To the Graduate Council:

I am submitting herewith a thesis written by Rachel Louise Glazener entitled "Determining the Activity of Three HDAC Variants in the Presence of Compounds Containing 1,2,3-and 1,2,4-Triazoles as Zinc Binding Groups." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Chemistry.

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Determining the Activity of Three HDAC Variants in the Presence of Compounds Containing 1,2,3- and 1,2,4-Triazoles as Zinc Binding Groups

A Thesis
Presented for
The Master of Science
Degree
The University of Tennessee, Knoxville

Rachel Louise Glazener
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Dedication

This master's thesis is dedicated to my dog Winson.
Who has loved me unconditionally and inspired me to continue to live life to the fullest.
He truly is the only thing I will *always* be able to count on.



Acknowledgements

I first want to thank my father for guiding me to become more than what I would have ever expected from myself. I would also like to thank my other family members for being supportive of all the decisions I've made in the past years and for the constant strength you have inspired in me.

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Abstract

Histone Deacetylase (HDAC) plays a vital role in cellular processes, for example gene expression, cell growth, and apoptosis. Finding drug candidates to inhibit the over activity of HDACs in cancer is a growing area of interest. Inhibitors, thus far, have three important motifs to be studied: the zinc binding group, a hydrophobic linker, and a cap group. By altering these groups on the inhibitor, not only can activity be increased but also selectivity within the classes of HDACs. We present the design of two novel sets of molecules that contain either a 1,2,3-triazole or 1,2,4-triazole. The 1,2,3-triazoles were synthesized using “click chemistry” with a novel pyridyl triazine catalyst. The 1,2,4-triazoles were synthesized utilizing substitution chemistry. This set of molecules was designed after suberoylanilide hydroxamic acid (SAHA) but replaced the hydroxamate with the triazole as the zinc binding group. The activity of these inhibitors against HDAC 1, HDAC 6, and SIRT 1 were tested using the Biomol *Fluor de Lys* in vitro kits. Though none of the synthesized compounds were strong activators or inhibitors of any of the classes of HDACs, trends were observed that could lead to the design of more potent inhibitors.

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Introduction

Background and Mode of Action of HDACs

The aberrant expression of various genes is crucial to the onset and progression of cancer. There is evidence that, not only can altering the Deoxyribonucleic acid (DNA) sequence directly cause the onset of cancer but also epigenetic changes can affect the cancer genome.[1] These epigenetic changes can be observed by controlling the 'open' and 'closed' states of chromatin, i.e.-chromatin remodeling. Chromatin is a regular array of nucleosomes and DNA linkers.[2-4] Nucleosomes are comprised of a histone octamer with double stranded DNA wrapping around the histones.[5] The histones serve as spindles for the DNA to be ordered and packaged around. They are paired with non-histone proteins and DNA linkers to string the nucleosomes together forming chromatin.[6] The 'open' and 'closed' states of chromatin refer to reversible modification of acetyl groups on the core histones. When the chromatin is 'open', transcription machinery will have access to the DNA, thus enabling the DNA to be read and gene expression to occur.[2,3] In the 'closed' form DNA is no longer accessible, and thus the gene expression is silenced.[3,4,6]

In Eukaryotic cells, these 'open' and 'closed' states are controlled by Histone acetylation or deactylation. Histone acetylation is maintained by histone deacetylases (HDACs) and histone acetyltransferases (HATs).[5] HATs transfer the acetyl group from acetyl-coenzyme A to the ε-amino groups of lysine residues at the N-terminal on core histones.[5,7] HDACs have an opposing role to remove the acetyl groups by hydrolysis

(Figure 1). This equilibrium process accounts for the above discussed gene expression, specifically chromatin remodeling. When HDACs are active this leads to hypoacetylated region of the chromatin, the amino groups are protonated and positively charged lysine can interact electrostatically with the phosphate groups of the DNA.[1,8] This causes the DNA to be bound more tightly to the chromatin and gene silencing will occur. The chromatin is unfolded when the HATs begin to reacetylate the lysine to removing the electrostatic interactions between the amine functionality and the DNA (Figure 2).[6,9]

Post-translational control of chromatin is an emerging area of anti-cancer drug design, focusing on HDAC regulation as a viable form of epigenetic and non-epigenetic control.[6] HDAC inhibition is the central focus over HATs due to the fact that HDACS are more structurally diverse. This leads to a diversity of function and a wider array of promising targets for drug discovery. Current research has shown that HDACs can also form various complexes with additional proteins, allowing for non-epigenetic modifications.

