Design, Synthesis and In vitro Evaluation of Novel Inhibitors and Degraders of Zn^{2+}-dependent Histone Deacetylases

DISSERTATION

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Abstract

Dysregulation of posttranslational modifications (PTMs) of histones and non-histone proteins can shift the balance of gene expression and cellular processes, therefore it is associated with the development of several diseases especially cancer. Reversible acetylation and deacetylation of histones and a variety of non-histone proteins are among the most studied PTMs. They are controlled by the two opposing enzyme groups histone acetyltransferases (HATs) and histone deacetylases (HDACs). The work presented in this dissertation focuses on the development of novel molecules that modulate HDAC6 and HDAC8 through inhibition or targeted degradation.

Because of the several advantages which a targeted protein degradation approach presents over the occupancy-driven strategy, the identification of novel heterobifunctional molecules to degrade HDAC6 and HDAC8 represents a promising therapeutic approach in different cancer forms including neuroblastoma. Based on benzhydroxamate inhibitors, degraders targeting HDAC6 and HDAC8 were designed, synthesized, and tested against human HDAC1, -6 and -8 for their inhibitory activity and in cultured neuroblastoma cells to determine their degradation profile. From the sixteen degraders designed to target HDAC8, two exhibited degradation of HDAC8 with good selectivity over HDAC1 and HDAC6. In addition, they induced neural differentiation and exhibited weak to no cytotoxic effects against human kidney-derived HEK293 cells. On the other hand, one out of the four degraders synthesized to target HDAC6 showed strong degradation of HDAC6 with no cytotoxic effects against HEK293 cells.

Epigenetic modulators can also be targeted in human parasites as a therapeutic approach for parasitic diseases. As *Schistosoma mansoni* HDAC8 (smHDAC8) seems to possess an important functional role in the different stages of the parasite's life cycle, it is the most targeted epigenetic regulator in the parasite. Optimization of previously reported benzhydroxamates using structure-based drug design led to the identification of novel small molecule inhibitors for smHDAC8. The most effective inhibitor synthesized showed pronounced dose-dependent decrease in the larvae viability, killing almost 98% of the schistosomula. In addition, it exhibited nanomolar inhibition of both sm- and hHDAC8 and low cytotoxicity against human HEK293 cells.

Moreover, the binding modes and binding free energy of a pool of synthesized benzhydroxamate derivatives targeting smHDAC8 were studied using a variety of computational methods in order to generate a QSAR model with a reliable predictive ability to predict the activity of future benzhydroxamates towards smHDAC8.

Key words: epigenetics, HDAC, cancer, proteolysis targeting chimera, PROTAC, targeted protein degradation, hydrophobic tagging, benzhydroxamate, neuroblastoma, antiparasitic, schistosomiasis, smHDAC8, quantitative structure–activity relationship, QSAR, docking, binding free energy calculation.

Kurzfassung

Die Dysregulation posttranslationaler Modifikationen von Histonen und Nicht-Histon-Proteinen kann das Gleichgewicht der Genexpression und zellulärer Prozesse verschieben und ist daher mit der Entwicklung mehrerer Krankheiten, insbesondere Krebs, verbunden. Reversible Acetylierung und Deacetylierung von Histonen und einer Vielzahl von Nicht-Histon-Proteinen gehören zu den am meisten studierten posttranslationalen Modifikationen. Sie werden von den gegensätzlichen Aktivitäten zweier Enzymgruppen, nämlich Histon-Acetyltransferasen (HATs) und Histon-Deacetylasen (HDACs), kontrolliert. Die in dieser Dissertation vorgestellte Arbeit konzentriert sich auf die Entwicklung neuartiger Moleküle, die die Hemmung und den gezielten Abbau von HDAC6 und HDAC8 bezwecken.

Der gezielte Proteinabbau bietet mehrere Vorteile gegenüber der traditionellen belegungsbasierten Hemmung durch kleine Moleküle. Daraufhin stellt die Identifizierung neuer heterobifunktioneller Moleküle zum gezielten Abbau von HDAC6 und HDAC8 einen vielversprechenden, therapeutischen Ansatz beim Neuroblastom dar. Basierend auf Benzhydroxamat-Inhibitoren wurden Degrader entworfen, synthetisiert und gegen die humanen HDAC1, -6 und -8 Isoenzyme auf ihre inhibitorische Aktivität getestet und in kultivierten Neuroblastomzellen ihr Abbauprofil bestimmt. Von den sechzehn Degradern, die HDAC8 als Zielprotein hatten, zeigten zwei einen Abbau von HDAC8 mit guter Selektivität gegenüber HDAC1 und HDAC6. Darüber hinaus induzierten sie eine neuronale Differenzierung und zeigten schwache bis keine zytotoxischen Wirkungen gegen HEK293-Zellen. Andererseits zeigte einer von den vier Degradern, die gegen HDAC6 synthetisiert wurden, eine starke Abbauwirkung gegenüber HDAC6, ohne zytotoxische Wirkungen gegen HEK293-Zellen zu haben.

Epigenetische Modulatoren können auch bei humanpathogenen Parasiten als therapeutischer Ansatz für parasitäre Infektionskrankheiten eingesetzt werden. Da *Schistosoma mansoni* HDAC8 (smHDAC8) eine wichtige funktionelle Rolle in den verschiedenen Stadien des Lebenszyklus des Parasiten zu spielen scheint, gilt es als ein wichtiges therapeutisches Angriffsziel. Die Optimierung von zuvor beschriebenen Benzhydroxamaten unter Verwendung von strukturbasierten Arzneimitteldesign, das sich an verfügbaren Kristallstruktur- und Docking-Studien orientierte, führte zur Identifizierung neuartiger, nanomolarer Inhibitoren für smHDAC8. Der effektivste synthetisierte Hemmstoff zeigte eine bemerkenswerte dosisabhängige Abnahme der Lebensfähigkeit der Larven und tötete fast 98 % der Schistosomulae. Darüber hinaus zeigte er eine nanomolare Hemmung sowohl von sm- als auch von hHDAC8 und eine schwache zytotoxische Wirkung gegen HEK293-Zellen.

Basierend auf einem Pool von synthetisierten Benzhydroxamat smHDAC8 Inhibitoren wurde mithilfe von Computermethoden ein Modell der quantitativen Struktur-Aktivitäts-Beziehung erstellt und verbessert, um mit einer hohen Verlässlichkeit die Aktivität zukünftiger Benzhydroxamate gegenüber smHDAC8 vorhersagen zu können.

Schlagwörter: Epigenetik, HDAC, Krebs, Proteolyse-Targeting-Chimären, PROTAC, gezielter Proteinabbau, hydrophobes Tagging, Antiparasitika, Benzhydroxamate, Neuroblastom, antiparasitär, Schistosomiasis, smHDAC8, quantitativen Struktur-Aktivitäts-Beziehung, QSAR, Docking, freie Bindungsenergieberechnungen.

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Dedication

I dedicate this work to my beloved **Mother** without whom I would have never been able to realise my dreams. You are my lifelong supporter and my backbone who always believes in me and always pushes me outside my comfort zone to follow my ambitions and dreams. You will always be my role model with your strong character and devotion to your work and your family despite the hardships. Without your unconditional parental love, advice, care, motivation and prayers I would have never developed into the woman I am today.

"Everything is theoretically impossible, until it is done."

-Robert A. Heinlein

Table of contents

List of Figures

List of Tables

List of Abbreviation

Abbreviation	Full Phrase
5mC	5-methylcytosine
AML	acute myeloid leukemia
Boc3Arg	tert-butyl carbamate-protected arginine
CD	catalytic domain
CGIs	cytosine-guanine islands
CRBN	cereblon
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
ERRα	estrogen-related receptor α
EZH2	zeste homolog 2
FDA	U.S. Food and Drug Administration
HAT	histone acetyltransferase
HCC	human hepatocellular carcinoma
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HDM	histone demethylase
HEK293	human embryonic kidney 293 cells
hHDAC	human histone deacetylase
HMT	histone methyltransferase
Hsp	heat shock proteins
HyT	hydrophobic tagging
IAP	inhibitor of apoptosis protein
IC_{50}	half maximal inhibitory concentration
IKZFs	ikaros family of zinc fingers
KDAC	lysine deacetylase
lncRNA	long non-coding RNA
Lys	lysine
MDM2	mouse double minute 2
miRNA	microRNAs
MM	multiple myeloma
NAD	nicotinamide adenine dinucleotide
ncRNA	non-coding RNA
NES	nuclear export signal

NLS	nuclear localization signal
piRNA	piwi-interacting RNA
POI	protein of interest
PPI	protein-protein interaction
PROTAC	proteolysis targeting chimera
PTCL	peripheral T cell lymphoma
PTM	posttranslational modification
RAI1	retinoic acid induced1
RNA	ribonucleic acid
rRNA	ribosomal RNA
SAM	S-adenosylmethionine
SARD	selective androgen receptor degrader
SERD	selective estrogen receptor degrader
siRNA	small interfering RNA
Sirt	sirtuin
SMC3	structural maintenance of chromosomes 3
SmHDAC8	*Schistosoma mansoni* HDAC8
snoRNA	small nucleolar RNA
snRNA	small nuclear RNAs
TPD	targeted protein degradation
tRNA	transfer ribonucleic acid
UPS	ubiquitin-proteasome system
VHL	von Hippel Lindau
WHO	World Health Organization
ZBG	Zn^{2+}-binding group
Znf-UBP	zinc finger ubiquitin binding domain

Chapter I

Introduction

1 Epigenetics

Since the introduction of the term "Epigenetics", the definition has undergone many changes due to the rapid development of genetics. Nowadays it is defined as the study of the changes in gene expression that can be inherited through mitosis and/ or meiosis, but do not involve changes in the DNA sequence **[1,2]**. The main epigenetic mechanisms encompass posttranslational histone modifications, DNA methylation and modulation by non-coding RNAs (**Figure 1**) **[3]**.

In the eukaryotic cell nucleus, chromatin is the packaged form of the genomic DNA. Its basic packaging unit is the nucleosome which is composed of four pairs of the highly conserved histone proteins (H3, H4, H2A and H2B) forming a disc-shaped unit around which 147bp of DNA is wrapped. Short linker DNA connects the nucleosomal cores forming a structure similar to beads on a string. The linker histone protein H1 binds to the surface of the nucleosome at both the entry and exit sites of the DNA forming the chromatosome and leading to higher-order compaction of chromatin. Both core and linker histones in the nucleosome and the chromatosome interact with the DNA through electrostatic interactions formed between the negatively charged phosphates of the DNA and the positively charged arginine and lysine residues in the histones **[4-7]**.

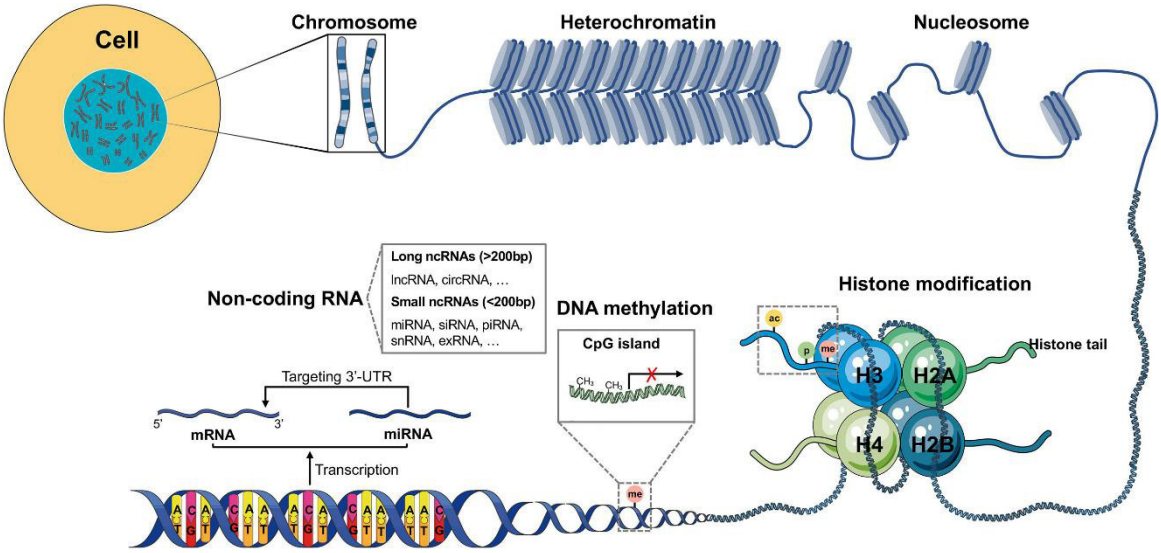


Figure 1 Schematic model of epigenetic regulation. Different epigenetic processes are present to control gene transcription and expression including histone posttranslational modifications, DNA methylation and non-coding RNA Reprinted with permission from **[8]**.

1.1 Chromatin remodelling and the "histone code"

The packaging of DNA into nucleosomes and higher order structures acts as a physical barrier to regulators binding to the DNA, thus is inhibitory to all DNA dependent processes such as gene transcription, DNA replication and DNA damage repair. Accessibility to nucleosomal DNA is controlled by chromatin remodelling enzymes through structural modification, compositional alteration and mobilization of nucleosomes **[4,9]**.

The four core histones are composed of a conserved central globular domain participating in histone-histone interaction and flexible N- and C-terminal tails **[6]**. The N-terminal tail of histones, in addition to some positions in the globular domain undergo several posttranslational modifications (PTMs) such as lysine acetylation, ubiquitination, lysine and arginine methylation, and serine, tyrosine and threonine phosphorylation. The "histone code" hypothesis suggests that unique combination or sequence of histone PTM on the same or different N-terminal tail, form patterns leading to certain biological events through induction of distinct sets of protein **[10,11]**. These reversible histone PTM are regulated by several enzyme groups and can affect each other. They are catalysed by "writers" such as histone acetylases (HAT) and histone methyltransferases (HMT) and are removed by "erasers" such as histone deacetylases (HDACs), histone demethylases (HDMs) and phosphatases. "Readers" which are recruited through specific binding domains, translate these modifications into biological events **[12]**.

1.2 DNA methylation and RNA-associated silencing

In mammals the predominant DNA modification is the methylation at the C-5 position of cytosine ring in a CpG dinucleotide forming 5-methylcytosine (5mC). In humans, cytosine-guanine islands (CGIs), which are characterized by a high CG content, are associated with at least 50% of gene promoters. Formation and maintenance of the DNA methylation pattern is mediated by three members of DNA methyltransferases (DNMT1, DNMT3a and DNMT3b), which catalyse the transfer of a methyl group from S-adenosylmethionine (SAM) to the C-5 position of a cytosine ring in a CpG dinucleotide, and an accessory protein (DNMT3L). Methylation can be either actively or passively reversed. Gene transcription repression can be affected by DNA methylation in three ways which are discussed in depth in multiple papers and reviews **[13-15]**.

At present, it is generally known that most of the transcribed RNA do not encode functional proteins. However, these non-coding RNAs (ncRNAs) play a vital role in the epigenetic regulation of gene expression. Generally, non-coding RNAs can be divided into housekeeping ncRNAs (snRNA, tRNA, rRNA and snoRNA) which are important for normal cellular function **[16]**, and regulatory ncRNAs that moderate cellular processes as chromatin remodelling, transcription, PTM and signal transfer **[17]**. The latter group is divided based on their size into short chain (including siRNAs, miRNAs and piRNAs) and long non-coding RNAs (lncRNAs) **[3,18]**. The following references, among others, provide further details on the mechanisms of action of the different ncRNAs **[3,18-22]**.

2 Histone deacetylases as therapeutic targets

2.1 Histone acetylation and deacetylation

Reversible acetylation and deacetylation are the most interesting PTMs, as they control the chromatin structure through affecting the ionic interactions between the positively charged histones and the negatively charged DNA; thereby influencing the accessibility of the DNA to the regulatory factors. These PTMs are controlled by two enzyme groups of opposing activities, namely histone acetyltransferases (HATs, now generally categorized as lysine acyltransferases) and histone deacetylases (HDACs, also known as lysine deacylases or KDACs).

While HATs catalyze the transport of an acetyl group from acetyl-co-A to the ε-amino site of a specific lysine leading to neutralization of the positive charge on histones and the opening of the chromatin structure; HDACs remove the acetyl group resulting in a closed chromatin structure repressing transcription. In addition to transcriptional regulation through reversible acetylation of histones, HATs and HDACs affect many cellular processes by dynamic acetylation and deacetylation to a variety of non-histone proteins such as transcription factors (p53), nuclear import factors and cytoskeletal proteins (α-tubulin) **[23-26]**.

2.2 Histone deacetylases

According to their primary homology to yeast HDACs, the 18 mammalian HDACs known to date are divided into four classes: class I HDACs (HDACs 1, 2, 3, and 8), class II HDACs (HDACs 4, 5, 6, 7, 9, and 10), class III (sirtuins: sirt1-sirt7) and class IV (HDAC 11).

Based on the presence of a conserved deacetylase domain and their dependence on specific cofactors, HDACs can be divided into two families namely the "classical" histone deacetylase family and the sirtuins or Sir2-like deacetylases. While the "classical" HDACs family including classes I, II and IV are Zn^{2+}-dependent enzymes, class III (sirtuins) members are Zn^{2+}-independent and require nicotinamide adenine dinucleotide (NAD) for their enzymatic activity (**Table 1**) **[25,27,28]**. Moreover, HDACs family members have a highly conserved catalytic domain of approximately 390 amino acid **[28]**, whereas sirtuin deacetylases possess a conserved catalytic domain composed of approximately 275 amino acids **[29]**.

Table 1 Classification of Histone deacetylases (HDACs).

Classical Zn^{2+}-dependent			Sirtuins NAD-dependent	Classical Zn^{2+}-dependent
Class I	Class II		Class III	Class IV
HDAC1	IIa	HDAC4	Sirt1	HDAC11
HDAC2		HDAC5	Sirt2	
HDAC3		HDAC7	Sirt3	
HDAC8		HDAC9	Sirt4	
	IIb	HDAC6	Sirt5	
		HDAC10	Sirt6	
			Sirt7	

The catalytic pocket of classical HDACs can be divided into multiple parts, namely the main pocket and the sub-pockets. While the former encompasses the acetate binding cavity, the substrate binding tunnel with a zinc ion at its bottom and the edge of the pocket, the latter include the side pocket, the lower pocket and the foot pocket **[30-33]**. In contrast to the main pocket which is present in all crystal structures of HDACs, the sub-pockets being open or closed depends on the HDAC isoform and the bound ligand **[34,35]**. Along with the first catalytic domain, HDACs might possess a second catalytic or pseudocatalytic domain, C- or N-terminal extensions and other domains **[36-38]**.

As HDACs lack intrinsic DNA binding activity, they are recruited to act on specific genomic sites through the direct interaction with target-specific transcriptional regulators, or through their inclusion into large multiprotein transcriptional complexes **[37,39]**.

2.3 Histone deacetylase inhibitors for cancer treatment

Due to the involvement of HDACs in a broad range of processes such as different intracellular functions, gene expression, DNA replication and repair, cell-cycle progression, and cytoskeletal reorganization, inhibition of these enzymes can influence a number of downstream biological pathways important for cellular proliferation, angiogenesis, differentiation, and survival. Abnormalities in HDACs' expression and activity have been associated with the development of several diseases such as cancer **[27,40-43]**, numerous neurodegenerative disorders **[44-47]**, and cardiac diseases **[48-50]**. Consequently, therapeutic inhibition of these enzymes has been the focus of many studies and research groups.

