testing of more HDAC inhibitors, we aimed to develop a direct assay to measure HDAC activity. Some modified peptide substrates containing thioacetyl and thiotrifluoroacetyl groups were synthesized to enable us to monitor the hydrolysis reaction via UV absorbance or fluorescence. The results showed that HDAC8 is especially capable of hydrolyzing both groups efficiently, while HDAC1 did not accept the modified substrates. Highly efficient thiotrifluoroacetyled HDAC8 substrates were identified, and the scalability of our assay was proved, enabling highly effective microtiter plate-based inhibitor screening projects. While it is intended to use this assay system for future testing of HDAC8 inhibitors, it would be interesting to develop a similar assay for other HDAC isoforms. Interestingly, HDAC11 was shown to be efficiently inhibited by an *N*-methylated derivative of a myristoylated substrate. That could also be the starting point to develop new inhibitors for this isoform.

7- Docking studies were performed on balsalazide—a previously reported Sirt5 hit—to get an idea about its mode of binding and different derivatives were then synthesized to study their structure-activity relationship. Although the new compounds were not as active as the original hit, the essential functional groups of the compounds were identified. Balsalazide was also shown to be selective for Sirt5 over other sirtuin isoforms. Since the crystal structure of this isoform is available, further structure-guided optimization could be carried out to identify more potent derivatives. These could then be used as probes to study the effect of Sirt5 inhibition in different biological systems.

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7. Appendix

Personal information

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Work experience

10/2020-present **Wissenschaftlicher Mitarbeiter**

Institute of Pharmacy, Martin-Luther-University (Halle-Wittenberg), Germany

- Synthesis of novel inhibitors for RNA binding proteins
- Practical courses of qualitative inorganic chemistry

10/2019-09/2020 **Wissenschaftliche Hilfskraft mit Abschluss**

Institute of Pharmacy, Martin-Luther-University (Halle-Wittenberg), Germany

- Synthesis of novel inhibitors for RNA binding proteins
- Practical courses of qualitative inorganic chemistry

02/2010–09/2015 **Research and Teaching Assistant**

Faculty of Pharmacy, Alexandria University, Alexandria (Egypt)

- Synthesis of dual inhibitors for HCV and HCC
- Practical courses of medicinal chemistry and computer-aided drug design

09/2009–09/2011 **Pharmacist**

"Mona Adel" Community Pharmacy, Alexandria (Egypt)

Education and training

04/2016–09/2019 **Research stay in the Medicinal Chemistry group**

Institute of Pharmacy, Martin- Luther University, Halle (Saale) (Germany)

Design and synthesis of novel inhibitors of HDACs and bromodomains

06/2015–03/2016 **German Language course**

Goethe-Institute, Alexandria (Egypt) & InterDaF e. V. am Herder-Institut der Universität Leipzig, Leipzig (Germany)

09/2009–04/2014 **M.Sc. of pharmaceutical science in pharmaceutical chemistry**

Faculty of pharmacy-Alexandria university, Alexandria (Egypt)

Design and synthesis of novel inhibitors against Hepatitis C virus and liver carcinoma

09/2004–06/2009 **Bachelor of pharmaceutical science (Distinction Honor)**
Faculty of pharmacy-Alexandria university, Alexandria (Egypt)

2001–2004 **General secondary certificate with grade 100% (GPA 4.00)**
"Gamal Abdelnaser" secondary school, Alexandria (Egypt)

Personal skills

Mother tongue: Arabic

Foreign languages

	Listening	Reading	Speaking	Writing
English	C1	C1	C1	C1
German	B2	B2	B2	B2

Communication skills
- Very good communication skills (work with students and collaborators)
- Networking (collaboration with different international working groups)
- Presentation skills (Posters and oral presentations in conferences)

Organisational skills: member of the organising committee of second and third annual scientific conferences of pharmaceutical chemistry department, faculty of pharmacy, Alexandria University