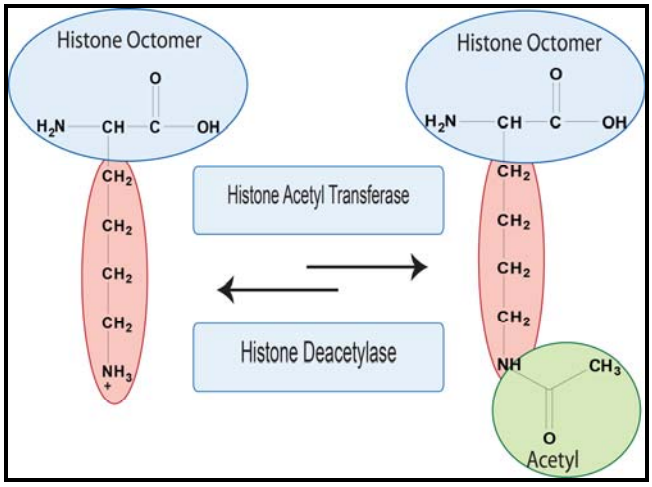


Figure 1:Histone Acetylation and Deacetylation with HAT and HDAC [8]

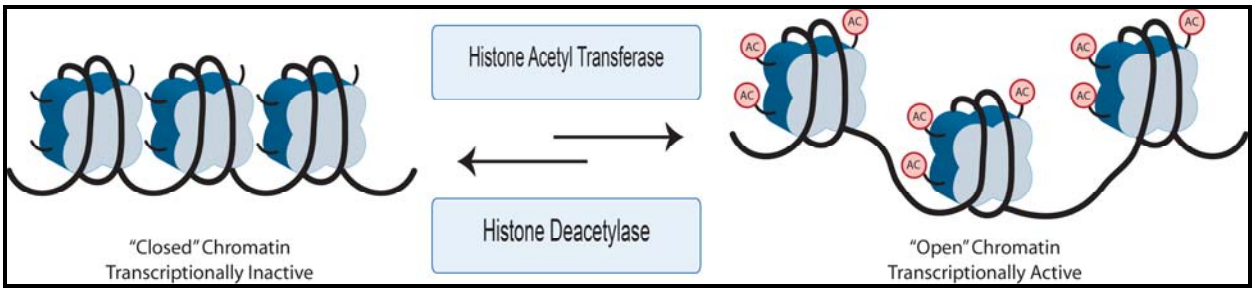


Figure 2: "Closed" vs "Open" chromatin during chromatin remodeling using HAT and HDAC [9,10]

HDAC can modify gene expression not only by altering chromatin but by forming complexes with other proteins and by recruiting other transcription factors. A few examples of HDAC complexes that still involve histone methylation or acetylation are the Sin3 and NuRD (nucleosome remodeling and deacetylating) complexs.[6] Sin3 contains RpAp46/48 with HDAC 1 and 2. The HDACs are recruited to the DNA which leads to gene silencing by altering the DNA methylation/acetylation. A similar complex is formed with NuRD with HDAC1 and HDAC2 and again mediates DNA methylation by recruiting DNA methyltransferase1 (DNMT).[6,11] Examples of non-histone modification include direct complexation with transcription factors such as STAT3, tumor suppressor genes such as p53, and cell cycle regulators such as RB (retinoblastoma protein).[12] STAT3 can be directly acetylated and inactivated by HDAC1. Other coenzymes needed for STAT3 transcription function can also be acetylated by HDAC1 or HDAC3 and cause inactivation.[6,12] Tumor suppressor gene p53 is also deacetylated by HDAC1, HADC2 and/or HDAC3. P53 plays an important role in cell proliferation and gene transcription by relying on its ability to bind DNA with a sequence specificity to activate transcription. The down-regulation of the p53 activity is very dependent on which region

of p53 is acetylated and is HDAC dose dependent.[13] RB also interacts with HADC similarly to p53, by interacting with RB and other proteins that complex with RB that can directly affect chromatin remodeling.[6,14] There are several ways, epigenetic and non-epigenetic, HDACs can alter gene transcription leading to cancer regulation. But the primary challenge in targeting HDACs is finding inhibitors that can have HDAC specificity amongst the various classes.