As a result of the Zn^{2+}-dependency of the catalytic activity of classical HDACs (**Figure 2**), suppressing the activity of these metalloenzymes is achieved by targeting the zinc pocket catalytic domain by natural product-derived or synthetically derived small-molecule inhibitors. Generally, the pharmacophore of the majority of these inhibitors is composed of three elements: a Zn^{2+}-binding group (ZBG), a linker mimicking the *N*-alkyl side chain of lysine and a cap group which interacts with amino acids at the rim of the pocket and serves as a peptide substrate recognition element therefore greatly affecting the isoform selectivity **[51-53]**.

Currently, HDAC inhibitors (HDACi) can be categorized either by the chemical structure of their Zn^{2+}-binding group into hydroxamic acids, carboxylates, benzamides, epoxyketones, cyclic peptides and hybrid molecules; or by their selectivity preferences for HDAC isoforms into pan-inhibitors and isoform selective inhibitors **[54-56]**.

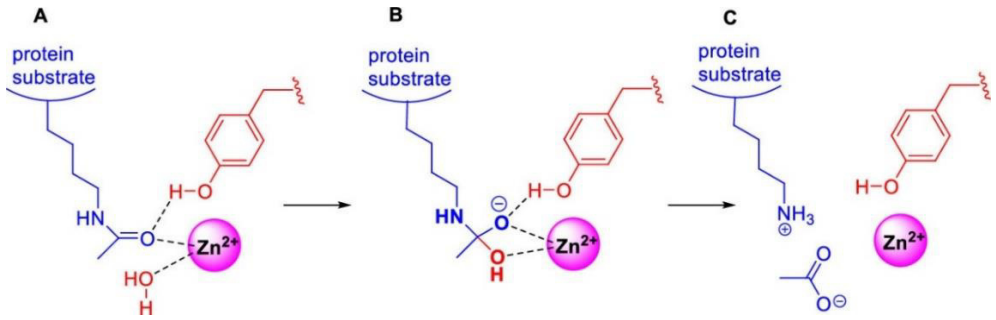


Figure 2 Simplified mechanism of catalysis of deacetylation by classical HDACs. A. The acetyllysine chain fits in the narrow hydrophobic tunnel of the active site. Interaction with a tyrosine residue enables hydrogen bonding with the C=O group; **B.** Tetrahedral oxyanion intermediate formation through nucleophilic attack of the zinc-bound water molecule on the carbonyl carbon; **C.** Breakdown of the intermediate to yield acetate and the deacetylated lysine bearing protein substrate Reprinted with permission from **[57]**. Copyright 2020 American Chemical Society.

Till now, five inhibitors with confirmed HDAC-mediated mode of action have been approved, namely vorinostat (hydroxamic acid), belinostat (hydroxamic acid), romidepsin (cyclic depsipeptide), panobinostat (hydroxamic acid) and tucidinostat (benzamide) (**Figure 3B-C**). While the first four HDAC inhibitors are approved by the U.S. Food and Drug Administration (FDA) for use as anti-cancer agents, tucidinostat has only been approved by China's National Medical Products Administration in 2014 for the treatment of PTCL and in 2019 as part of a combination therapy in postmenopausal advanced breast cancer patients **[58]**.

All currently marketed HDACi and most developed HDACi present various off-target side effects because of the hydroxamic acid group, which coordinates a wide range of transition metal ions including zinc, iron and nickel. Consequently, they are able to interact with off-target metalloproteins **[59-62]**. Also compounds containing hydroxamic acid group and its derivatives were reported to be mutagenic, therefore the therapeutic application of hydroxamic acid based HDACi is limited **[63]**. Moreover, the three approved hydroxamate pan-HDACi vorinostat, panobinostat and belinostat are associated with poor pharmacokinetics and several off-target interactions leading to dose-limiting toxicities **[64-69]**. Hence, continuous efforts are made to introduce novel zinc binding chemical groups that exert potent HDAC inhibition **[53,70,71]**.

Interestingly, Beshore et al. **[52]** were able to develop a new class of HDACs inhibitors lacking the zinc binding motif and exhibiting an efficacy in biochemical and cell-based assays comparable to vorinostat and belinostat. These developed inhibitors

(examples in **Figure 3D**), which do not interact with the zinc ion in the catalytic core domain, showed a selectivity preference for HDACs 1, 2, 3, 10, and 11.

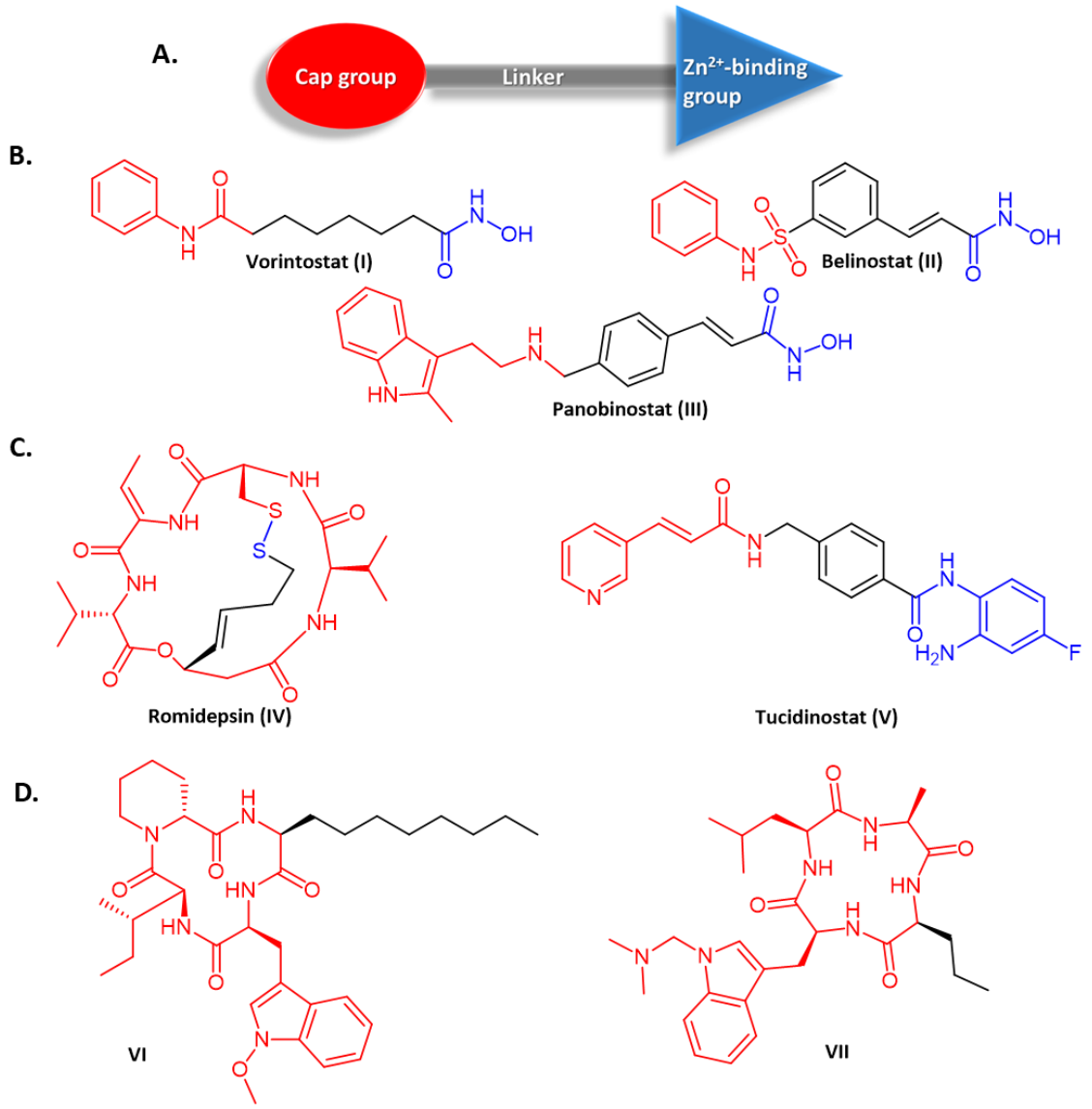


Figure 3 Structures of marketed HDACi in clinical use. A. Schematic representation of the classical pharmacophore model of HDACi. **B.** Structures of the hydroxamic acid approved HDACi showing the different structural elements of the pharmacophore. **C.** Structures of the non-hydroxamic acid approved HDACi showing the different structural elements of the pharmacophore. Structures as per ref. **[58]**. **D.** Examples of novel HDAC inhibitors with enzymatic inhibitory activity that lack a zinc-binding moiety. Structures as per ref. **[52]**. ZBG - blue, linker - black, cap group – red

When used alone in clinical trials, HDAC inhibitors showed low response rate as anti-cancer agents. Therefore, the use of a combination of different chromatin modifying agents such as HDACi with the classical anti-cancer therapy including chemotherapeutic agents, phototherapy or radiotherapy, seems to be a promising treatment strategy against cancer. Another promising approach, especially for hematologic diseases, is the use of a combination of chromatin modifying agents such as DNA-demethylating agents and HDACi. **[72]**.

2.3.1 Targeting histone deacetylase 6 (HDAC6) to control cancer

HDAC6 is a class IIb member that is highly expressed in the heart, liver, kidney and pancreas **[73]**. The distinctiveness of HDAC6 lies in the fact that it is the only isozyme of the HDAC family with two homologous catalytic domains (**Figure 4**) **[73]**. HDAC6 is actively maintained in the cytoplasm due to the presence of the nuclear export signal (NES) and the cytoplasmic retention signal termed SE14 motifs **[74-76]**. Although *in vitro* HDAC6 is capable of deacetylating histones, its predominant cytoplasmic location indicates that this enzyme *in vivo* mainly targets unique cytoplasmic non-histone proteins not related to transcription **[73]**. Studies reported that HDAC6 deacetylates cytoplasmic proteins including α-tubulin **[77-79]**, cortactin **[80]** and Hsp90 **[81]**, thereby playing a role in protein trafficking and degradation, cell shape and migration **[82]**. As a result, aberration in HDAC6 activity and expression is connected with a variety of diseases including cancer **[83-86]**, neurodegenerative diseases **[87-90]**, cardiac diseases **[91-94]** and pathological inflammatory disorders **[95-98]**.

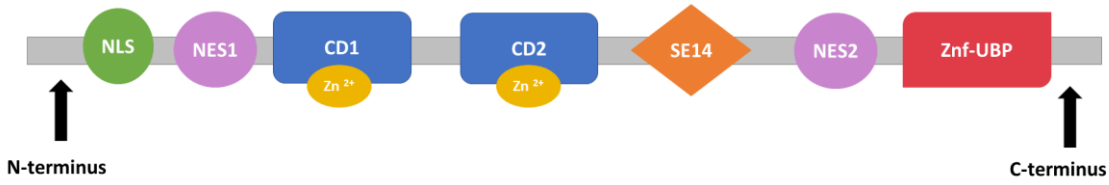


Figure 4 Schematic representation of human HDAC6 functional domains. From the N-terminus to the C-terminus, the protein domains present on this enzyme are: a nuclear localization signal (NLS); nuclear export signal 1 (NES1); two catalytic domains CD1 and CD2; a cytoplasmic retention signal termed SE14; nuclear export signal 2 (NES2) and a zinc finger ubiquitin binding domain (Znf-UBP).

While some studies report that both conserved domains (CD1 and CD2) are catalytically active with different substrate selectivity **[73,99,100]**, other examinations indicate that only CD2 possesses deacetylase activity **[101]**. In addition, some studies demonstrate the absence of domain-domain interactions **[73,101]**, while other research groups' work shows that both domains are necessary for intact activity and any change in the linker region between CD1 and CD2 has a great impact on the catalytic activity of the enzyme **[102]**. In the development of selective HDAC6 inhibitors to act as therapeutic agents for various diseases such as cancer and neurodegenerative diseases, CD2 is the targeted catalytic domain as it is responsible for tubulin deacetylation **[79,103]**.

Moreover, posttranslational modifications such as acetylation and phospohrylation regulate the activity of HDAC6. While acetylation of HDAC6 is associated with decreased tubulin deacetylation consequently reducing cell motility **[75,104]**, phosphorylation of HDAC6 on specific sites by kinases such as glycogen synthase kinase 3β **[105]**, Aurora A **[106]**, G protein-coupled receptor kinase 2 **[107]** and extracellular

signal regulated kinase **[108]**, enhances the deacetylation activity of the enzyme towards its cytoplasmic substrate α-tubulin hence promoting cell migration.

According to the cancer cell subtype, HDAC6 expression may be up- or downregulated. The enzyme was found to be highly expressed in breast cancer **[83]**, advanced primary acute myeloid leukemia (AML) **[84]**, primary oral squamous **[85]** and laryngeal squamous cell carcinoma **[86]**. It was also reported that upon inhibition of the activity of HDAC6 or downregulation of its expression, the invasion and migration of neuroblastoma in some cell lines profoundly decreased **[109,110]**. Additionally, Subramanian et al. **[111]** demonstrated that inhibiting or knocking down HDAC6 in neuroblastoma activates Bax-dependant cell death.

However, HDAC6 is downregulated in human hepatocellular carcinoma (HCC). Ectopic overexpression of HDAC6 lead to the suppression of tumor cell growth and proliferation in different liver cancer cells **[112,113]**.

As HDAC6 is involved in the development of a variety of human diseases especially cancer; specifically targeting it has attracted interest over the past decades. Selective HDAC6 inhibitors as therapeutic agents should help avoid the undesirable off-target effects that can result from the developed pan-HDACi in clinical practice **[114,115]**.

Since, in contrast to the catalytic domain, the surface of the active site rim is not conserved among HDACs, many researchers concentrate at modulating the cap group to design isozyme selective inhibitors with high affinity. Alteration of the ZBG and the linkers has also been employed to achieve inhibitor selectivity.

The hydroxamic acid which constitutes the ZBG in most HDAC6 inhibitors was reported to adopt different zinc-binding modalities in the active site of the enzyme due to its unique three dimensional shape. Porter et. al reported in 2017 that sterically bulky phenylhydoxamate inhibitors interact with the Zn^{2+} ion at the bottom of HDAC6 active site in a unique monodentate binding mode, as they are too bulky to bind more deeply in the active site as needed for bidentate coordination (**Figure 5**). This alternative Zn^{2+}-binding mode that can take place in the HDAC6 active site is disfavored in the active sites of HDACs1-3, resulting in higher selectivity of these bulky aromatic linker containing hydroxamate inhibitors towards HDAC6. Concomitantly, the short aromatic linker prevents the cap group from interacting efficiently with the L1 loop of the enzyme which seems essential for effective binding to HDACs1-3 than to HDAC6 **[116]**.

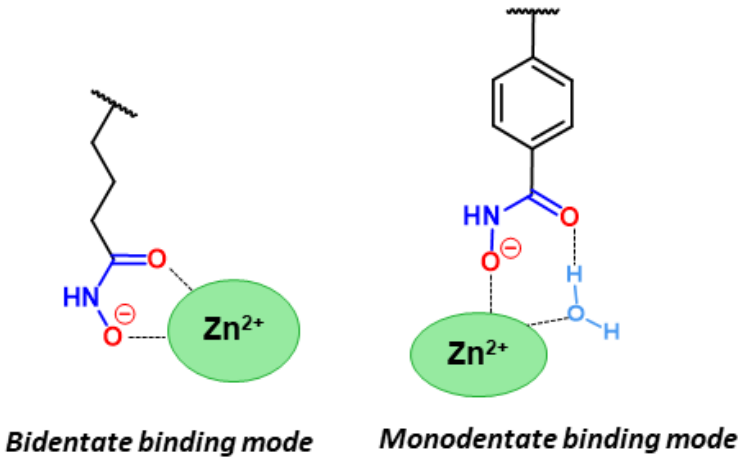


Figure 5 Schematic representation of bidentate (left) and monodentate (right) zinc-binding modes noticed in the coordination complexes of hydroxamate HDAC inhibitors with HDAC6 [116].

Based on these findings, the hydoxamate bearing HDAC6 inhibitors can be subdivided into inhibitors possessing flexible, slender aliphatic linkers such as citarinostat (ACY-241) and ricolinostat (ACY-1215) and inhibitors comprising a rigid bulky aromatic linker such as HPOB, HPB, Nexturastat A and Tubastatin A (**Figure 6**) **[117]**. As shown by Butler et al. **[118]** in the development of Tubastatin A, combining a bulky cap group with an aromatic linker increases the selectivity towards HDAC6. Intrestingly, changing the aromatic linkers to n-pentyl linkers maintained high HDAC6 selectivity over HDAC1, emphasizing the contribution of the nature of cap group to the selectivity towards HDAC6.

To avoid the serious side effects of the hydroxamic acid based HDAC6 inhibitors, researchers try to develop novel non-hydroxamate selective HDAC6 inhibitors to target central nervous system disorders. Lv et al. **[119]** were able to develop indole-based (XIV) and quinoline-based (XV) mercaptoacetamides that potently inhibited HDAC6 (IC_{50} 11.4 and 2.79 nM respectively) with remarkable selectivity against HDAC1. Disulfide prodrugs were also prepared which resulted in a dose-dependant increase in tubulin acetylation in HEK293 cells *in vitro*.

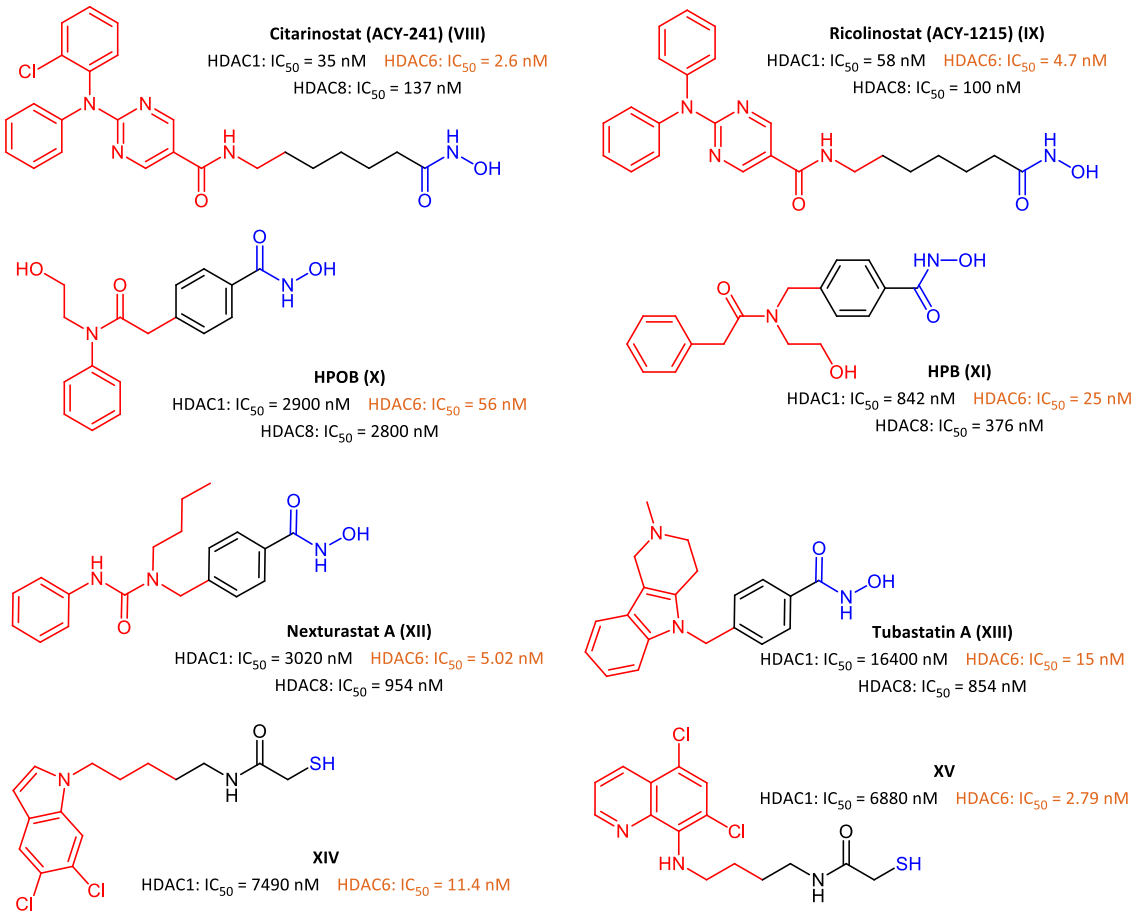


Figure 6 Structures and HDAC inhibition data of selected examples of HDAC6 inhibitors. ZBG - blue, linker - black, cap group – red. Structures as per ref. **[117]**.