Additional information

Projects
- Design, synthesis and molecular modeling study of novel benzofurans and their bioisosteres as HCV and tumor inhibitors (2011-2013)
- A-PARADDISE: Anti-Parasitic Drug Discovery in Epigenetics (2016-2017)

Scholarships: German Egyptian Research Long-term Scholarships (GERLS): scholarship of DAAD for PhD study at MLU, Halle, Germany (2015-2019)

Conferences
- Scientific conference of medicinal chemistry department, faculty of pharmacy, Alexandria University (2013, 2014).
- Epigenetic Drug Design for Neglected Parasitic Diseases, Rome, 2016
- Drug Innovation in Academia, Heidelberg, 2016
- 13th German Peptide Symposium, Erlangen, 2017
- Annual conference of the German society of pharmacy, 2017 & 2018
- 28th Annual Meeting of the German Society for Parasitology, Berlin, 2018
- 2nd RSC Anglo Nordic Medicinal Chemistry Symposium, Snekkersten, Denmark, 2019
- International Conference on “Pharmaceutical and Healthcare Sciences, Alexandria, Egypt, 2019

List of publications

Publications related to the thesis

Structure-based design and biological characterization of selective HDAC8 inhibitors with anti-neuroblastoma activity

J. Med. Chem. **2017,** *60* (24), 10188-10204.

One-Atom-Substitution Enables Direct and Continuous Monitoring of Histone Deacylase Activity

Biochemistry **2019,** *58* (48), 4777-4789.

Design, synthesis, and biological evaluation of novel hydroxamates as dual targeting inhibitors of histone deacetylase 6/8 and bromodomain BRPF1

Eur J Med Chem **2020,** *200*, 112338.

Histone deacetylases inhibitors as new potential drugs against Leishmania braziliensis, the main causative agent of New World Tegumentary Leishmaniasis

Biochem. Pharmacol. **2020,** *180*, 114191.

The potential for histone deacetylase (HDAC) inhibitors as cestocidal drugs

PLoS neglected tropical diseases **2021,** *15* (3), e0009226.

Identification of the subtype-selective Sirt5 inhibitor balsalazide through systematic SAR analysis and rationalization via theoretical investigations

Eur J Med Chem **2020,** *206*, 112676.

Binding free energy (BFE) calculations and quantitative structure-activity relationship (QSAR) analysis of Schistosoma mansoni histone deacetylase 8 (smHDAC8) inhibitors

Molecules **2021,** *26* (9), 2584.

Synthesis, structure-activity relationships, cocrystallization and cellular characterization of novel smHDAC8 inhibitors for the treatment of schistosomiasis.

Eur J Med Chem **2021,** *225*, 113745.

Other publications not related to the thesis

Dual inhibitors of hepatitis C virus and hepatocellular carcinoma: design, synthesis and docking studies.

Future Sci OA **2018,** *4* (1), FSO252.

Patent applications

Novel inhibitors of insulin-like growth factor 2 mRNA binding proteins

S. Hüttelmaier, W. Sippl, N. Bley, S. Müller, D. G. Martínez, E. Ghazy

Application No EP20211807.1

Identification of novel HDAC and DHFR dual-targeting inhibitors as potent antimalarials

E. Ghazy, C. Pierrot, M. Abdelsalam, J. Khalife, W. Sippl

Patent application submitted May 2021

Selbstständigkeitserklärung/Declaration of Authorship

Hiermit erkläre ich, dass ich die vorliegende Dissertationsschrift selbständig und ohne fremde Hilfe angefertigt, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und die aus ihnen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe. Die Arbeit wurde ausschließlich der Mathematisch-Naturwissenschaftlichen Fakultät der MartinLuther-Universität Halle-Wittenberg vorgelegt und an keiner anderen Universität oder Hochschule weder im In- und Ausland zur Erlangung des Doktorgrades eingereicht.

I hereby declare that I am the sole author of this thesis and that I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this thesis at any other institution in order to obtain a degree.

Halle (Saale), 22.10.2021