Classes and Functions of HDACs

There are four major classes of HDACs, class I, IIa and IIb, III, and IV. Within the four classes are 18 isozymes.[3,14] Class I HDACs include HDAC1, HDAC2, HDAC3, HDAC8. Class I HDACs are generally localized in the nucleus and are related to the Rpd3 gene product.[10] Class II HDACs can be split into two sub-classes: IIa which includes HDAC4, HDAC5, HDAC7 and HDAC9 and sub-class IIb which includes HDAC6 and HDAC10. Class IIa has a conserved C-terminal catalytic domain similar to the HDAC1 yeast protein but the N-terminal domain has no similarity to HDACs in other classes. Class IIb has an extra acetylase domain when compared to the HDAC1 yeast protein. Class II HDACs primarily localize in the cytoplasm but can migrate from the nucleus to the cytoplasm.[6,14] Class III HDACs are the sirtuin family, these include SIRT1 through SIRT7. Class IV HDACs include HDAC 11 and are located in the cytoplasm and nucleus but not known to shuttle between the two like class II HDACs.[14] HDAC class IV is the newest class of HDACs and has features of both class I and II HDACs.[2] The various HDAC classes also exhibit different functions as well as different localities within the cell. (Table 1). [8,10,14]

Table 1: HDACs expression sites and functions in cancer [2,9,15,16]

HDAC Class	HDAC Family	Expression Site	Function in Cancer
HDAC CLASS I	HDAC1	Nucleus	Inhibition of proliferation, induction of autophagy, cell cycle arrest, apoptosis
	HDAC2	Nucleus	Inhibition of proliferation, apoptosis, growth arrest, differentiation
	HDAC3	Nucleus/ cytoplasm	Induces differentiation and disrupts cell cycle.
	HDAC8	Nucleus	Induces, differentiation, cell cycle arest and reduces proliferation
HDAC CLASS IIA	HDAC4	Nucleus/ cytoplasm	Represses differentiation genes, inhibits some gene expression
	HDAC5	Nucleus/ cytoplasm	Shuttles between nucleus and cytoplasm during differentiation
	HDAC7	Nucleus/ cytoplasm	Inhibition changes morphology and migration ability
	HDAC9	Nucleus/ cytoplasm	no data
HDAC CLASS IIB	HDAC6	Nucleus/ cytoplasm	Inhibition leads to depletion of pro-growth chaperones, involved in several cancer regulating proteins
	HDAC10	Nucleus/ cytoplasm	Inhibition leads to down regulating of a few cancer regulating proteins
HDAC CLASS III	SIRT1 to 7	Nucleus/ cytoplasm/ mitochondria	Inhibition disrupts the cell cycle, gene expression, and metabolism
HDAC CLASS IV	HDAC11	Nucleus/ cytoplasm	No Data

HDAC class III isoforms are not related to class I and II in structure or function. HDAC class I and II both contain zinc containing amide hydrolases and are zinc dependent in their function. HDAC class III relies on NAD^+ dependent amide hydrolases and are zinc independent. Of the four HDAC classes, class I is the primary anti-cancer drug target because it not only has epigenetic features but important non-epigenetic control abilities as well. Examples include degrading the tumor suppressor gene p53, which is active in apoptosis, DNA repair and overall cell cycle control.[2,14]

Types of HDAC Inhibitors

HDAC inhibitors have various anti-cancer functions and work on several different forms of cancer. Because of their broad spectrum of function, HDAC class I and II

inhibitors have been subdivided into four different classes: hydroxamates, aliphatic acids, benzamides and cyclic peptides.[17] There are a few molecules in particular that have been highly studied in each class of inhibitors (Figure 2). Trichostatin A (TSA) and Suberoyl anilide hydroxamic acid (SAHA, also known as vorinostat) are known HDAC inhibitors in the hydroxamate family.[3] SAHA is the first HDAC inhibitor to be approved for clinical treatment.[15] TSA shows reduction in tumor weight but it is not selective enough to be approved by the FDA for clinical trials. SAHA on the other hand showed a reduction in tumor weight, had a low abnormality in biochemical function rate, has cancer selectivity (for T cell lymphoma), and when used in combination with other drugs could enhance other forms of cancer therapy such as tumor radiation therapy.[3,6] Valproic acid (VPA) is an example of the aliphatic acid inhibitors, MGCD0103 is an example of the benzamide inhibitors and lastly FK-228 is a cyclic peptide inhibitor.[2,15] The aliphatic acid inhibitors, benzamide inhibitors and the cyclic peptides have not shown cancer selectivity in trials yet but are being tested in clinical trials for various types of cancers.[3] All of these inhibitors have shown therapeutic potential as monotherapy or in combination with other anti-tumor drugs and are only active on HDAC class I and II inhibition. HDAC class III (sirtuin inhibition) is dependent on NAD^+.[7] A common Class III inhibitor is nicotinamide but this has issues similar to TSA, it is not very selective against cancer types, treatments, or sirtuin isoforms.