2.3.2 Targeting HDAC8 in human diseases

HDAC8, the only isoform linked to the X-chromosome, is a Zn^{2+}-dependant class I member of the HDAC family that is ubiquitously expressed. It is relatively smaller in size than the other members of its class and is primarily localised in the nucleus. Despite being a member of class I, several differences can be noticed between HDAC8 and HDAC1-3. One prominent difference is the inability of HDAC8 to incorporate into multiprotein complexes due to the absence of a protein-binding domain at the C-terminus (**Figure 7**) **[120]**.

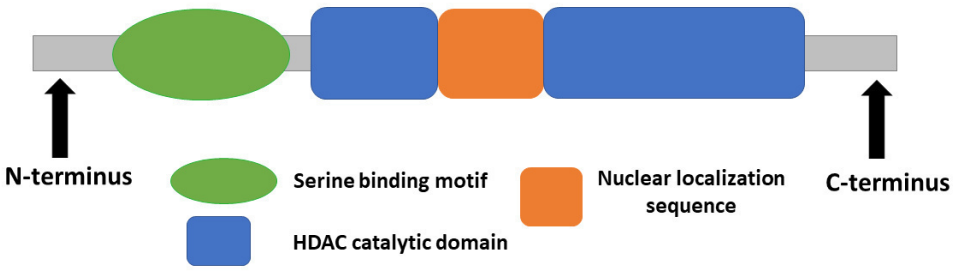


Figure 7 Schematic representation of human HDAC8 functional domains.

In 2018, Marek et al. **[121]** confirmed the presence of a distinct HDAC8-specific pocket formed by the catalytic tyrosine and L1 and L6 loops. Compared to HDAC isozymes 1-3, 6, and 10, the L1 loop in HDAC8 is shorter and more flexible, while the L6 loop is less protrusive. The formed pocket allows the binding of L-shaped molecules that cannot bind in the active site of other isozymes (HDAC 1-3,6,10) due to the steric hindrance resulting from the L1-L6 lock, formed by the interaction of the two loops' residues (**Figure 8**).

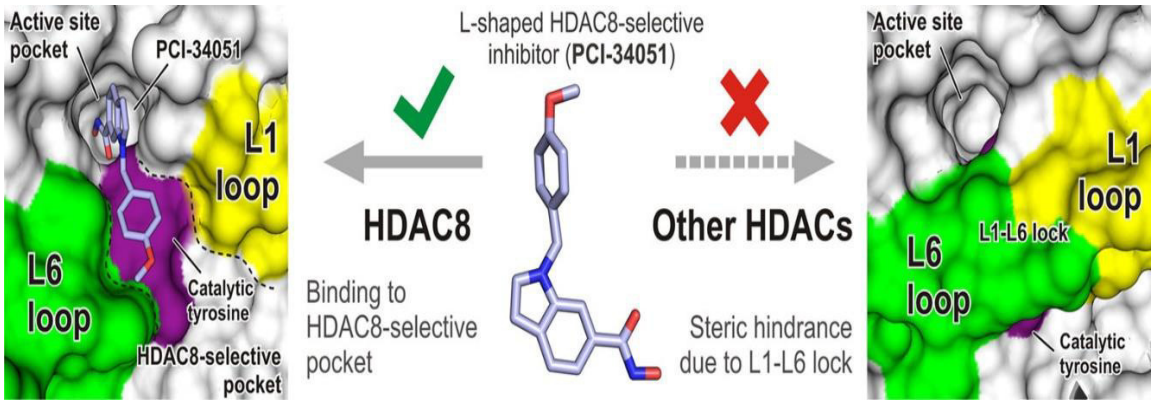


Figure 8 Comparison between the surfaces of the active sites of HDAC8 (left) and other HDACs (right). In the case of HDAC8, the active site pocket accommodates the linker and capping groups of the HDAC8-selective inhibitor PCI-34051, as it adopts a L-shaped conformation to bind to the pocket formed by the catalytic tyrosine (purple) and L1 (yellow) and L6 (green) loops. In HDACs 1-3, 6, and 10, L1 and L6 loop residues interact and form a L1-L6 lock over the pocket preventing L-shaped inhibitors from binding. Reprinted with permission from **[121]**. Copyright 2018 American Chemical Society.

In 2004, Lee et al. **[122]** reported the negative regulation of HDAC8 through posttranslational phosphorylation of Ser39 which is a non-conserved residue among class I HDACs. This modification takes place both *in vivo* and *in vitro* by cAMP-dependent protein kinase A (PKA). The decrease in the deacetylase activity of HDAC8 upon phosphorylation take place by several proposed mechanisms such as possible alteration in enzyme`s confirmation and possible changes in the subcellular localization among other mechanisms **[122,123]**.

As several non-histone proteins such as the structural maintenance of chromosomes 3 (SMC3) subunit of cohesin protein complex **[124]**, retinoic acid induced 1 (RAI1) **[125]**, estrogen-related receptor α (ERRα) **[126]** and p53 **[127]** are targets of HDAC8, the enzyme plays an important role in the regulation of various biological processes. Overexpression or dysregulation of HDAC8 was associated with several cancers and disorders. Wu et al. **[127]** reported that HDAC8 was significantly overexpressed in hepatocellular carcinoma cell lines and tumour tissues. In addition, the enzyme was

reported to be overexpressed in breast cancer **[128]**, gastric cancer **[129]** and adult T-cell leukemia/lymphoma **[130]**.

In their work, Oehme et al. **[131]** reported the role that HDAC8 plays in the regulation of proliferation, clonogenic growth, and neuronal differentiation of neuroblastoma cells. They also showed that the enzyme is downregulated in 4S group, which is a subgroup of metastatic neuroblastoma characterised by increased spontaneous incidence of regression and high survival rate despite metastasis into liver, skin and bone marrow **[131-133]**. Consequently, targeting HDAC8 using selective small-molecule inhibitors is a promising therapeutic approach which can result in the inhibition of cell proliferation and clonogenic growth and in the induction of neuronal differentiation in treated cultured cells **[131,134]**.

To obtain HDAC8 selective inhibitors, researchers tend to optimize the linker and the nature and the position of the cap group in the designed compounds in order to achieve good activity and selectivity through interaction of the cap group with the HDAC8-specific side pocket. Studies show that hydroxamic acid-based L-shaped inhibitors with bulky cyclic aromatic **[135-139]** and nonaromatic linkers **[140]** show obvious isoform selectivity towards HDAC8 or HDAC6/HDAC8 depending on the cap group. On the contrary, slim linkers are able to fit well into the substrate binding tunnel in all studied HDACs therefore are non-selective **[141]**.

Balasubramanian et al. **[136]** developed PCI-34051 (**Figure 9**), a potent HDAC8 inhibitor with an *N*-substituted indolyl-6-hydroxamic acid core. It showed a >200-fold selectivity over HDAC1 and HDAC6 and >1000-fold selectivity over HDAC2, HDAC3 and HDAC10. The inhibitor XVII (**Figure 9**), which is >100-fold selective towards HDAC8 over HDAC1 and 6 with submicromolar IC_{50} value, was rationally designed based on the malleability of the HDAC8 active site and its ability to form a large side pocket allowing bulky, linkerless hydroxamates to access the catalytic Zn^{2+}. Other HDAC isoforms hinder the chelation of the Zn^{2+} by these compounds due to their rigid active sites **[142]**. NCC149 (**Figure 9**) (IC_{50} 0.07 μM) was synthesized using click chemistry. Through the triazole ring, the phenylthiomethyl group and the hydroxamate group are fixed in an orientation allowing favourable interactions with the side pocket and the catalytic Zn^{2+}. It is selective over other class I HDACs (HDAC1>500-fold and HDAC2 >1400-fold), in addition to class IIa (HDAC4>600-fold) and class IIb (HDAC6 34-fold) HDACs **[137]**. Interestingly, substitution of the triazole ring with an inversed triazole or other aromatic rings also generated HDAC8-selective derivatives **[143]**. Other examples of developed selective hydroxamate HDAC8 inhibitors are shown in **Figure 9** (XIX-XXII) and are discussed in details in the following references **[139,144-146]**.

Due to the pharmacokinetic challenges and the side effects of the inhibitors bearing hydroxamic acid group, researchers aim to develop non-hydroxamate selective HDAC8 inhibitors containing structural motifs such as carboxylic acids and β-lactams, with effective inhibitory activity and good selectivity over the other HDAC isozymes.

An example of the non-hydroxamate HDAC8 inhibitors is the amino acid derivative XXIII (**Figure 9**) which showed good activity against HDAC8 (IC_{50} 200 nM) and exhibited good selectivity over HDAC1, HDAC2 and HDAC6 **[147]**.

From the series of β-lactams synthesized by Galletti et al., compound XXIV (**Figure 9**), which possesses an *N*-thiomethyl group, showed the best activity against HDAC8 (IC_{50} 4.53 µM) and good selectivity over HDAC6 (IC_{50} > 1000 µM) **[148]**.

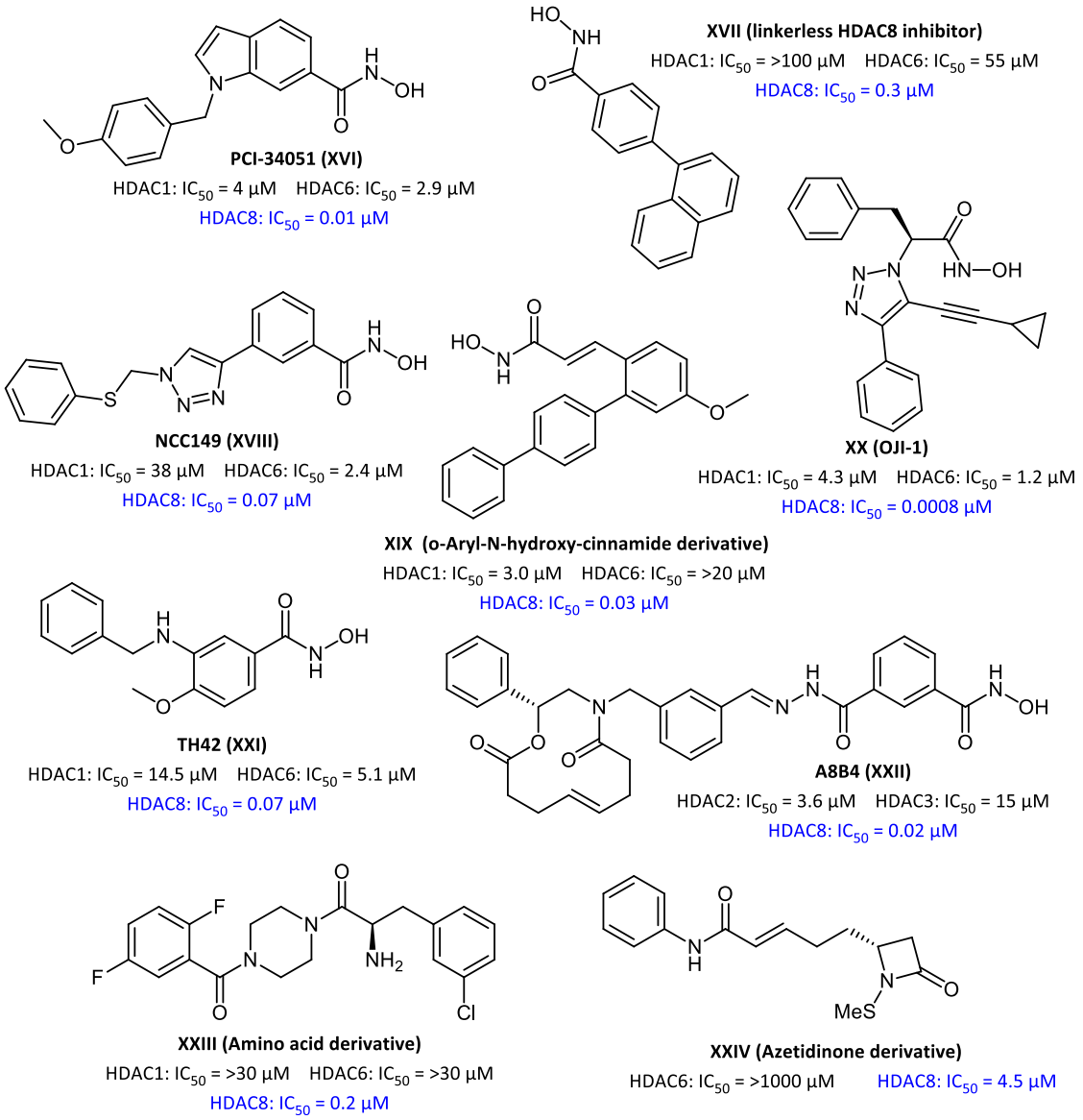


Figure 9 Chemical structures of reported HDAC8 inhibitors.

2.4 Role of histone deacetylases in Schistosomiasis

Schistosomiasis is one of the most important neglected tropical diseases caused by the Schistosoma trematode. *Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium* are the three main disease-causing species of the genus Schistosoma, able to infect humans. While *S. mansoni* is mainly found in South America, Africa, and the Caribbean, *S. japonicum* is present in China, Philippines and Indonesia and *S. haematobium* in Africa and in the Middle East **[149]**.

At present, according to the World Health Organization (WHO), praziquantel is the first line drug used for the treatment of schistosomiasis; especially due to its wide spectrum of activity against the major species infecting humans. Additionally, it is of low cost, causes mild side effects and is orally bioavailable. The extensive metabolism it undergoes in the liver and its low efficiency against the juvenile forms in comparison to the mature parasite forms, represent the main disadvantages of the drug. Till now full clarification of the mechanism of action of praziquantel could not be achieved **[150,151]**.

Oxamniquine is another drug developed to treat schistosomiasis. It is a prodrug that is activated in the parasite via the sulfotransferase mechanism. Although it shares the advantages of praziquantel, being administered orally and possessing mild side effects, its use is limited because of its narrow spectrum of activity, as it is active against *S. mansoni* but not against *S. japonicum* and *S. haematobium*. Due to the occurrence of resistance or tolerance to both drugs, the development of new therapies aiming novel targets with different mechanisms of action is of great interest **[151]**.

Studies and genetic analysis of schistosomes so far suggest the importance of epigenetic mechanisms in the different stages of the parasites' life-cycle. Marked effects on the development and differentiation of the parasite were obtained upon treatment of schistosomes with HDAC inhibitors **[152-154]** and HAT inhibitors **[155]**; therefore schistosomal HDACs and HATs present interesting targets for drug discovery.

However, developing inhibitors for a parasite's epigenetic modifier is very difficult due to several reasons. First, the catalytic domains of these enzymes are evolutionarily conserved, therefore developed inhibitors must exert high selectivity to the parasite's enzymes in order to avoid side effects from interacting with corresponding human orthologs. Secondly, the selected target in the parasite must be crucial to its survival with no mechanism compensating its inhibition. Therefore, target validation and structural studies of the targeted catalytic pocket is important **[156]**.

2.4.1 SmHDAC8 as a promising target in schistosomes

Studies reported that *Schistosoma mansoni* class I HDACs are expressed in all stages of the schistosome's life-cycle **[157]**. Among these, *S. mansoni* HDAC8 (smHDAC8) represents an interesting target for drug discovery of anti-schistosomal drug candidates. On the contrary to normal human tissues where hHDAC8 transcript is much less expressed than those of hHDAC1 and hHDAC3 **[158]**, smHDAC8 shows high levels of expression compared to smHDAC1 and smHDAC3 at all life-cycle stages except schistosomula. This expression pattern points the important functional role of this enzyme in the different stages of the parasite's life cycle **[157]**. The high level of smHDAC8 transcript expression in the schistosome is similar to the noticeable upregulation of hHDAC8 which is found in some cancerous cell lines and tissues **[158]**.

Moreover, characterization and alignment of smHDAC catalytic domains' sequences with their mammalian orthologues' sequences indicate that smHDAC8 possesses a highly conserved catalytic domain compared to its human counterpart.**[157,159]**. As a result of the high degree of similarity between the human and schistosome HDAC8 enzymes, the design of a selective inhibitor against smHDAC8 enzyme is difficult.

Several studies investigated the effects of pan-HDAC inhibitors on the parasite. *In vitro* treatment of *S. mansoni* miracidia with the pan-HDAC inhibitor Trichostatin A (TSA) by Azzi et al. **[152]** resulted in a reversible metamorphosis arrest preventing the transformation of the miracidia into sporocysts. In another study by Dubois et al. **[154]** the three HDAC inhibitors trichostatin A (TSA), valproic acid (VPA) and suberoylanilide (SAHA) were tested on cultured larvae and adult worms. While the three inhibitors inhibited the HDAC activity at all life stages, only TSA and VPA were able to kill the schistosomula and adults. Additional studies are referenced here **[160,161]**.

Multiple attempts to design and synthesize selective smHDAC8 inhibitors that show preference for the parasitic HDAC8 enzyme over the human HDAC isoforms especially hHDAC8 have been reported over the last decades. In 2016, Heimburg et al. **[32]** reported the synthesis of 3-amidobenzohydroxamates (TH65, **Figure 10**) that exhibited significant dose-dependent killing of the schistosomula *in vitro*, in addition to marked decrease in egg laying and separation of the adult worm pairs. These inhibitors were designed as open ring analogues of J1038 (**Figure 10**) **[162]**. Although selectivity towards the human orthologue needs to be improved, these compounds showed high selectivity over hHDAC1 and hHDAC6 **[32]**. The same effect was achieved by other hydroxamate inhibitors developed by Bayer et al. (XXVIII and XXIX, **Figure 10**) **[163]** based on J1075 (**Figure 10**), which is a micromolar hit discovered by a target-based virtual screening campaign using a homology model of smHDAC8 **[162]**. Further work to obtain potential scaffolds of hydroxamate based inhibitors of smHDAC8 (XXX-XXXIII, **Figure 10**) is discussed in the following published reports **[164-167]**.

As the hydroxamic acid-based inhibitors are associated with several pharmacokinetic and pharmacodynamic problems limiting their use, the development of

non-hydroxamic acid inhibitors that show the same effectiveness of hydroxamates is of great interest. Stolfa et al. **[168]** identified a thiol derivative (XXXIV, **Figure 10**) of the hydroxamate inhibitor SAHA. Although it exhibited decreased potency compared to its parent compound, it demonstrated higher selectivity to the smHDAC8 over the human orthologue hHDAC8. The difference in the activity and the selectivity between both inhibitors may be the result of the different binding mode with which the thiol-based compound binds in the catalytic pocket. Additionally, the thiol ester prodrug (XXXV, **Figure 10**) showed antiparasitic activity on cultured schistosomes in a dose-dependent manner.

Through screening a library of class I HDAC inhibitors against the schistosome larval stage, Guidi et al. **[169]** obtained several hit compounds that reduced the viability of both the schistosomula and adult form of *S. mansoni*. From these, SmI-148 and SmI-558 (**Figure 10**), resulted in changes in the reproductive system of the mature female worms and decreased the number of eggs laid *in vitro*.

In another study, a non-hydroxamic acid-based benzothiadiazine dioxide derivative NSC163639 (**Figure 10**) was identified as an smHDAC8 inhibitor with potential activity by means of a virtual screening of the NCI Diversity Set V database. Biological testing indicates that the benzothiadiazine dioxide moiety is a promising scaffold that can be optimized to develop smHDAC8 inhibitors **[170]**.

Applying a new docking based virtual screening protocol to scan a library of 550,000 molecules led to the identification of eight *N*-(2,5-dioxopyrrolidin-3-yl)-*n*-alkylhydroxamate derivatives with low micromolar activity against smHDAC8 *in vitro*. Among the tested compounds J1036 (**Figure 10**) demonstrated the highest inhibitory activity against smHDAC8 **[33]**.

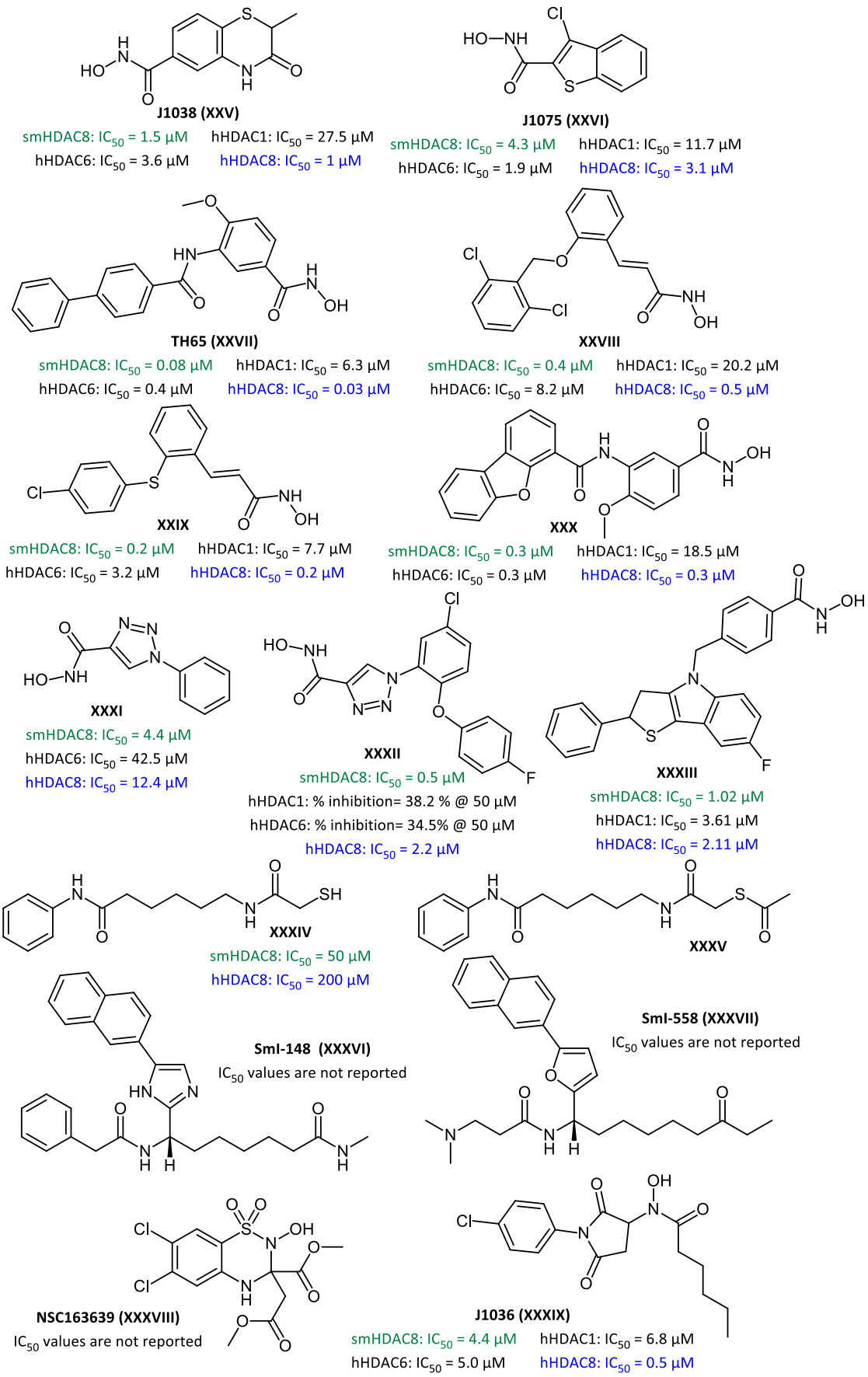


Figure 10 Examples of reported hydroxamic and non-hydroxamic acid derived smHDAC8 inhibitors.

3 Targeted Protein Degradation

The conventional drug discovery strategy aims at the identification of small molecules with high binding affinity to the targeted protein's binding site, thereby regulating its function. This occupancy-driven strategy requires the presence of well-defined active or allosteric site to achieve its goal. In addition, this therapeutic approach is usually associated with high risk of off-target adverse effects resulting from the high systemic drug exposure required to accomplish sufficient site occupancy *in vivo*. Also, resistance to these therapies very likely develop **[171]**.

Contrastingly, the approach of targeted protein degradation (TPD) is based on using small molecules to trigger the degradation of specific proteins through redirecting the endogenous protein degradation machinery towards them thereby reducing their cellular levels. This event-driven strategy offers several advantages over the classical occupancy-driven approach. The catalytic mode of action of non-covalent degraders leads to less total drug exposure to achieve therapeutic efficacy. Consequently, less side effects can be expected. Furthermore, degrader molecules can bind to any site on the targeted protein of interest (POI), whether catalytically or non-catalytically, to trigger degradation, in contrast to conventional inhibitors which must stoichiometrically bind to the active site or in some cases an allosteric site to achieve inhibition. As they do not require an active site for binding, degraders can target proteins without enzymatic nor receptor functions, which were considered undruggable by the occupancy-driven approach **[172,173]**. In addition, longer duration of action in comparison to the conventional inhibitors can be achieved by degraders, as regaining of the activity requires the resynthesis of the protein **[171].**

Innate protein degradation in the cell takes place either by the ubiquitin-proteasome system (UPS) or the autophagy-lysosome pathway **[174]**. In this dissertation the degradation by the UPS as a potential therapeutic pathway will be focused on. In this degradation pathway, the proteins are marked with a polyubiquitin chain formed by a repeated sequence of reactions mediated by E1, E2 and E3 enzymes **[175]**. There are multiple possibilities with which the individual ubiquitin units can be linked. According to the ubiquitin code, Lys48 and Lys11 linkages mediate proteasomal degradation by 26S proteasome **[176]**.

E3 ligases, the main component of the ubiquitination cascade, are encoded by over 600 genes in the human genome indicating specificity of the ubiquitination process **[177]**. However, till now only a small number of E3 ligases have been exploited in targeted protein degradation, with Cullin-RING E3 ligases being the main E3 ligases utilized. Nowadays, cereblon (CRBN) and von Hippel Lindau (VHL) which are the substrate recognition subunits of two biologically important Cullin-RING E3 ligase complexes are largely used in TPD, followed by the inhibitor of apoptosis protein (IAP) and the E3 ligase mouse double minute 2 (MDM2) **[177,178]**.

Under the umbrella of the targeted protein degradation strategies based on the UPS, proteolysis targeting chimeras (PROTACs) and molecular glues are the two major

technologies. Furthermore, several PROTAC-based technologies including Hydrophobic tagging (HyT), TF-PROTAC, and dual-PROTAC, have been developed **[179]**. In the following part PROTAC and HyT technologies will be discussed.

3.1 Proteolysis targeting chimeras technology

Proteolysis targeting chimeras (PROTACs) are heterobifunctional molecules composed of a protein targeting warhead, an E3 ligase ligand and a linker. They can bind both the protein targeted for degradation and the E3 ligase simultaneously, allowing posttranslational ubiquitination of the accessible lysine residues on the surface of the targeted protein. Consequently, the marked protein is recognized and degraded by 26S proteasome **[180,181]** (**Figure 11**).

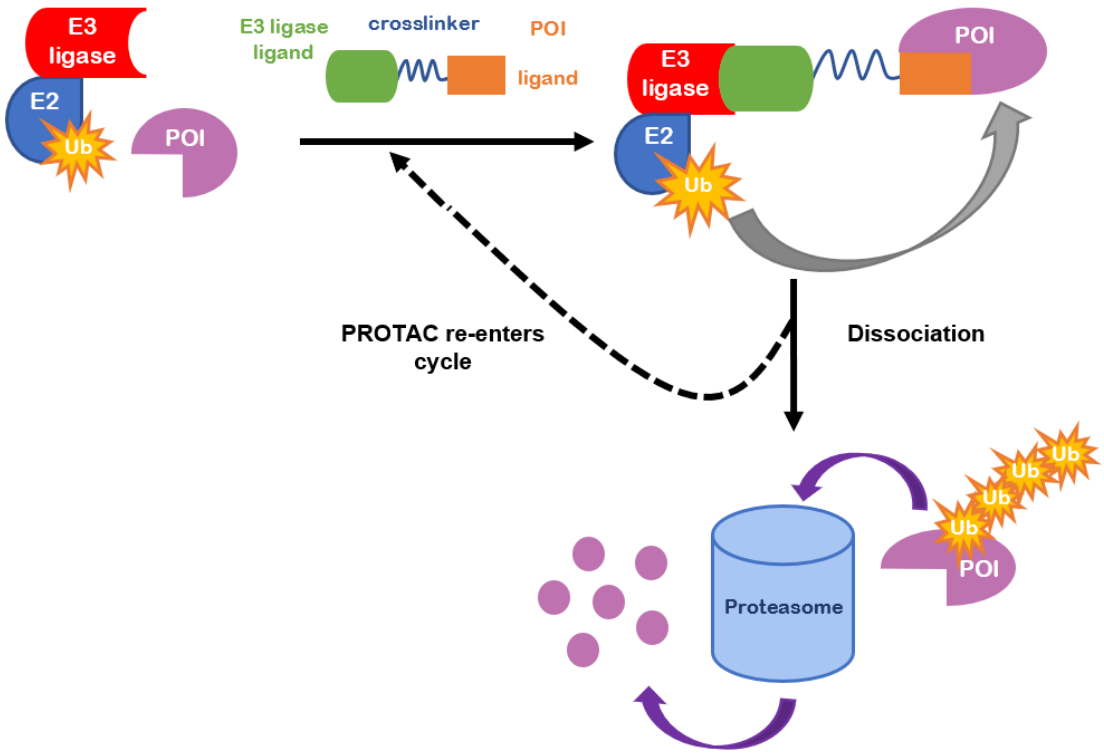


Figure 11 The catalytic mechanism of action of target degradation via PROTACs. The heterobifunctional PROTAC binds to the target protein and the E3 ligase simultaneously leading to the formation of a ternary complex. In the presence of a linker with proper flexibility and length, the two bound biomacromolecules are brought in proper vicinity and alignment allowing posttranslational introduction of a ubiquitin chain as degradation marker on the target protein. Afterwards the labeled protein is recognized and degraded by 26S proteasome.

Early PROTAC molecules included a peptide-based ligand for the E3 ligase. These negatively affected the stability, the drug-likeness and the pharmacokinetics of the developed bifunctional molecules among others **[182,183]**. Great step forward was

achieved upon the development of an all-small molecule PROTAC by Schneekloth et al. **[184]**. Afterwards, several small molecules of E3 ligases were identified **[185,186]**.

PROTACs' development is based on the successful pairing of an E3 ligase recruiting ligand with a POI targeting ligand and linking both with a suitable linker. The E3 ligase to be recruited must be ligandable and available in the tissues and subcellular location of interest. Generally, recruitment of E3 ligases demonstrating tissue-selective expression should lead to tissue-specific degradation of POI **[187,188]**. In addition, it must result in a good degradation profile towards the targeted protein upon incorporation into the PROTAC **[189]**. While the most known E3 ligase ligands including thalidomide-based immunomodulatory ligands, hydroxyproline-based ligands, nutlins and ligands for cIAP bind reversibly to the corresponding E3 ligase **[171,190]**, several small molecules have been reported that employ an irreversible covalent mode of binding to the E3 ligases **[191-193]**. In 2020, Tong et al. reported the first covalent and reversible E3 ligase recruiting ligand. The latter mode of binding promises prolonged engagement of the E3 ligase without permanent modification of the degradation machinery, thereby sustaining the catalytic nature of PROTACs **[194]**.

Although the warhead of the chimeric PROTAC molecules do not have to be functional, most reported PROTACs till now have been developed based on established inhibitors which occupy the active site (examples are discussed in the following references **[195-197]**); whereas only a small number were developed using allosteric inhibitors **[198,199]**. Most reported PROTACs bind to the targeted protein in a reversible noncovalent mode **[200,201]**. Employing covalent POI binders can enhance selectivity and improve the degradation profile **[202]** especially in the case of proteins lacking well-defined pockets or possessing high affinity natural substrates **[203]**. However, they can negatively affect the degradation ability of the chimeric molecule due to the abolishment of the catalytic nature of its mechanism **[204]**. Additionally, off-target toxicity can result from irreversible covalent modification of not targeted biomolecules **[205]**.

Reversible covalent PROTACs theoretically form a compromise between both modes of binding offering enhanced potency, selectivity and sustained duration of action associated with the covalent bond formation, without negating the catalytic mode of binding of PROTACs nor permanent protein modification **[194,206,207]**.

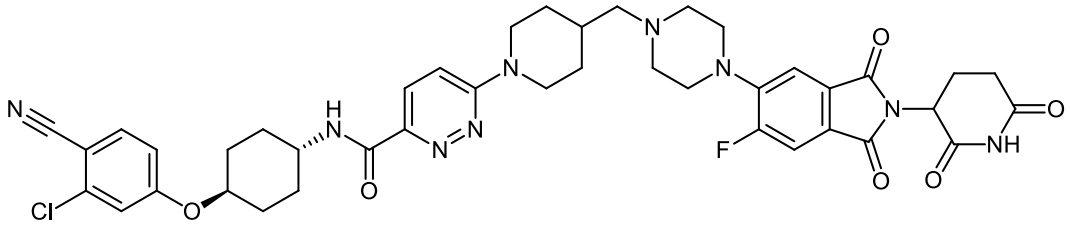
For the polyubiquitination of the POI to take place, the PROTAC molecule must bind to both the POI and the E3 ligase complex simultaneously, and bring them into the proximity and the orientation required for favorable protein-protein interaction (PPI) to take place. This is only possible via a linker of suitable flexibility, length, and chemical composition. This molecular entity is attached to both parts of the chimeric molecule at solvent exposed points, not involved in target binding **[180,208]**. The ternary complex formation is highly affected by the employed linker **[209-211]**.

Because of their polarity and flexibility, polyethylene glycol chains are the most commonly used linker type **[197,201,208,212,213]**, however lipophilic alkyl chains **[197,213,214]** in addition to rigid linkers containing heterocycles **[195,215]** have been used in multiple studies. The following references elucidate the impact of the different

linker characteristics and design strategies on the activity and selectivity of PROTACs **[216-220]**.

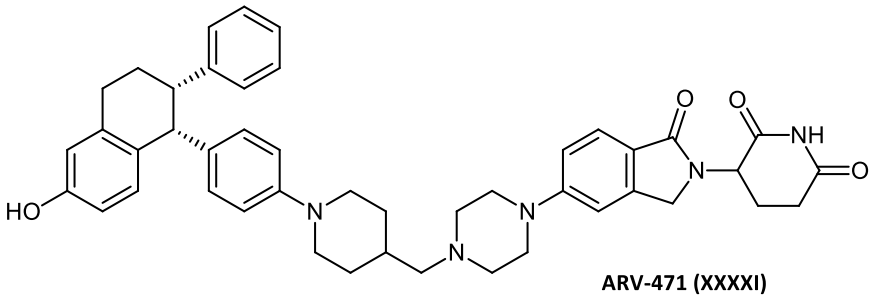
In 2019, ARV-110 and ARV-471 (**Figure 12**) were the first PROTACs to enter in-human clinical trials. ARV-110 is an orally bioavailable heterobifunctional degrader targeting androgen receptors aimed to treat prostate cancer **[221]**. The early reported data demonstrate antitumor activity with acceptable safety and tolerability **[222,223]**.

On the other hand, ARV-471 targets estrogen receptor alpha. This PROTAC is administered orally and should be used in the treatment of breast cancer **[224]**. Like ARV-110 it possesses an acceptable safety profile. As a result of their promising results these two PROTACs are currently in phase 2 clinical trials **[223]**.



ARV-110 (XXXX)

DC_{50} < 1 nM



ARV-471 (XXXXI)

DC_{50}= ~ 2 nM

Figure 12 First PROTACs to enter in-human clinical trials. Structures obtained from https://www.guidetopharmacology.org (last accessed on: 28.8.2022).

3.1.1 PROTACs targeting HDAC6

As previousely stated, HDAC6 regulates protein trafficking and degradation as well as cell shape and migration **[82]** through modulation of several cytoplasmic proteins including α-tubulin **[77-79]**, cortactin **[80]** and Hsp90 **[81]**. Consequently, irregulation in its expression and activity is associated with cancer **[83-86]** and neurodegenerative diseases **[87-90]** among other diseases. Besides exhibiting upregulation in some cancer types such as breast cancer **[83]**, and advanced primary acute myeloid leukemia (AML) **[84]**, its knockdown results in reduction of migration and invasion of some neuroblastoma cell lines **[109,110]**. Hence, HDAC6 degradation is an interesting therapeutic strategy in several diseases.

In 2018, Yang et al. **[225]** reported the development of the first small molecule HDAC6 degraders. They used a non-selective HDAC inhibitor as POI warhead and pomalidomide as E3 ligase ligand. The two entities were connected with linkers of

various lengths. Degrader XXXXII (**Figure 13**) showed higher degradation ability of HDAC6 compared to the other degraders. Its degradation activity was dose-dependant and spared the other tested HDACs 1, 2 and 4. In further work, they seeked the development of dual degraders targeting HDAC6 and ikaros family of zinc fingers (IKZFs), the neo-substrates of immunomodulatory drugs. This concept is based on the knowledge that the simaltaneous use of HDAC6 inhibitors and CRBN ligands results in a synergistic effect and increase the antiproliferation of multiple myeloma (MM) **[226]**. Therefore, they decided to conjugate Nexturastat A **[227]**, a selective HDAC6 inhibitor as the POI targeting ligand and CRBN ligands. Among the synthesized bifunctional molecules, XXXXIII (**Figure 13**) was able to achieve three tasks as proposed; HDAC6 inhibition by Nexturastat A, degradation of IKZFs through the pomalidomide moiety and HDAC6 degradation through ability of the molecule to form a successful ternary complex. IKZF degradation can be considered as an off-target effect for CRBN-based PROTACs **[226]**.