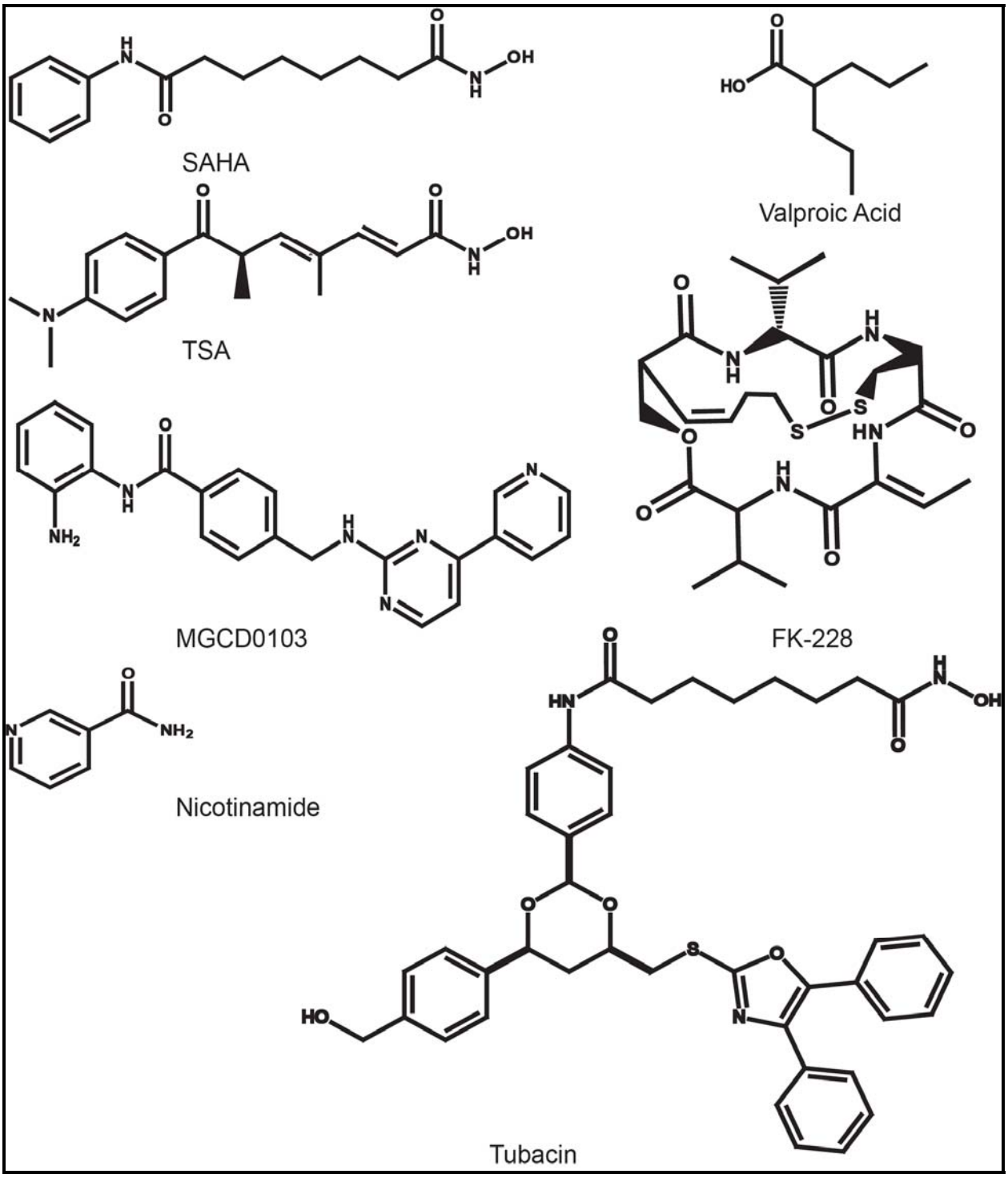


Figure 3: Commonly known HDAC inhibitors [2,6,18]