Also using Nexturastat A as the POI ligand and pomalidomide, An and his team developed CRBN-based PROTACs to target HDAC6 **[228]**. From the synthesized bifunctional molecules, NP8 (**Figure 13**) showed the most potent HDAC6-specific degradation without affecting the other HDACs (HDACs 1, 2 and 4) taken into consideration. Additionally, MM.1S multiple myeloma cell line showed the best sensitivity to the degrader compared to other tested cell lines. As continuation of the work, NH2, an analouge of NP8, was synthesized **[229]**. In NH2 the linker was introduced to the benzene ring of Nexturastat A instead of conjugating it to the end of the aliphatic chain of the ligand. The resulted degrader exhibited comparable degradation activity as NP8, therefore proving that different protein-protein interaction interfaces due to different E3 ligase ligand anchoring positions can lead to equivalent degradation activity.

In 2020, the Tang lab reported the development of the first cell-permeable HDAC6 selective bifunctional degraders engaging VHL E3 ubiquitin ligase **[230]**. XXXXVI (**Figure 13**) was the most potent among them. These degraders lacked any known neo-substrates which is an advantage over CRBN-based PROTACs.

Recently, Cao et al. **[231]** reported the development of an CRBN-based HDAC6 degrader with low cytotoxicity and with the capability to attenuate NLRP3 inflammasome activation. The HDAC6 targeting ligand is derived from the natural product indrubin. The degrader XXXXVII (**Figure 13**) selectively decreased HDAC6 expression in several tested cell lines.

All the aforementioned HDAC6 PROTACs contain a hydroxamic acid as zinc-binding group (ZBG). Lately, Keuler et al. **[232]** reported the development of the first non-hydroxamate, selective HDAC6 PROTACs that possess difluoromethyl-1,3,4-oxadiazole warheads as ZBGs (XXXXVIII and XXXXIX**, Figure 13**). Both heterobifunctional molecules potently and selectively degraded HDAC6 with the half-degrading concentrations (DC_{50}) of 131 nM and 171 nM, respectively.

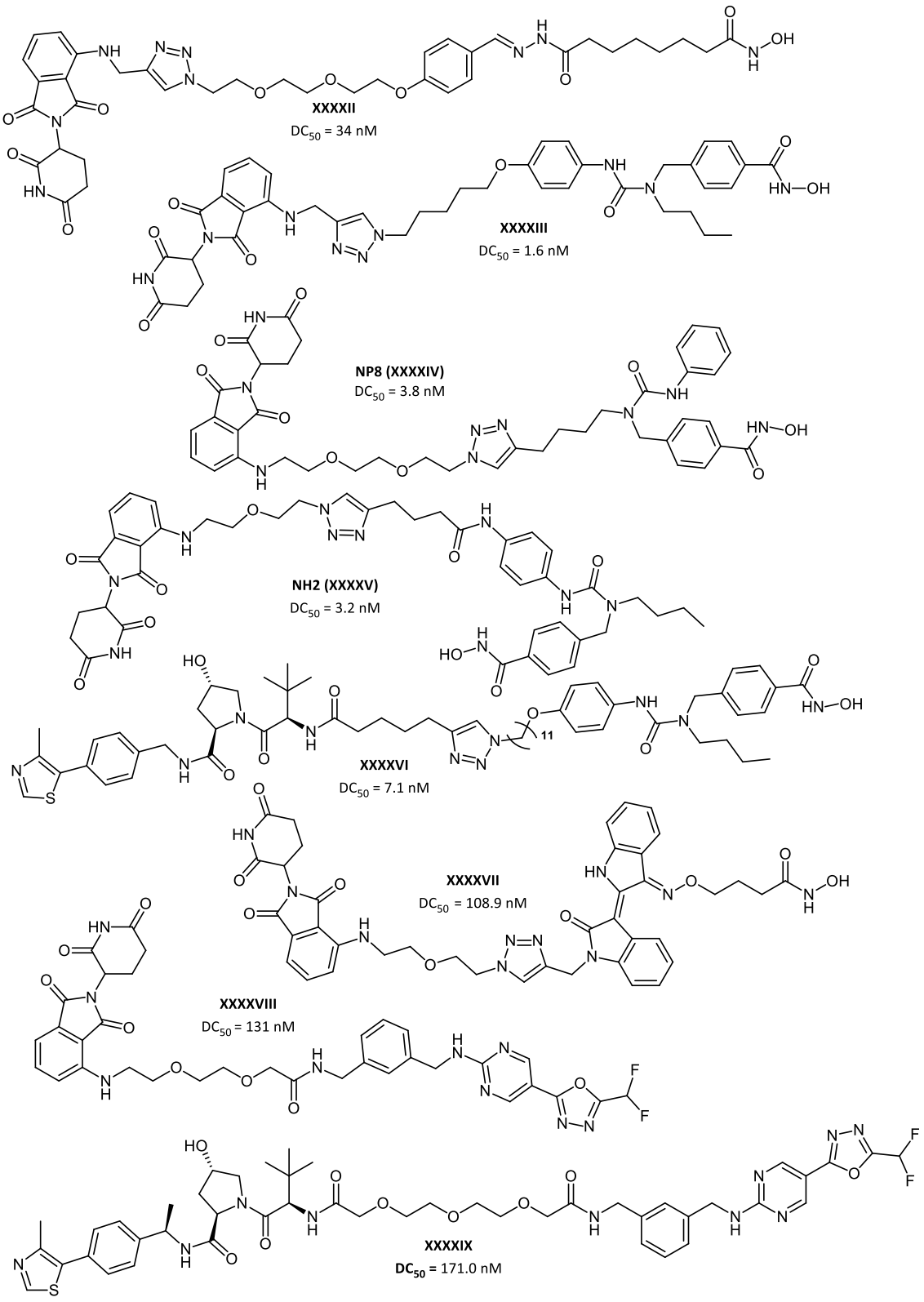


Figure 13 Chemical structures of reported HDAC6 bifunctional degraders.

3.1.2 PROTACs targeting HDAC8

As mentioned before, HDAC8 is involved in multiple cellular processes as a result of having several non-histone proteins such as SMC3 **[124]**, retinoic acid induced1 (RAI1) **[125]**, estrogen-related receptor α (ERRα) **[126]** and p53 **[127]** as biological substrates. Various types of cancer and disorders e.g. hepatocellular carcinoma **[127]**, breast cancer **[128]**, gastric cancer **[129]**, adult T-cell leukemia/lymphoma **[130]** and childhood neuroblastoma **[131]**, demonstrate overexpression or dysregulation of the enzyme. Therefore, degradation of HDAC8 and thereby abolishing all its functions, whether scaffolding or catalytic, is considered a promising therapeutic approach.

Only in 2022, the development of a first-in-class HDAC8 PROTAC was reported. Chotitumnavee et al. **[233]** synthesized three degraders using NCC149 analogue **[143]** as the POI warhead combined with pomalidomide as the E3 ligase ligand through three different lengths of aliphatic linker. Compound XXXXX (**Figure 14**) of the three degraders exhibited efficient degradation of HDAC8 enzyme via PROTAC-mediated UPS in T-cell leukemia Jurkat cells. In addition, it showed stronger inhibition of Jurkat cells growth than its parent HDAC8 inhibitor. The levels of HDACs1,2 and 6 were not affected.

In another recent study, Sun et al. **[234]** developed a series of CRBN-based PROTACs employing an HDAC6/8 dual inhibitor as POI targeting ligand. From the synthesized degraders ZQ-23 (XXXXXI, **Figure 14**) significantly and selectively degraded HDAC8 in HCT-116 cells with DC_{50} of 147 nM. Moreover, this degrader showed no degrading effects on HDAC1 and HDAC3 at all the concentrations tested. However, ZQ-23 was able to degrade HDAC6 at high concentrations (DC_{50} 4.95 μM).

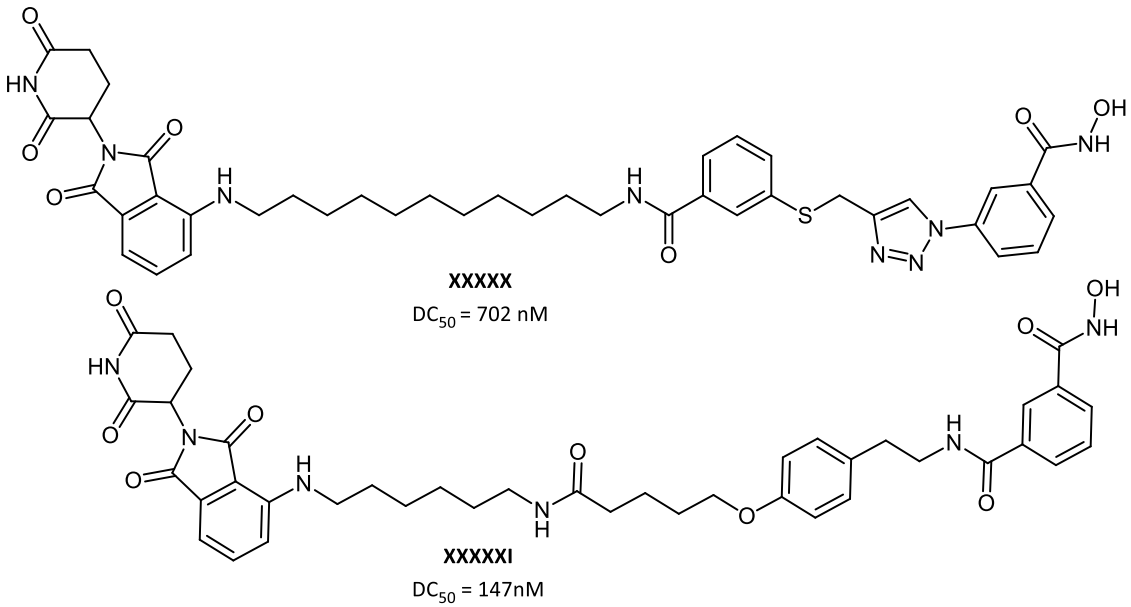


Figure 14 Chemical structures of reported first-in-class HDAC8 PROTACs.

3.2 Hydrophobic tagging (HyT) technology

In eukaryotic cells, the protein degradation machinery recognizes misfolded proteins and degrades them to protect itself from the toxic effects due to their accumulation. Exposed hydrophobic residues seem to be a feature used by the protein quality control system to distinguish the misfolded proteins from their normal correctly folded counterparts **[235,236]**. Based on these findings, Neklesa et al. **[237,238]** proved that covalently attaching a hydrophobic group such as the hydrophobic adamantyl group to the POI can induce its degradation by the cell's quality control machinery. Furthermore, Long et al. **[239]**, demonstrated that non-covalent binding of the hydrophobic tags can also induce proteasomal protein degradation. They also presented tert-butyl carbamate-protected arginine (Boc3Arg) moiety as a hydrophobic fragment able to induce efficient degradation. Generally, the bifunctional hydrophobic tagging molecule is composed of a hydrophobic group and a ligand of the POI linked together through a linker **[171,238,240]**.

Two mechanisms have been proposed through which proteasomal degradation is initiated. In the first one, the hydrophobic tag leads to POI destabilization, resulting in recruitment of chaperones to the misfolded protein followed by proteasomal degradation. This mode has been associated with the adamantane-based hydrophobic tags **[171,238,241]**. However, in the other mechanism of action the hydrophobic mark is directly recognized by chaperones mediating proteasomal degradation of the tagged POI. Boc3Arg was reported as a hydrophobic tag not leading to protein destabilization **[171,242]**.

Different processes for the proteasomal degradation of the hydrophobic tagged proteins have been reported. In one approach, Hsp70 and its co-chaperone mediate ubiquitination of the tagged destabilized POI by the E3 ligase, followed by its degradation by the 26S proteasome **[241,243]**.

On the other hand, when Boc3Arg was employed as the hydrophobic tag, a unique approach was noticed. For Boc3Arg-induced degradation, ubiquitination is not required nor is 26S proteasome involved. This hydrophobic tag seems to lead to direct localization of the tagged POI to 20S proteasome for degradation as a result of direct non-covalent interaction between Boc3Arg and 20S proteasome **[239,242]**.

Over the past decade, several degraders were developed based on the hydrophobic tagging technology. Among them is the first-in-class enhancer of zeste homolog 2 (EZH2) selective degrader MS1943 (**Figure 15**). It was designed by linking a non-covalent inhibitor of targeted protein to the bulky hydrophobic adamantyl group. The developed effective EZH2 degrader exerted intense cytotoxic effect *in vivo* in multiple triple-negative breast cancer cell lines without affecting normal cells **[244]**.

On the other hand, Xie et al. reported the synthesis of the bifunctional hydrophobically tagged degrader TX2-121-1 (**Figure 15**) based on TX1-85-1 which was disclosed as the first selective small molecule Her3 ATP-competitive ligand. The degrader TX2-121-1 covalently modified the targeted epidermal growth factor receptor

tyrosine kinase, leading to its partial degradation and reduction of Her3-dependant signalling **[245]**.

Fulvestrant (ICI 182,780) (**Figure 15**) is the only selective estrogen receptor degrader (SERD) clinically used in the treatment of breast cancer, despite its poor pharmacokinteic properties **[246-249]**. Based on the success of SERDs, development of selective androgen receptor degraders (SARDs) has been attempted. In their work, Gustafson et al. **[243]** explain how small-molecule ligands of androgen receptor can induce its degradation upon linkage to hydrophobic tags (e.g. SARD279, **Figure 15**). Other examples of degraders employing the hydrophobic tagging strategy are discussed in the following references **[239,250,251]**.

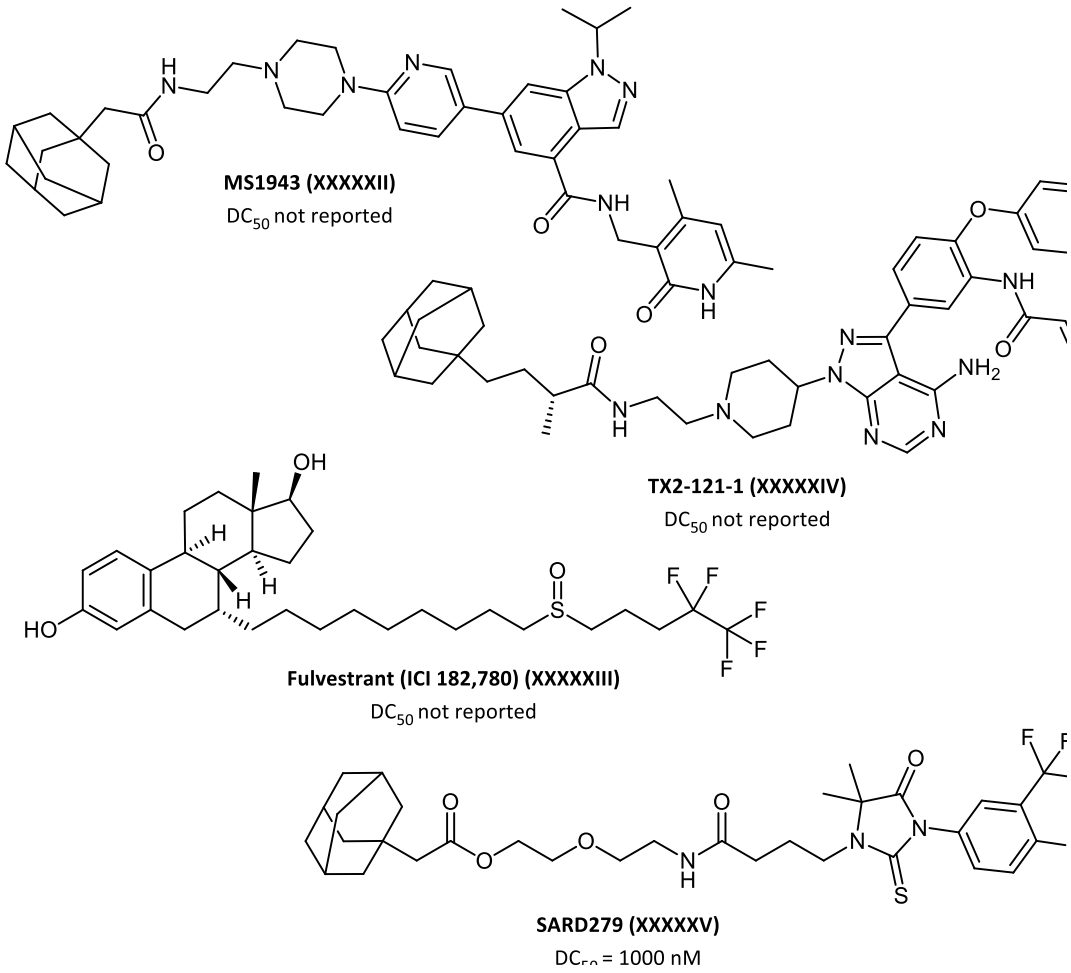


Figure 15 Examples of reported bifunctional hydrophobically tagged molecules.

Chapter II

Aims and Objectives

Among several posttranslational processes, reversible acetylation and deacetylation of histone tails highly influence gene expression. While acetylation is catalyzed by histone acetyltransferases (HATs), the removal of the acetyl mark is catalyzed by histone deacetylases (HDACs). In addition to histones, these opposing enzymes regulate many cellular processes through dynamic acetylation and deacetylation of non-histone proteins such as transcription factors, nuclear import factors and cytoskeletal proteins. Abnormal acetylation/acylation of histones and non-histone proteins has been found to greatly contribute to the development of various diseases **[23,25,252]**. This dissertation comprises two research projects that address the regulation of histone deacetylases as epigenetic targets.

The first project will focus on targeting both human HDAC6 (hHDAC6) and human HDAC8 (hHDAC8) through targeted protein degradation. Both enzymes are involved in the regulation of several cellular processes as each possesses several non-histone proteins as biological substrates **[77-82,124,127]**. Hence, irregulation in their expression and activity is associated with multiple diseases including childhood neuroblastoma. Several reports indicated that inhibition or downregulation of HDAC6 in some neuroblastoma cell lines led to a decrease in the degree of invassivness and migration of the malignant cells **[109,110]**. Other studies indicated that the knockdown of HDAC8 results in the inhibition of cell proliferation and clonogenic growth and in the induction of neuronal differentiation in treated cultured cells **[131]**. Therefore, degradation of both enzymes is considered a promising therapeutic approach to childhood neuroblastoma.

While the conventional drug discovery strategy aims at the identification of small molecules that can bind to the protein's binding site with high affinity instead of its natural substrate thereby modulating the protein of interest (POI), targeted protein degradation (TPD) approach is based on hijacking the cellular protein degradation machinery and directing it to degrade the POI. Nowadays the TPD strategy is of great interest as it offers many advantages over the classical occupancy-driven drug discovery approach including catalytic mode of action (in case of non-covalent degraders), less side effects, longer duration of action and the ability to target the until now undruggable proteome **[171-173]**.

In the work presented in this dissertation two targeted protein degradation technologies will be pursued, namely proteolysis targeting chimeras (PROTACs) and hydrophobic tagging (HyT). Heterobifunctional molecules based on both technologies will be designed and synthesized. As protein targeting warhead, substituted benzhydroxamate-based inhibitors with good inhibition and selectivity profiles towards their respective target (hHDAC6 or hHDAC8) will be utilized. These will be linked to the E3 ligase ligand or the hydrophobic group through a variety of linkers. All synthesized bifunctional molecules will be screened for their inhibitory activity using human recombinant HDACs namely 1, 6 and 8 and an enzymatic assay. Furthermore, selected compounds will be tested in different cell lines to determine their cytotoxicity and their ability to degrade the target enzymes.