HDAC Typical Design Features

There are three typical requirements that need to be considered when designing an HDAC inhibitor for class I or II: 1-) a zinc-binding group, capable of fitting into the catalytic site and bind zinc(II), 2-) a linker group able to occupy the hydrophobic channel, and 3-) a cap group to interact with sites on the surface of the HDAC (Figure 3).[19] One or more of these features can be changed to try to maximize isozyme selectivity and overall HDAC inhibition. Common changes to the known inhibitors TSA or SAHA used to make them more selective include: making the cap group more bulky by adding more ring groups or a cyclic peptide at the end of the linker, adding substituents within or on the linker region, and using heteroaromatic substituents as the zinc binding group.[20] Some of these modified compounds did show more selectivity such as depsipeptide (FK-228) and tubacin. Depsipeptide is a cyclic peptide and is selective towards HDAC1 and 2.[3,6] Tubacin is very similar to SAHA but has a much bulkier cap group and is selective towards HDAC6. By adding more cyclic groups, there is an increase of π-π stacking allowing for a stronger interaction with phenylalanine and tyrosine residues that are present in the linker and cap region of the HDAC binding site.[20]

The major HDAC inhibitors SAHA and TSA have the hydroxamate moiety as the zinc binding group. The reason that hydroxamates can inhibit HDACs is a salt-bridge can be formed from the hydroxamate to the positively charged zinc.[6] Also, there are two histidines, two tyrosines and an aspartic acid, that within the pocket interacts with the hydroxamate.[21,22]

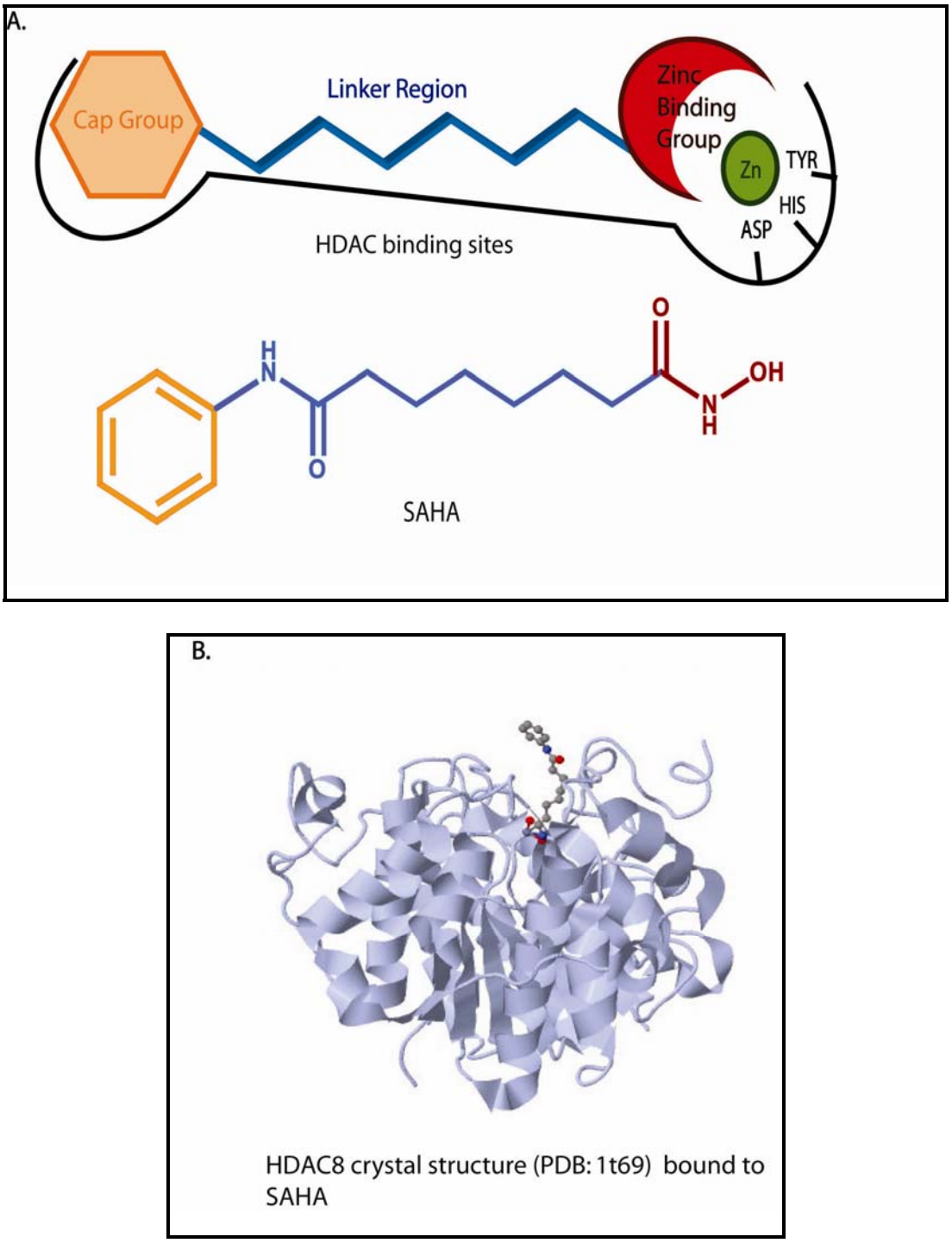


Figure 4: HDAC binding site: A: Drawing representing the HDAC binding sites with SAHA. B: Crystal structure of HDAC8 bound to SAHA

The histidine to aspartic acid interaction is a charge-relay system that is common in active sites. These charge relays bind to the water adjacent to the zinc molecule. The tyrosine is located directly next to the zinc atom and may also contribute to hydrogen bonding. The hydroxamate can also hydrogen bond with the charge relay system and the tyrosine hydroxyl group. The hydroxamate also replaces the water from the zinc and interacts with the zinc using its carbonyl oxygens and the hydroxamate hydroxyl.[6,21,22]

SAHA Based Inhibitor Design

In designing molecules for this project SAHA was used as a template molecule (Figure 3). It is a well known HDAC inhibitor and is currently being used in clinical studies.[3] The HDAC protein is the most flexible in the cap group region. Altering the cap group is, in turn, the easiest part of the inhibitor to modify but does not lead to significant isozyme selectivity because of the flexibility of the outside of the protein.[23] Inside the pocket is a much more rigid environment. Studies have shown that the linker is not directly involved in the binding mechanism but by changing the linker or shape, inhibition can be affected.[23] If the linker is too long the cap group and/or zinc binding group will not fit into their recognition site properly or at all.[6,24]

To improve the selectivity, the zinc binding group was the primary focus. SAHA has a hydroxamate as its zinc binding group. Hydroxamates are easily hydrolyzed under biological conditions, thus making them inaccessible to the zinc group in the HDAC binding pocket, making it a poor drug candidate. While keeping the straight chain alkane as the linker and a phenyl cap group, the zinc binding hydroxamate was replaced by a 1,2,3-triazole with various substituents. Triazoles have been

proposed to be a non classical bioisostere of amides.[25] A triazole binding group would not be hydrolyzed under biological conditions. Also, the nitrogens in the triazole would be better able to bind the zinc atom and to interact with the charge relay system.

Although the linker remains a straight alkyl chain, the length can be altered to better fit the channel in between the cap group and the zinc binding group. An azide-alkyne click reaction catalyzed by a novel catalyst (5,6-diphenyl-3-(pyridine-2-yl)-1,2,4-triazine) with copper (I) was used to synthesize the 1,2,3-triazoles. Solution phase synthesis was employed. The molecules were tested for inhibition activity purified or in solution under click conditions against the following HDACs.

Designing Class Selective Inhibitors

Part of the aim of this project was to focus on the inhibition of each class of histone deacetylases. HDAC1 (class I), HDAC2 (class II) and SIRT1 (Class III) were chosen to experimentally represent the inhibition abilities of various molecules. HDAC6 being a class IIb HDAC is found in the cytoplasm.

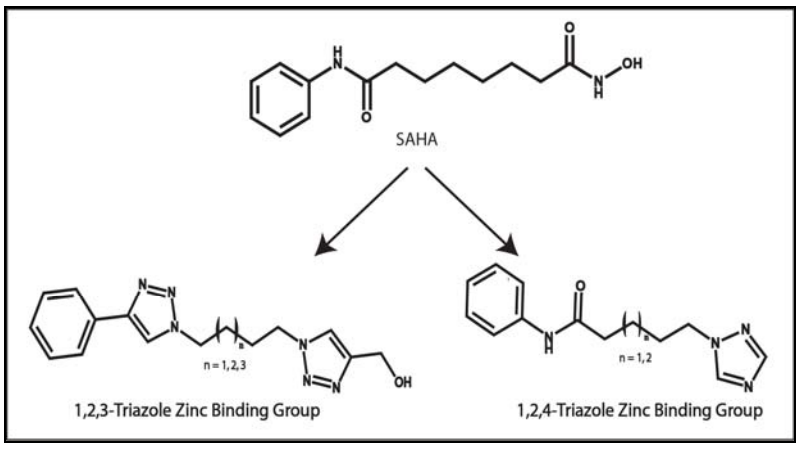


Figure 5: SAHA as an Inhibitor Model: Replacing the zinc binding groups with 1,2,3-triazoles or using the classical hydroxamate zinc binding group