On the other hand, the aim of the second project is to design and synthesise novel *Schistosoma mansoni* histone deacetylase 8 (smHDAC8) inhibitors that exhibit

selectivity for smHDAC8 over major human HDAC (hHDAC) isoforms, especially hHDAC1 and hHDAC6. *Schistosoma mansoni* is one of the three main species of the genus Schistosoma, able to infect humans causing schistosomiasis which is a major neglected tropical disease affecting millions **[149]**. At present the control strategy consists of mass treatment with the drug of choice available praziquantel which lacks efficacy against the juvenile stages of the parasite. However, reports on tolerance and resistance to the drug make the development of new multi-stage therapies of great interest **[150,151]**. Studies suggest the importance of epigenetic mechanisms including deacetylation **[152-154]** in the different stages of the complex life cycle of the parasite therefore, schistosomal HDACs present interesting targets for drug discovery. In *S. mansoni, sm*HDAC8 is the most targeted epigenetic regulator as it exhibits high levels of expression compared to smHDAC1 and smHDAC3 at all life-cycle stages except schistosomula, indicating an important functional role in the different stages of the parasite's life cycle.

To identify novel small molecule inhibitors for smHDAC8, the structure of previously reported benzhydroxamate-based smHDAC8 inhibitors will be optimized. This structure-based drug design will be guided by available crystal structures and docking studies on smHDAC8. Also, we are interested in obtaining novel inhibitors exhibiting a better solubility profile than former developed compounds by our group, whose solubility constituted a drawback. The developed compounds will be screened for their inhibitory activity against both schistosomal and human HDAC isoforms. Additionally, their potential to kill cultured larvae and adult worms will be tested. Moreover, the binding modes of a pool of synthesized and tested benzhydroxamate derivatives targeting smHDAC8 will be studied using a variety of computational methods, in order to generate a QSAR model with a reliable predictive ability to predict the activity of future benzhydroxamates towards smHDAC8.

Chapter III

Results and Discussion

The results of the work presented in this thesis are reported in the following scientific manuscripts.

1 Design, Synthesis and Biological Characterization of Histone Deacetylase 8 (HDAC8) Proteolysis Targeting Chimeras (PROTACs) with Anti-Neuroblastoma Activity

Salma Darwish, Ehab Ghazy, Tino Heimburg, Daniel Herp, Patrik Zeyen, Rabia Salem-Altintas, Johannes Ridinger, Dina Robaa, Karin Schmidtkunz, Frank Erdmann, Matthias Schmidt, Christophe Romier, Manfred Jung, Ina Oehme and Wolfgang Sippl

Int. J. Mol. Sci. **2022**, 23, 7535.
https://doi.org/10.3390/ijms23147535

Abstract

In addition to involvement in epigenetic gene regulation, histone deacetylases (HDACs) regulate multiple cellular processes through mediating the activity of non-histone protein substrates. The knockdown of HDAC8 isozyme is associated with the inhibition of cell proliferation and apoptosis enhancement in several cancer cell lines. As shown in several studies, HDAC8 can be considered a potential target in the treatment of cancer forms such as childhood neuroblastoma. The present work describes the development of proteolysis targeting chimeras (PROTACs) of HDAC8 based on substituted benzhydroxamic acids previously reported as potent and selective HDAC8 inhibitors. Within this study, we investigated the HDAC8-degrading profiles of the synthesized PROTACs and their effect on the proliferation of neuroblastoma cells. The combination of *in vitro* screening and cellular testing demonstrated selective HDAC8 PROTACs that show anti-neuroblastoma activity in cells.

2 Synthesis, Biochemical and Cellular Evaluation of HDAC6 targeting proteolysis targeting chimeras

Salma Darwish, Tino Heimburg, Johannes Ridinger, Daniel Herp, Matthias Schmidt, Christophe Romier, Manfred Jung, Ina Oehme, Wolfgang Sippl

The manuscript "Synthesis, Biochemical, and Cellular Evaluation of HDAC6 Targeting Proteolysis Targeting Chimeras", Methods Mol. Biology, in press.

DOI: https://doi.org/10.1007/978-1-0716-2788-4_12

Abstract

Histone deacetylases are considered promising epigenetic targets for chemical protein degradation due to their diverse roles in physiological cellular functions and in the diseased state. Proteolysis-targeting chimeras (PROTACs) are bifunctional molecules that hijack the cell's ubiquitin-proteasome system (UPS). One of the promising targets for this approach is histone deacetylase 6 (HDAC6) which is highly expressed in several types of cancers and is linked to the aggressiveness of tumours. In the present work we describe the synthesis of HDAC6 targeting PROTACs based on previously synthesized benzhydroxamates selectively inhibiting HDAC6 and how to assess their activities in different biochemical *in vitro* assays and in cellular assays. HDAC inhibition was determined using fluorometric assays, while the degradation ability of the PROTACs was assessed using western blot analysis.

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://doi.org/10.1016/j.ejmech.2021.113745

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 of smHDAC8 activity by the highly potent inhibitor 5o. Structure-based 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.

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, 2584.
https://doi.org/10.3390/molecules26092584

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 IC_{50} values. Thus, the generated models can be considered as interesting tools for the identification of novel smHDAC8 inhibitors.

Chapter IV

Summary of the results

1 Design and Synthesis of bifunctional molecules targeting the degradation of HDACs as epigenetic modulators

The development of heterobifunctional degraders has enabled the selective targeting of any potential protein of interest (POI) via degradation even those proteins that were considered undruggable. Generally, PROTAC degraders are heterobifunctional molecules composed of two specific moieties namely the POI targeting ligand and the E3 ubiquitin ligase recruiting moiety, linked together by a flexible linker. According to the mechanism of action, the PROTAC molecule binds to both its targeted E3 ligase and the POI simultaneously forming a ternary complex that will allow polyubiquitination to take place which should lead to selective and rapid degradation of the POI **[180,181]**. In the design of the degraders several factors including the recruited E3 ligase **[171,185-188,190-193,208,253]**, the type and the length of the linker **[208,211,218,219]**, as well as the point of linker attachment on each of the recruiting units **[208,217,220]** influence the selectivity profile of the degrader molecule as they influence the formation of the ternary complex.

From the point of view of the design, the bifunctional molecule employed for hydrophobic tagging (HyT) is similar to PROTACs as it is composed of a hydrophobic group and a POI ligand linked together through a linker **[171,238,240]**. The hydrophobic label can initiate the proteasomal degradation via different modes **[171,238,241,242]**. This part briefly summarizes the results obtained from studies 1 and 2 in which heterobifunctional molecules were developed to regulate HDAC6 and HDAC8, respectively.

1.1 Design, synthesis and biological characterization of bifunctional degraders targeting histone deacetylase 8

The unique class I zinc-dependent HDAC8 overexpression was significantly correlated with the advanced stage and metastasis of neuroblastoma **[131]**. However, in 4S neuroblastoma cases which are characterized by increased spontaneous incidence of regression and high survival rate despite metastasis into liver, skin and bone marrow, HDAC8 was found to be downregulated. In addition, several studies demonstrated that the knockdown of HDAC8 in cultured neuroblastoma cells resulted in inhibition of proliferation and induction of cell cycle arrest and differentiation **[131-134]**. Therefore, selective HDAC8 degradation represents a promising therapeutic approach in neuroblastoma.

In study 1, we developed bifunctional molecules that were designed to act through PROTAC or HyT technology to achieve selective and potent degradation of HDAC8 in neuroblastoma cells, without affecting the activity of the other HDAC isozymes. Furthermore, the *in vitro* activity of the synthesized compounds against human HDAC enzymes as well as on SK-N-BE(2)-C neuroblastoma cells were determined.

In the developed degraders, the POI targeting ligands were based on previously published HDAC8 inhibitors by our group possessing IC_{50} values in the low nanomolar range **[32,146]**. Based on previous crystallographic studies as well as molecular docking studies **[121]**, the *para*-position of the phenyl capping group was chosen as an appropriate point for the attachment of the linker for the designed bifunctional molecules.

To increase the likelihood of HDAC recruitment to a ligase, two different E3 ligase ligands that are most commonly utilized in degrader development, namely the cereblon ligand pomalidomide which recruits the $CRL4^{CRBN}$ and a VHL ligand that recruits $CRL2^{VHL}$, were chosen. On the other hand, an adamantyl derivative was chosen as the hydrophobic tag in the HyT based degraders. Both binding moieties were linked using a variety of linkers, including PEG- and hydrocarbon-based linkers with varying lengths, in addition to triazole ring-containing linkers. In total sixteen heterobifunctional degraders were synthesized (

Figure 16).

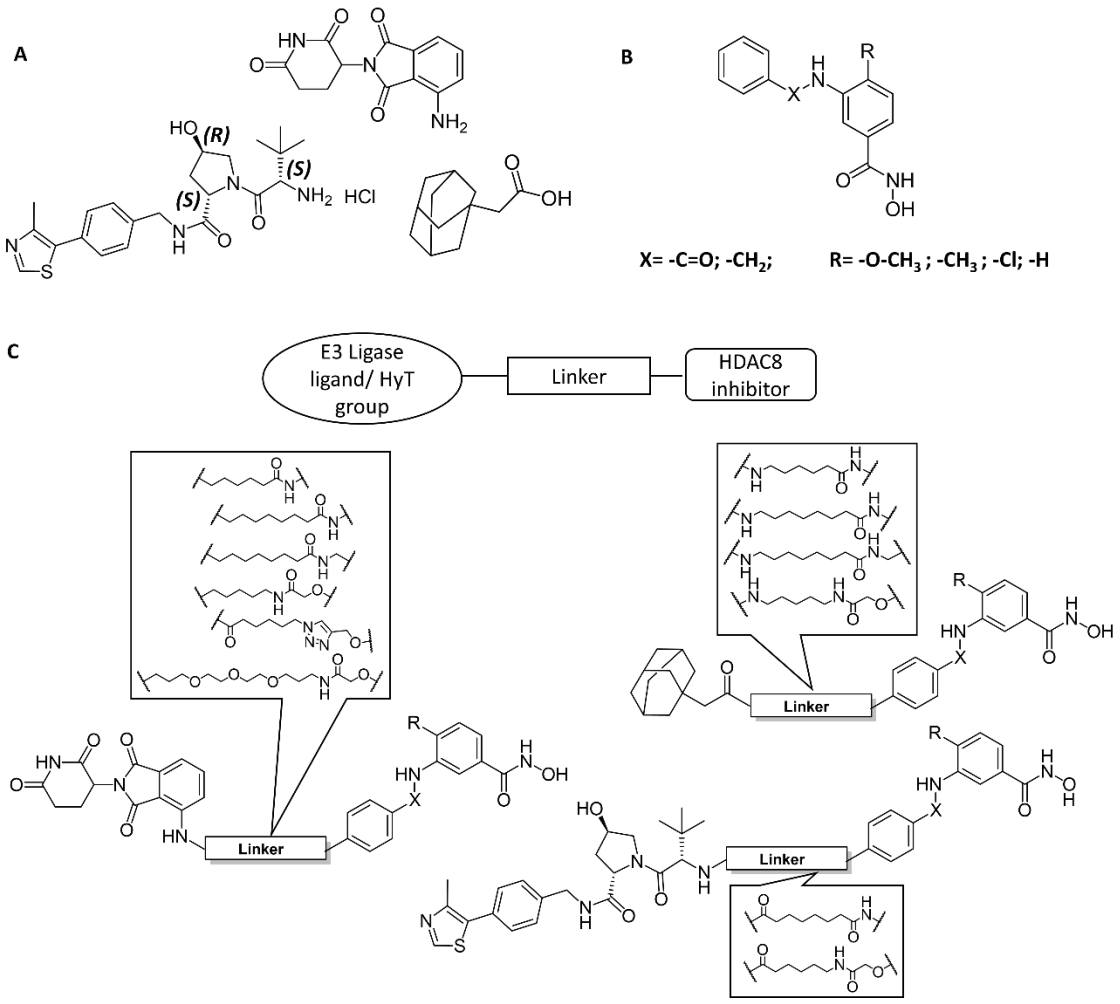


Figure 16 A. Structure of the E3 ligase ligands pomalidomide, and VHL-ligand, and adamantyl derivative as HyT group. **B.** General structure of HDAC8 inhibitors. **C.** Schematic representation of designed HDAC8 degraders.

While the synthesized VHL- and HyT-based PROTACs did not show significant HDAC8 degradation, two CRBN-based PROTACs, **CRBN_1b** and **CRBN_1e**, resulted

in strong HDAC8 degradation connected with hyperacetylation of its substrate SMC3 in SK-N-BE(2)-C cells after 6 h treatment with a concentration of 10 µM (**Figure 17A-B**). Testing of the active PROTACs **CRBN_1b** and **CRBN_1e** against HDAC1 and HDAC6 showed no degradation (at max. concentration of 10 µM) indicating the good selectivity of these PROTACs (**Figure 17C**).

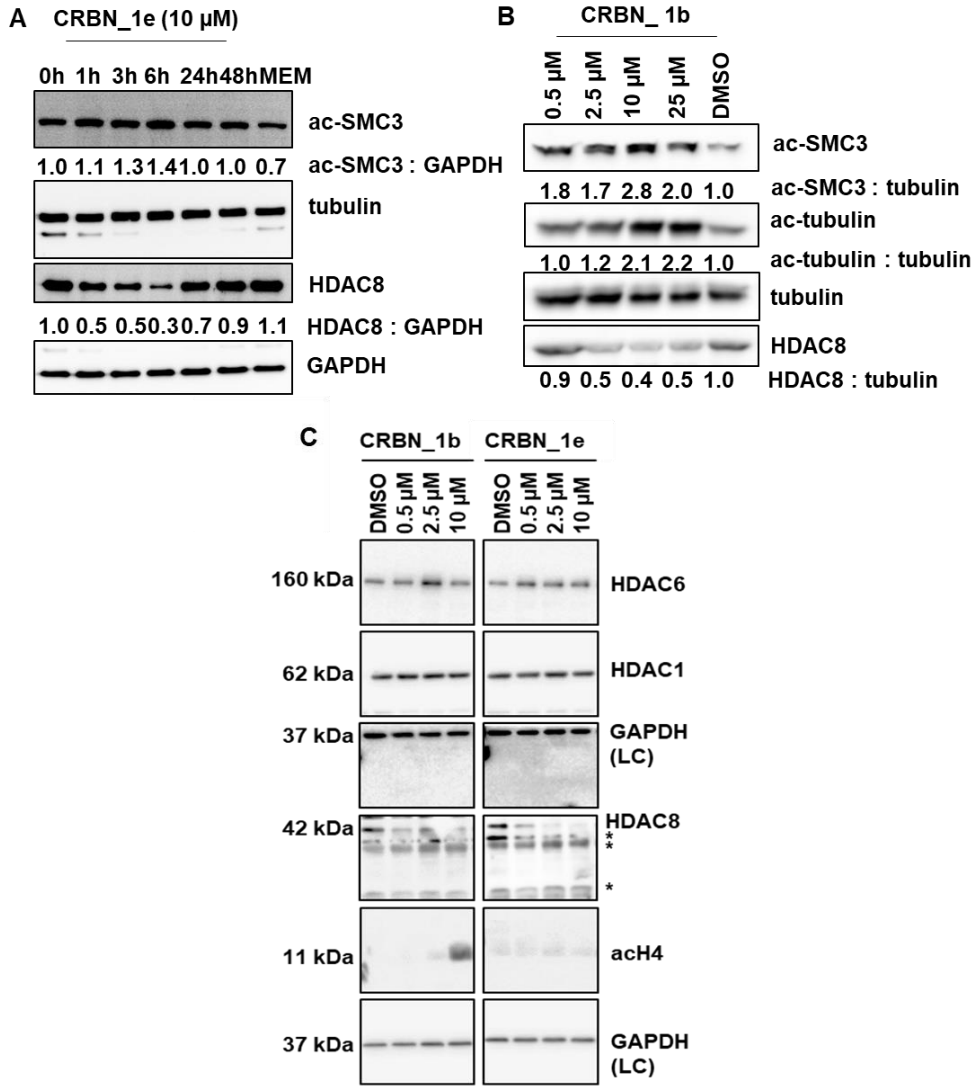


Figure 17 A. SK-N-BE(2)-C neuroblastoma cells were treated for indicated time points with 10 µM of CRBN_1e. Degradation of HDAC8 and acetylation of HDAC8 target was analysed via Western blot. **B.** SK-N-BE(2)-C neuroblastoma cells were treated for 6 h with indicated concentrations of CRBN_1b. Acetylation of HDAC8 target SMC3 and HDAC6 target tubulin, as well as total HDAC8 levels, were assessed by Western blot. Quantified ac-SMC3 or ac-tubulin expression, respectively, was normalized to the respective tubulin loading control and to the solvent control (DMSO). This quantification is reflected by the numbers below each blot. **C.** SK-N-BE(2)-C neuroblastoma cells were treated for 6 h with indicated concentrations of PROTACs CRBN_1b and CRBN_1e. Total HDAC6, total HDAC1, total HDAC8, and acetyl-histone H4 expression levels, were assessed by Western blot. Total GAPDH protein levels served as a loading control (LC). * unspecific bands obtained through reprobing of the membrane.

Signs of neuronal differentiation, such as neurite-like outgrowths in neuroblastoma cells can be induced by HDAC8 inhibition **[134]**. Treatment of SK-N-BE(2)-C cells with **CRBN_1b**, **CRBN_1e**, and PCI-34051 (potent and selective HDAC8 inhibitor) for 6–10 days demonstrated the development of neurite-like outgrowths. For comparison, the cells were treated with the known neuronal differentiation inducer retinoic acid (ATRA) which is a known drug applied for neuroblastoma treatment under some circumstances. A combination of **CRBN_1e** with ATRA enhanced the differentiation phenotype (**Figure 18A**). These results agree with the published differentiation enhancement effect **[134]**.