It is currently of interest because not only does HDAC6 deacetylate histone proteins in chromatin to regulate transcription but it can also deacetylate some nonhistone proteins, such as α-tubulin and HSP90.[6,26] As mentioned earlier, these nonhistone proteins are involved with cell signaling, cell growth, and tumor suppression.[6,24] These are all processes which need to be regulated during the course of cancer. HDAC6 is thought to have two catalytic domains that work independently from each other. These differing domains allow for more isozyme selectivity when it comes to drug design due to the fact that the only other HDAC that may have two domains is HDAC10. One of the binding domains is for the histone deacetylation and one for the nonhistone deacetylation. Recently, HDAC6 has been shown to have a great effect on myeloma cells. Thus, not only is the selective inhibition of HDAC6 of interest because of its biological functions but also for its antitumor drug candidate possibilities. HDAC6 overexpression has mainly been observed in breast cancer. Inhibition of HDAC6 leads to the depletion of pre-growth and survival of chaperone proteins in cancer cells.[2,6,16]

HDAC1 is in the class I HDACs and is one of the more commonly studied HDACs. HDAC1 is found in the nucleus and is directly related to proliferation, gene regulation and apoptosis.[24] Most class I HDACs are involved in proliferation and chromatin remodeling but HDAC1 differs in the fact that it helps to regulate apoptosis by deacetylating protein p53.[24,27] HDAC1 over expression has been observed in prostate, gastric, colon and breast cancer. Inhibition of HDAC1 in cancer cells results in inhibition of proliferation and induction of autophagy. HDAC 6 and HDAC1 are strongly inhibited

by TSA and SAHA. But only HDAC1 is inhibited strongly by valproic acid, MGCD0103, and FK-228 when compared to the inhibition of HDAC6, which is only weakly inhibited by these known inhibitors.[2]

SIRT1 is a class III HDAC inhibitor and differs from class I or II due to the fact that it is dependent on NAD^+. SIRT1 not only has histone deacetylase abilities but is also known for its ADP-ribosyltransferase activity.[2] ADP-ribosyltransferase is involved in DNA repair and apoptosis, as well as histone modification. Sirtuin activity is linked to NAD hydrolysis which forms nicotinamide.[17] Sirtuin is thus its own regulator because it forms its own inhibitor while acting in the cell. Sirtuin activity not only can be inhibited to down regulate deacetylation but it can also be up regulated. This is caused by a molecule that blocks the receptor site for nicotinamide, thus allowing sirtuin activity to increase. The diversity in the molecules that can cause inhibition or activation of SIRT1 makes SIRT1 a good cancer target. The above HDACs are all readily available in a fluorometric assay kit, Fluor de Lys, from biomol. [16,17]

Fluor de Lys Assay Background

The Fluor de Lys assay is a one pot assay kit that is convenient and sensitive enough to detect even slight HDAC inhibition. The assay is carried out in two easy steps. During the first step the Fluor de Lys substrate, which has an acetylated lysine side chain, is incubated with HDAC1 or HDAC 6 and the inhibitor being studied at 37 ^oC for 30 minutes. For SIRT1 NAD^+ is included in this first incubation step. When the substrate is deacetylated it can then react with the developer (incubated at room temperature for 45 minutes) in the second step to create a fluorophore. The samples

were excited at 360 nm and the emitted light was detected at 440 nm. This assay can be performed in a 96 well plate or in microcentrifuge tubes and be transferred to an appropriate cell for the fluorometer.

Design and Synthesis of 1,2,3- and 1,2,4-Triazoles as Zinc Binding Groups

A series of SAHA-like compounds (**3a-c, 4 a, 6a-b –** Figure 7 and Figure 8) were synthesized to determine if 1,2,3- or 1,2,4-triazoles could be used efficiently as zinc binding groups. These compounds have the typical inhibitor layout: a cap group, alkyl linker group, and a zinc binding group. Using a triazole as a zinc binding group should offer a more robust moiety that can withstand biological conditions and allow for optimal zinc binding.