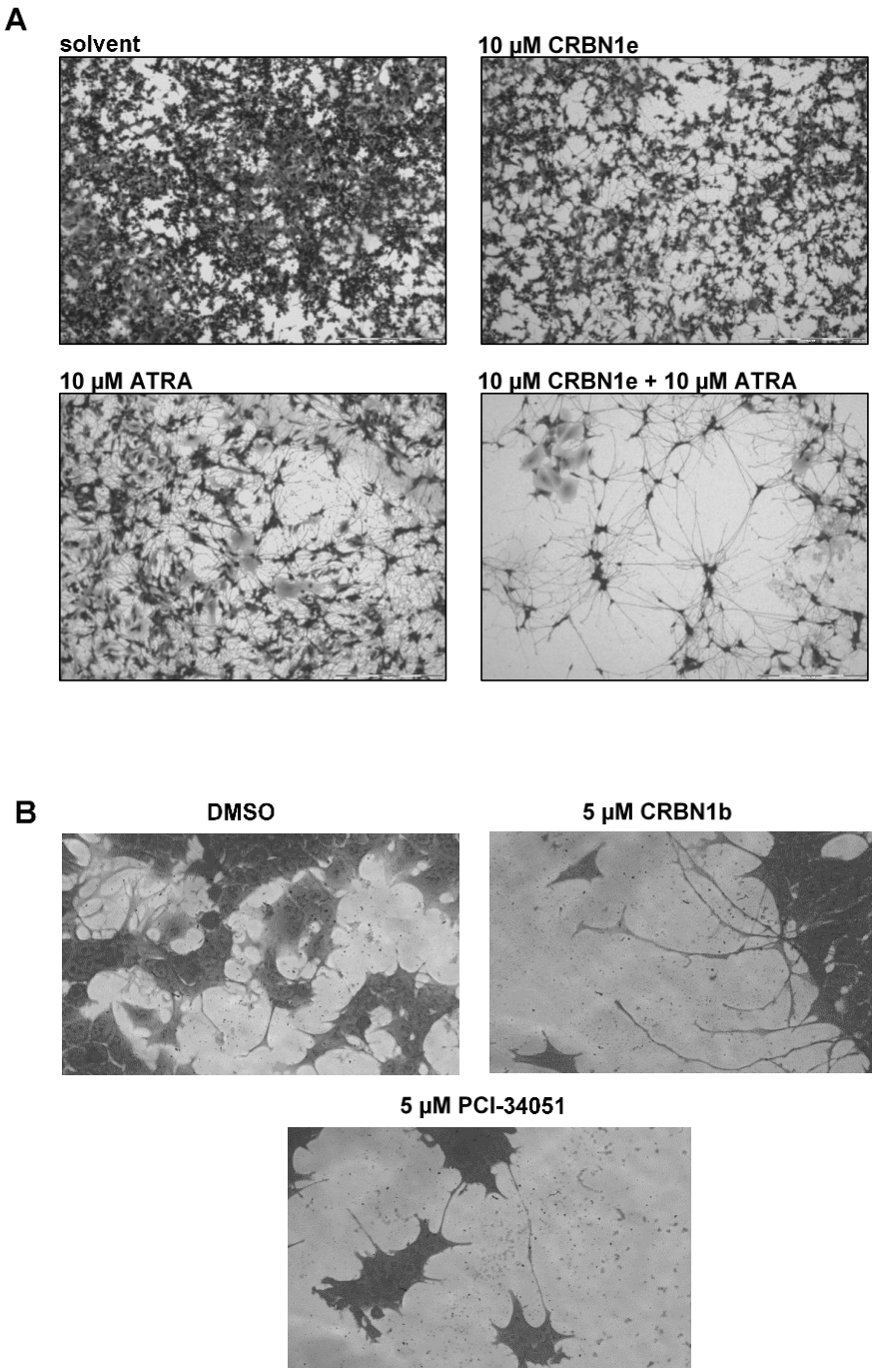


Figure 18 A. SK-N-BE(2)-C cells, treated for 10 days. Scale bar: 500 µm. Stained with crystal violet. **B.** SK-N-BE(2)-C cells, treated for 6 days. Stained with crystal violet.

In conclusion, sixteen degraders were designed and synthesized to target HDAC8. Two of which, **CRBN_1b** and **CRBN_1e**, exhibited degradation towards the POI with good selectivity over HDAC1 and HDAC6. Moreover, they induced neural differentiation. These heterobifunctional molecules showed weak to no cytotoxic effects against human kidney-derived HEK293 cells at the concentration of 50 μM. The developed and validated PROTACs can be used in future studies to analyze the role of HDAC8 knockdown in other cancer cells.

1.2 Design, Synthesis and Biological Evaluation of histone deacetylase 6 targeting degraders

HDAC6 is a member of class IIb HDACs that is highly expressed in the heart, liver, kidney and pancreas **[73]**. Because of its role in protein trafficking and degradation as well as cell shape and migration **[82]**, it is associated with a variety of human diseases including cancer **[83-86]** and neurodegenerative diseases **[87-90]**.

The development of HDAC6 degraders that act as anti-neuroblastoma agents has attracted our interest based on the different reports that demonstrate a decrease in the invasion and migration of some neuroblastoma cell lines upon inhibition or downregulation of HDAC6 **[109,110]**. Moreover, activation of Bax-dependant cell death in neuroblastoma was reported following HDAC6 inhibition **[111]**.

In study 2, we designed and synthesized CRBN-based PROTACs (TH170, SD46, SD64 and SD100) using two HDAC6-selective benzhydroxamate inhibitors, namely TH74 (**Figure 19A**; IC_{50}= 130 nM) and SD100NC (**Figure 19B**; IC_{50}= 140 nM), as HDAC6 recruiting warhead. Furthermore, pomalidomide was chosen as the E3 ligase recruiting ligand. To link the two warheads, hydrocarbon-based linkers with varying lengths and triazole ring-containing linkers were used (**Figure 19**). The negative control compound TH170E was obtained by replacing the essential zinc-binding group (hydroxamic acid) of **TH170** by methyl ester group thereby preventing the binary engagement with the targeted enzyme. After synthesis, the bifunctional molecules were characterized by spectroscopic analyses. All synthesized bifunctional molecules were screened for their inhibitory activity using human recombinant HDACs namely 1, 6 and 8 and an enzymatic assay. Furthermore, selected compounds were tested in different cell lines to determine their cytotoxicity and their ability to degrade the target enzyme.

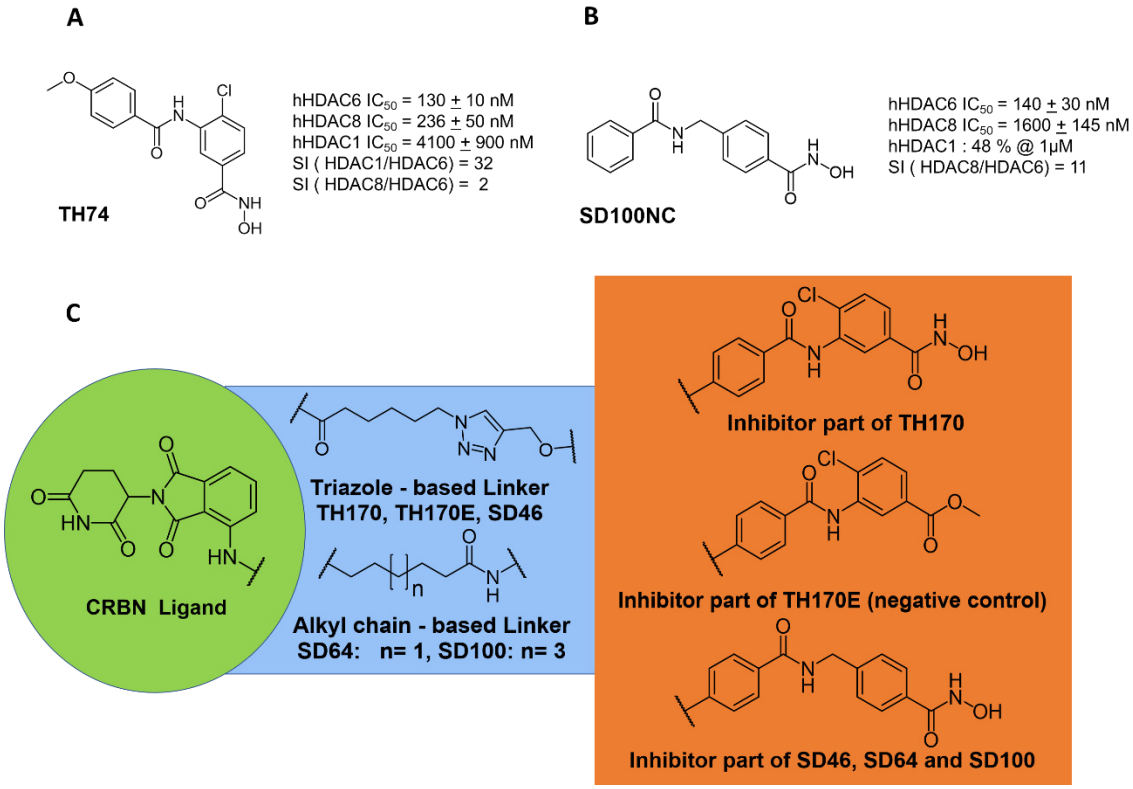


Figure 19 A. and B. TH74 and SD100NC are potent HDAC6 selective inhibitors used for the design of HDAC6 PROTACs. **C.** Synthesized PROTACs based on TH74 and SD100NC.

The obtained *in vitro* testing results of the inhibitory activity showed that TH170 exhibited potent HDAC6 inhibition with good selectivity (**Table 2**). Moreover, the western blot analyses in SK-N-BE(2)-C cells (**Figure 20A-B**) demonstrated that TH170 induced a notable decrease in the HDAC6 protein levels. Additionally, an increase in the level of the acetylated HDAC6 substrate acetyl-tubulin, which remained for 48 h after the start of the test but at a lower rate, was noticed.

However, the other synthesized PROTACs demonstrated weaker degradation effects. SD46 which resembled **TH170** in all features except the HDACi part showed a stronger inhibitory effect on the enzyme but resulted in a weaker degradation effect (**Table 2, Figure 20D**). Similar degradation effect was observed for SD100. While both SD64 and SD100 contain the HDACi part from SD46, changes in the linker type and length were made. However, these changes did not further enhance the degradation effect and in the case of SD64 degradation of HDAC6 was only observed at the highest tested concentration (10 µM) (**Figure 20C-E**). Degradation of the related HDAC10 was not noticed (**Figure 20D-E**).

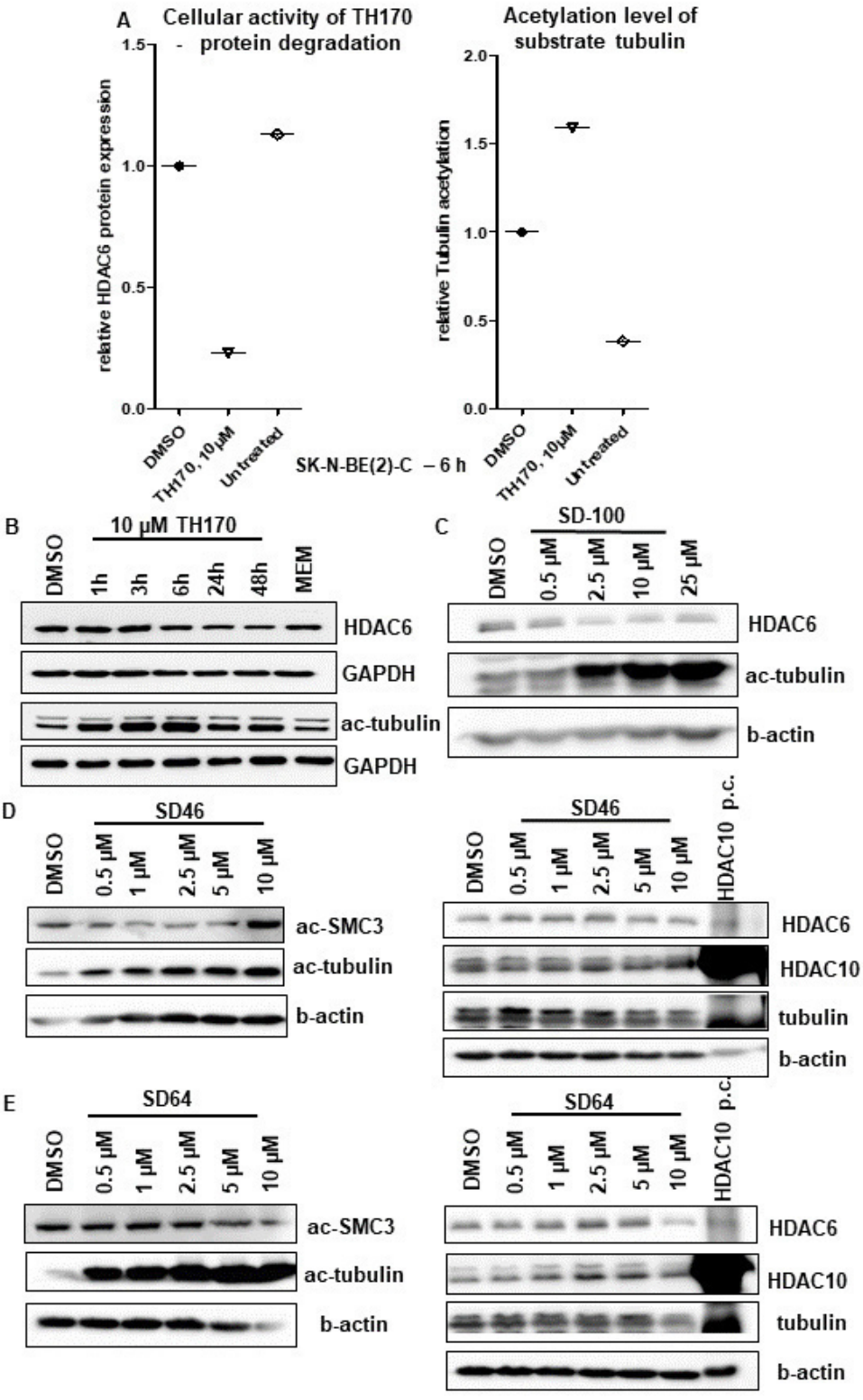


Figure 20 Degradation effect of HDAC PROTACs on HDAC6 and hyperacetylation of tubulin. A. SK-N-BE(2)-C cells were treated for 6 hours with 10 µM TH170 and checked for expression of HDAC6, as well as for acetylation of the HDAC6 target tubulin, via western blot. **B.** Western blot analysis of protein expression after treatment of SK-N-BE(2)-C cells for indicated timepoints with 10 µM HDAC6 PROTAC TH170. **C-E.** Western blot analysis of protein expression after treatment of SK-N-BE(2)-C cells with HDAC6 PROTACs SD100, SD46 and SD64, respectively. SK-N-BE(2)-C were treated for 6h with the indicated concentrations of HDAC6 PROTACs

Table 2 In vitro and cellular evaluation of the inhibitory activity and protein degradation of the HDAC6 PROTACs

ID	hHDAC1 IC_{50} nM	hHDAC6 IC_{50} nM	hHDAC8 IC_{50} nM	SI_1	SI_2	HDAC6 degradation SK-N-BE(2)-C
TH170	**4700 ± 300**	**72 ± 6**	**1050 ± 130**	**65**	**15**	**strong effect**
TH170E	**n.i.**	**n.i.**	**n.i.**	**----**	**----**	**no effect**
SD46	**1640 ± 250**	**37 ± 8**	**990 ± 150**	**44**	**27**	**moderate effect**
SD64	**840 ± 260**	**6 ± 2**	**123 ± 26**	**140**	**21**	**weak effect (highest conc.)**
SD100	**3200 ± 210**	**27 ± 1**	**3500 ± 800**	**119**	**130**	**moderate effect**

SI_1: Selectivity index (HDAC1/HDAC6)
SI_2: Selectivity index (HDAC8/HDAC6)
n.i. no inhibition @ 10 µM

In conclusion, four degraders were synthesized to target HDAC6. One of them, **TH170**, showed strong degradation effect towards HDAC6. The other heterobifunctional molecules showed weaker degradation effects towards the POI. **TH170** exhibited no cytotoxic effects against HEK293 cells at the used concentration of 50 µM. The most potent PROTAC will be used for *in vitro* studies using other cancer cell lines in future work.

2 Design, synthesis and biological evaluation of novel smHDAC8 inhibitors as antischistosomal agents

Schistosomiasis is a major neglected tropical disease affecting millions worldwide and is mainly caused by *Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium* which are the three main species of the genus Schistosoma, able to infect humans **[149]**. At present the applied control strategy consists of mass treatment with the drug of choice praziquantel which lacks efficacy against the juvenile stages of the parasite. However, tolerance and resistance to the drug has been reported **[150,151]**. Therefore, development of new multi-stage therapies is of great interest.

Schistosoma mansoni HDAC8 (*sm*HDAC8) is the most targeted epigenetic regulator in the parasite as it seems to have an important functional role in the different stages of the parasite's life cycle shown by its high levels of expression at all life-cycle stages compared to smHDAC1 and smHDAC3 except schistosomula **[157]**.

In this part, the results obtained in studies 3 and 4 are briefly discussed. Both studies focus on the design, synthesis and biological evaluation of new smHDAC8 inhibitors that can be used as antischistosomal agents.

2.1 Development of novel smHDAC8 inhibitors for the treatment of schistosomiasis

Study 3 is a continuation of previous work in which our group with its collaborators identified and validated smHDAC8 as a potential target for anti-schistosomal agents **[157]** and identified small molecule inhibitors of smHDAC8 **[162]**.Using a combination of virtual screening and *in vitro* testing we have identified a benzhydroxamate template, based on which we can obtain compounds with smHDAC8 inhibitory activity *in vitro* and antischistosomal activity in cellular assays such as the lead compound TH65 **[32]**.

In the current study, we further investigated the structure-activity relationship of benzhydroxamates as inhibitors of smHDAC8 and developed novel benzhydroxamate inhibitors through chemical optimization of the previously reported TH65 (**Figure 21**) which possesses a promising inhibitory profile and is able to induce dose-dependent killing of the schistosomal larvae **[32]**. We used a combination of structure-based design strategies with chemical synthesis and *in vitro* testing against smHDAC8 and other human orthologues.

TH65 possesses a hydrophobic biphenyl capping group that is able to occupy a specific pocket in the HDAC8 enzyme and to interact extensively with the hydrophobic amino acids lining the pocket. As shown in **Figure 21**, derivatives possessing variable capping group including different substituted phenyl, biphenyl, bicyclic and tricyclic rings were designed and synthesized. Furthermore, we synthesized inhibitor molecules with inverted amide as a linker based on previously reported inhibitors showing potent inhibition of smHDAC8 **[146]**. A further focus was the development of more soluble smHDAC8 inhibitors, therefore polar capping groups were used for the synthesis of novel compounds.

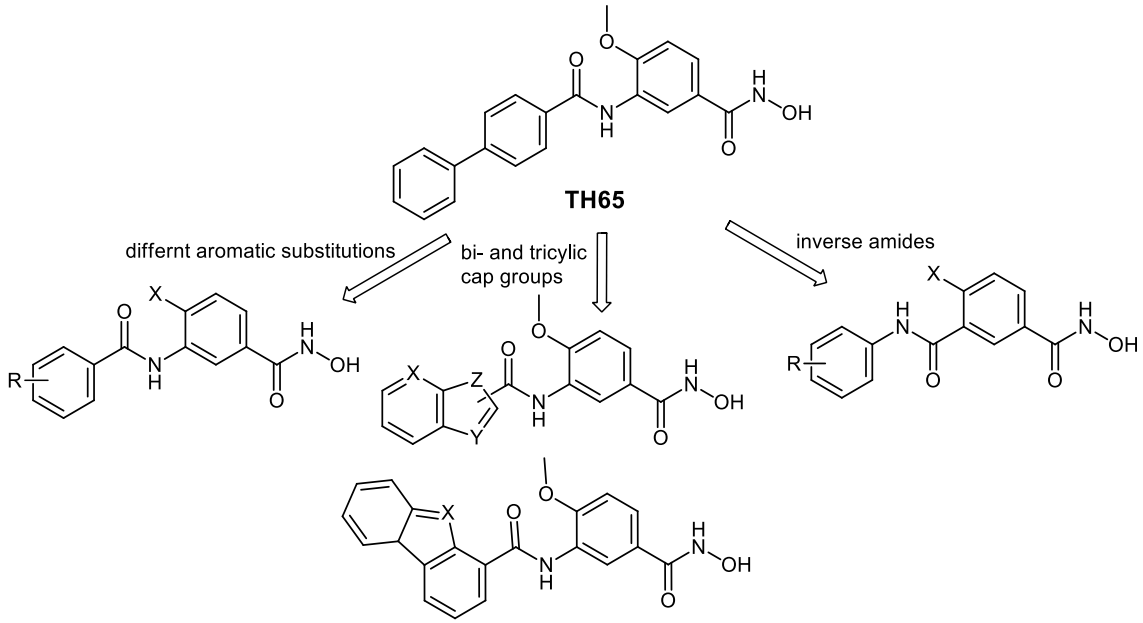
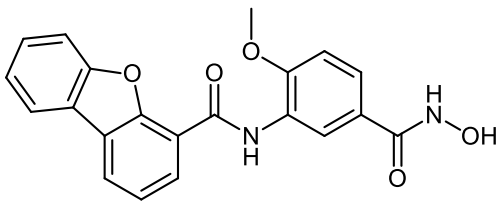


Figure 21 Schematic representation of the employed chemical optimization strategy to obtain novel smHDAC8 inhibitors based on TH65.

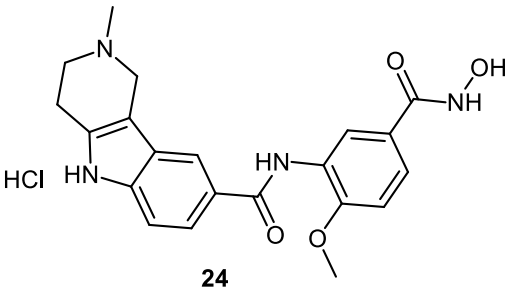
The synthesized compounds were tested against smHDAC8 and human isozymes HDAC1 and 6 in an *in vitro* assay. Generally, the developed derivatives showed nanomolar inhibition of both sm- and hHDAC8. Aside from a few exceptions, they exhibited decreased inhibitory activity towards HDAC1 and HDAC6. In addition, the developed inhibitors demonstrated a good safety profile against human HEK293 cells.

Furthermore, the developed compounds were tested for their toxicity towards the schistosomula using Alamar Blue-based viability assay using *in vitro* cultured parasites, along with the lead compound TH65, the reported HDAC8 selective inhibitor PCI-34051 and praziquantel which is clinically used for the treatment of schistosomiasis. Among the synthesized inhibitors, **5o** (**Figure 22**) showed the most pronounced dose-dependent reductions in the larvae viability, killing almost 98% of the schistosomula. Unfortunately, we were not able to test the compound *in vivo* in mice infected with *S. mansoni* due to its lipophilicity and poor solubility. Therefore, the more soluble analogue **24** was synthesized as a hydrochloride salt (**Figure 22**). Although this derivative exhibited almost the same activity on smHDAC8 as **5o**, it failed to exhibit toxicity towards the schistosomula. Therefore, it can be concluded that a good balance between lipophilicity and water solubility is crucial to obtain inhibitors capable of penetrating the parasite to affect *in vivo* activity.

Moreover, crystal structure of smHDAC8 in complex with **5o** confirmed the results of our docking studies and demonstrated the importance of the hydrophobic interactions between the hydrophobic capping group and the side pocket present in HDAC8.



5o
smHDAC8: IC_{50}= 0.27 µM
hHDAC8: IC_{50}= 0.32 µM
hHDAC1: IC_{50}= 18.51 µM
hHDAC6: IC_{50}= 0.29 µM
EC_{50}-value (schistosomula) : 3.5 µM



24
smHDAC8: IC_{50}= 0.20 µM
hHDAC8: IC_{50}= 0.29 µM
hHDAC1: IC_{50}= n.d.
hHDAC6: n.d.
negligible toxicity towards schistosomula

Figure 22 Comparison between 5o and its analogue 24 concerning the *in vitro* inhibitory activity and the activity on schistosomula.

2.2 Using computational methods to predict the activity of novel benzhydroxamates as smHDAC8 inhibitors

In a previous work **[32]** we identified *m*-substituted benzhydroxamates bearing an amide linker at the *meta*-position as promising lead structures, that upon chemical optimization resulted in the development of potent smHDAC8 inhibitors with good selectivity over the human orthologues HDAC1 and 6. These inhibitors were able to kill the larvae in a dose-dependent manner and impair egg laying of cultured adult worm pairs in cellular assays.

In study 4, we aimed to develop QSAR models that are capable of explaining the differences between the predicted and the experimental smHDAC IC_{50} values of 34 reported inhibitors **[32]** and subsequently, use the developed models to predict and evaluate the activities of newly designed benzhydroxamate derivatives as potential inhibitors for smHDAC8.

At first, docking studies were carried out to establish a docking protocol that can reproduce the binding poses of reported crystal structures and suggest the most probable binding pose for molecules with no crystal structures, based on confirmed reported interactions of the chemical scaffold in the binding site of smHDAC8. However, the Glide-SP docking scores of the selected docking poses failed to deliver a correlation between the predicted activity and the reported experimental activity. Consequently, the binding free energy (BFE) of each docking pose using different GB models was calculated to re-score the docking poses.

Based on the calculated BFEs, 3D-QSAR models were generated. The QSAR model (model 94) was further improved using a 2D descriptor and/or the removal of outliers to give models 95-97. These models were able to correlate between the predicted and the observed activities of the previously reported benzhydroxamates. The best model (model 97) could explain around 73 % of the variations of the reported experimental activity (**Figure 23**). Moreover, the predictive strength of these models (95-97) was validated using a set of newly designed molecules (some compounds in study 3 were part of this set). The predicted biological activities of these compounds agreed with the experimentally determined activities indicating the reliability of the predictive power of the generated models and their capability of suggesting new smHDAC8 inhibitors.

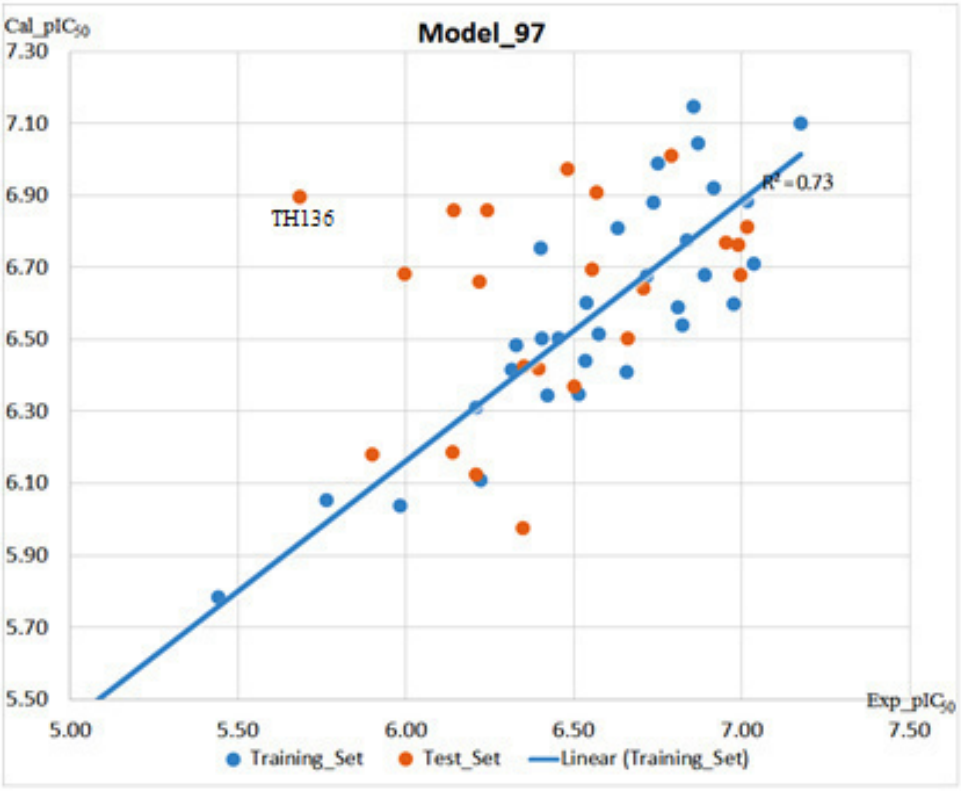


Figure 23 Correlation plot between the experimental pIC_{50} values (X-axis) and the calculated pIC_{50} values (Y-axis) using model 97 along the linear regression line. Training set molecules (blue points), test set molecules (orange points)

Chapter V

General Conclusion and Perspectives

Over decades of studies, accumulating evidence indicated that dysregulation of posttranslational modifications of histones and non-histone proteins is linked to the development of several diseases, especially cancer. Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are important epigenetic modulators that regulate the dynamic processes of acetylation and deacetylation of various proteins at lysine residues, thereby influencing a broad range of physiological processes. Therefore, HDACs are considered promising targets for therapeutic interventions in cancer and other diseases including human parasitic diseases.

In the first project, we wanted to develop heterobifunctional degraders that can effectively and selectively degrade human HDAC6 (hHDAC6) and human HDAC8 (hHDAC8) without affecting the other major HDAC isoforms. In total, four CRBN-based PROTACs targeting HDAC6 were developed based on two HDAC6-selective benzhydroxamate inhibitors. One of the synthesized degraders showed a strong degradation of HDAC6. On the other hand, a pool of sixteen degraders was designed and synthesized to target HDAC8 based on previously reported benzhydroxamate inhibitors with good activity and selectivity towards HDAC8. Several warheads were used to target the cellular degradation machinery. In addition, a variety of linkers connecting the POI-warhead to the E3 ligase-warhead were exploited. Two of the synthesized heterobifunctional molecules exhibited degradation of HDAC8 with good selectivity over HDAC1 and HDAC6. When selected degraders were tested for their cytotoxicity they exhibited weak to no cytotoxic effects against HEK293 cells at the used concentration of 50 µM.

Although some of the synthesized degraders showed an efficient degradation profile towards the protein of interest, they are not tissue-specific and might affect cells and tissues in a non-selective manner because they exploit E3 ligases with broad expression profiles. Unfortunately, till now the development of novel ligands targeting E3 ligases with restricted tissue distribution is challenging.

Tissue-specific degradation could help minimizing side effects and improve the therapeutic dose range for broad-spectrum proteolysis targeting chimeras (PROTACs) increasing their potential as therapeutic agents. Therefore, in future work, approaches for selective delivery of the efficient degraders presented in this work to diseased cells could be pursued. One example of approaches to increase the intrinsic tissue selectivity is the development of antibody-PROTAC conjugates in which the E3 ligase-directed degrader's activity is caged by an antibody linker which can be hydrolysed following internalization of the conjugate into the cell, releasing the active PROTAC in quantities sufficient to induce potent catalytic protein degradation. Recently, examples employing this strategy to induce enhanced pharmacokinetic properties and tissue selectivity of chimeric protein degraders have been reported **[254,255]**.

The PROTACs synthesized in the presented work, only utilize $CRL4^{CRBN}$ and $CRL2^{VHL}$ ligases to achieve degradation. In coming work, other E3 ligases as c-IAP and MDM2 can be employed to explore whether they will result in the degradation of our targeted proteins.

Generally, flexible alkyl chain or ethylene glycol derived linkers including those containing a triazole moiety are the most common linkers used in PROTACs' development. The linker's optimization process in PROTACs is mainly an empirical process, and the optimal linker's chemical structure and length depends on the used E3 ligase and the protein of interest's ligand. Therefore, in forthcoming work more linker lengths, chemical structures and points of attachment to both E3 ligase ligand and HDAC inhibitor should be tried, to investigate their effect on the degradation profile towards the targeted proteins, as these factors highly affect the formation of the ternary complex.

On the other hand, the degraders based on hydrophobic tagging technology presented in this work uses adamantyl derivatives as the hydrophobic fragment. As a continuation of this work, a tert-butyl carbamate-protected arginine (Boc3Arg) moiety can be employed as an alternative hydrophobic fragment to investigate whether its use will affect the degradation profile of the degraders.

In addition, to improve the selectivity of the degraders targeting HDAC8, the HDAC8 ligand can be modified by introducing HDAC8 foot pocket targeting groups. Due to differences in the foot pockets between class I HDACs 1-3 and HDAC8, selectivity over HDACs 1-3 can be maintained by selecting a suitable foot pocket targeting group. On the other hand, introducing a foot pocket targeting group to the ligand can increase the selectivity over HDAC6 which lacks a foot pocket.

Finally, other new technologies for protein degradation via autophagy-lysosome system can be employed to target hHDAC6 and hHDAC8 such as AUTOphagy-TArgeting Chimera (AUTOTAC) and autophagosome-tethering compound (ATTEC).

In the second project, the identification of novel small molecule inhibitors for *Schistosoma mansoni* histone deacetylase 8 (smHDAC8) was pursued. Through structure-based chemical optimization of previously reported benzhydroxamate-based inhibitors and docking studies, we designed and synthesized several new benzhydroxamates. These were tested for their inhibitory activity against both schistosomal and human HDAC isoforms and for their ability to kill cultured larvae and adult worms. One of the synthesized compounds showed potent inhibitory activity, in addition to dose-dependent decrease in schistosomula viability. The trial to improve the aqueous solubility resulted in the synthesis of a more soluble derivative that maintained the inhibitory activity of its parent compound but exhibited negligible toxicity towards the schistosomula. Moreover, the synthesized pool of compounds was used in the development of a QSAR model with a reliable predictive ability to predict the activity of future benzhydroxamates towards smHDAC8.

The best drug candidate developed during the study lacked the right balance between lipophilicity and water solubility and therefore could not be tested *in vivo* in mice infected with the *S. mansoni*. However, its water-soluble analogue did not possess the same parasiticidal activity. Therefore, further work should be directed towards the development of analogues with the right lipophilicity to allow the absorption of the drug upon administration and its penetration into the parasite, and good water solubility that allows *in vivo* administration.

Furthermore, cooperation with other groups in the pharmaceutical technology field can take place to design nano-scaled carriers such as nanostructured lipid carriers and solid lipid nanoparticles that can incorporate the effective lipophilic drug candidate and improve its bioavailability **[256]**.

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Appendix

Curriculum Vitae

PERSONAL INFORMATION

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Date of birth: 19 / 11 / 1987

|Nationality: Egyptian

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WORK EXPERIENCE

from April 2016 – to January 2018	Assistant Lecturer Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Alexandria University ▪ Preparation and teaching of the practical course of medicinal chemistry and computer aided drug design to fourth year undergraduate students and fifth year Clinical sector - undergraduate students
from April 2012 – to March 2016	Demonstrator and Researcher Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Alexandria University ▪ Preparation and teaching of the practical course of medicinal chemistry and computer aided drug design to fourth year undergraduate students and fifth year Clinical sector - undergraduate students ▪ Synthesis of pyrido[1,2-a]benzimidazoles containing pyridine and 2-pyridone nuclei to be evaluated for their in vitro anticancer activity.

EDUCATION AND TRAINING

January 2018- till present	Ph.D. student in the Medicinal Chemistry group (AG Sippl) Institute of Pharmacy, Martin- Luther University, Halle (Saale) (Germany) ▪ Design and synthesis of novel inhibitors and Degraders of zinc dependent HDACs.
Academic year 2016 / 2017	Studies as preparation for the Ph.D. Faculty of Pharmacy-Alexandria University, Egypt
March 2016	M.Sc. of pharmaceutical sciences in pharmaceutical chemistry Faculty of Pharmacy-Alexandria university, Egypt

	Thesis title: "Design, Synthesis and Biological Investigations of Some 2-Pyridones and Related Derivatives"
June 2011	Bachelor of pharmaceutical sciences (Distinction Honor) Faculty of Pharmacy-Alexandria university, Egypt
May 2006	Deutsches Abitur with grade: 1.4 Deutsche Schule der Borromäerinnen Alexandria

PERSONAL SKILLS

Mother tongue	Arabic
Foreign languages	German and English
German	Native speaker level (Deutsches Abitur)
English	Fluent, IELTS, Academic, overall band score 8.0
Communication skills	▪ Good communication skills with students, colleagues and advisors gained through my experience as demonstrator, assistant lecturer and researcher. Presentation skills (Posters and oral presentations in conferences and seminars)
Job-related skills	▪ Ability to work within a team: o member of a team responsible for practical course for undergraduate students o member of two conferences organizing committees, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Alexandria University, Egypt. ▪ Knowledge of Synthetic medicinal and organic chemistry (gained during masters and Ph.D. research)
Personal development courses	▪ "Science 2.0-How to accelerate your research" International graduate academy (InGrA) - Martin-Luther University of Halle-Wittenberg ▪ "Critical reasoning and logic" International graduate academy (InGrA)- Martin-Luther University of Halle-Wittenberg ▪ "Team communication: key roles in interdisciplinary and intercultural contexts" International graduate academy (InGrA) - Martin-Luther University of Halle-Wittenberg ▪ "Time- and self-management for doctoral canditates" International graduate academy (InGrA) - Martin-Luther University of Halle-Wittenberg ▪ "Rhetorik kompakt" International graduate academy (InGrA) - Martin-Luther University of Halle-Wittenberg ▪ "Design of academic posters" International graduate

academy (InGrA) - Martin-Luther University of Halle-Wittenberg

- "Public speaking and rhetoric" International graduate academy (InGrA)- Martin-Luther University of Halle-Wittenberg
- "Next level - E-poster design" International graduate academy (InGrA)- Martin-Luther University of Halle-Wittenberg

ADDITIONAL INFORMATION

Conferences and training schools

- Third scientific annual conference of pharmaceutical chemistry department, faculty of Pharmacy, Alexandria University, 2014
- Fifth scientific annual conference of pharmaceutical chemistry department, faculty of Pharmacy, Alexandria University, 2016
- BioVisionAlexandria, Bibliotheca Alexandrina, Alexandria, Egypt, 2014
- BioVisionAlexandria, Bibliotheca Alexandrina, Alexandria, Egypt, 2016
- COST Action: CM1406 Training School "From Molecule to Medicine", 2019, Ljubljana, Slovenia
- European School of Medicinal Chemistry (40^{th} Advanced Course of Medicinal Chemistry and "E. Duranti" National Seminar for Ph.D. Students), 2021, Virtual edition
- 3rd Alpine Winter conference on Medicinal and Synthetic Chemistry, 2022, Online event

Seminars

- Prepared and gave a seminar titled "Cancer Drug Resistance: An Overview" - pharmaceutical chemistry department, Faculty of Pharmacy, Alexandria University
- Prepared and gave a seminar titled "HDACs and HDAC Inhibitors: An Overview" - Pharmaceutical Chemistry Department, Faculty of Pharmacy, Alexandria University

List of publications

Publications related to the thesis

Design, Synthesis and Biological Characterization of Histone Deacetylase 8 (HDAC8) Proteolysis Targeting Chimeras (PROTACs) with Anti-Neuroblastoma Activity

Int. J. Mol. Sci. **2022**, 23, 7535.

Synthesis, Biochemical and Cellular Evaluation of HDAC6 targeting proteolysis targeting chimeras

Methods Mol. Biology, **in press**. DOI: https://doi.org/10.1007/978-1-0716-2788-4_12

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

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

Binding Free Energy (BFE) Calculations and Quantitative Structure–Activity Relationship (QSAR) Analysis of Schistosoma mansoni Histone Deacetylase 8 (smHDAC8) Inhibitors

Molecules **2021**, 26, 2584.

Other publications not related to the thesis

Potential Anticancer Agents: Design, Synthesis of New Pyrido[1,2-a]benzimidazoles and Related Derivatives Linked to Alkylating Fragments

Med Chem (Los Angeles) **2018,** 8: 86-095.

Selbstständigkeitserklärung/Declaration of Authorship

Hiermit erkläre ich, dass ich die vorliegende Dissertationsschrift selbständig und ohne fremde Hilfe angefertigt habe. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und habe die aus ihnen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht. Die Arbeit wurde ausschließlich der Naturwissenschaftlichen Fakultät I der Martin-Luther-Universität Halle-Wittenberg vorgelegt und wurde an keiner anderen Universität oder Hochschule im In- oder 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), den 1. September 2022