Design and Synthesis of 1,2,3-triazoles:

The 1,2,3-triazoles have been used in a previous paper as a replacement of the amide bond in SAHA that links the phenyl cap group to the alkyl linker (Figure 6).[19,25]

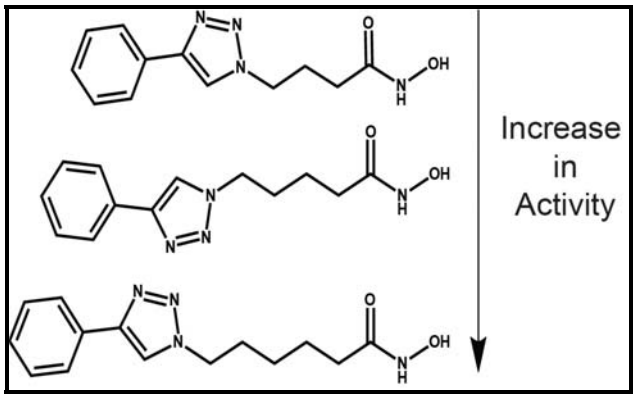


Figure 6: Known HDAC1 Inhibitors: from Chen et al.[19]

These papers show that the replacement of the amide bond did increase activity but the activity was dependent on the linker length and what substituents were used as the cap group.[19] The triazole replacement was thought to make the cap group interaction stronger with the various amino acids in the cap group region.[19] In compounds **3a-c** and **4a** (Figure 7) the amide bond was replaced with the 1,2,3-triazole to give the most advantageous cap group recognition region. In our research design, the 1,2,3-triazole was also used to replace the hydroxamate zinc binding group, as seen in SAHA. In addition to replacing the amide bond and the hydroxamate binding group, the linkers were varied to determine the optimal length needed to be active in the HDAC or SIRT pocket. The 1,2,3-triazoles offer two adjacent nitrogens, along with 'side-arms' with alcohols that can assist in zinc binding.

The key reaction in synthesizing the 1,2,3-triazoles (Figure 7) was the use of Cu(I) catalyzed "click chemistry" in the presence of 5,6-diphenyl-3-(pyridine-2-yl)-1,2,4-triazine (**7a**). This triazine ligand is a novel and readily used catalyst in the presence of base and Cu(I) in organic solvent. The use of this catalyst increases the 'sexiness' of "click chemistry" by resulting in a pure product. The column chromatography needed in these reactions is primarily to remove the catalyst.

Design and Synthesis of 1,2,4-triazoles:

The 1,2,4-triazoles (**6a, 6b –** Figure 8) offer two possibilities for zinc binding. The nitrogen atoms that are available are transversely placed which could allow for one nitrogen to directly interact with the zinc molecule and the other nitrogen to interact with the amino acids (such as histidine, aspartic acid, and tyrosine).[6] The other

option would allow both nitrogen atoms to be used for zinc binding. The 1,2,4-triazoles do not contain the 'side-arms' seen in the 1,2,3-triazoles. These 'side-arms' could cause extra bulk in the binding pocket and not allow the nitrogen to appropriately interact with the zinc molecule. With the differences between the 1,2,3- vs the 1,2,4-triazoles and by using a combination of different linkers, cap groups and zinc binding groups it was hypothesized that an isoform selective inhibitor would be designed.

The key reaction in forming the 1,2,4-triazoles (Figure 8) is a basic substitution reaction in N,N-dimethylformamide (DMF) and potassium carbonate (K_2CO_3) at room temperature. The substitution reaction is novel in the fact that, at room temperature there is a regioselective preference for the reaction to occur at the 1 position and not the 4 position in the triazole. Through trial and error, it was observed that when the reaction was heated (60-100 ^{o}C) there was a mixture of products with substitution at the one and four positions.

To test the ability of the triazoles to be adequate zinc binding groups an in vitro *Fluor de Lys* assay from BioMol was employed. This kit offers a convenient one pot assay that can be used with a variety of HDAC and SIRT enzymes. HDAC1, HDAC6, and SIRT1 were chosen to represent one of each type of the classes of HDACs, class I, II and II respectively.

Results and Discussion

Enyzme Inhibition

SAHA like compounds with the typical hydroxamate zinc binding group being

replaced with a 1,2,3- or 1,2,4-triazole were synthesized and tested in the *Fluor de Lys* assay. On initial evaluation, compounds **3a-c**, **4a**, and **6a-b** were the most active against inhibiting HDAC6 and can both activate and inhibit SIRT1.

HDAC1

There is no clear trend with the compounds in HDAC1 (Figure 9 and Figure 11). There was some 'negative' inhibition numbers included in the HDAC1 data. These numbers indicate that the enzyme was activated, an increase in activity was observed, by the compound instead of inhibited. It was slightly surprising the activity did not show more inhibiting properties. The compounds compared to the ones shown in Figure 6 are very similar. The only differences being the compounds have a hydroxamate instead of a triazole zinc binding group. This shows that for HDAC1 triazoles as the zinc binding group does not increase inhibition.

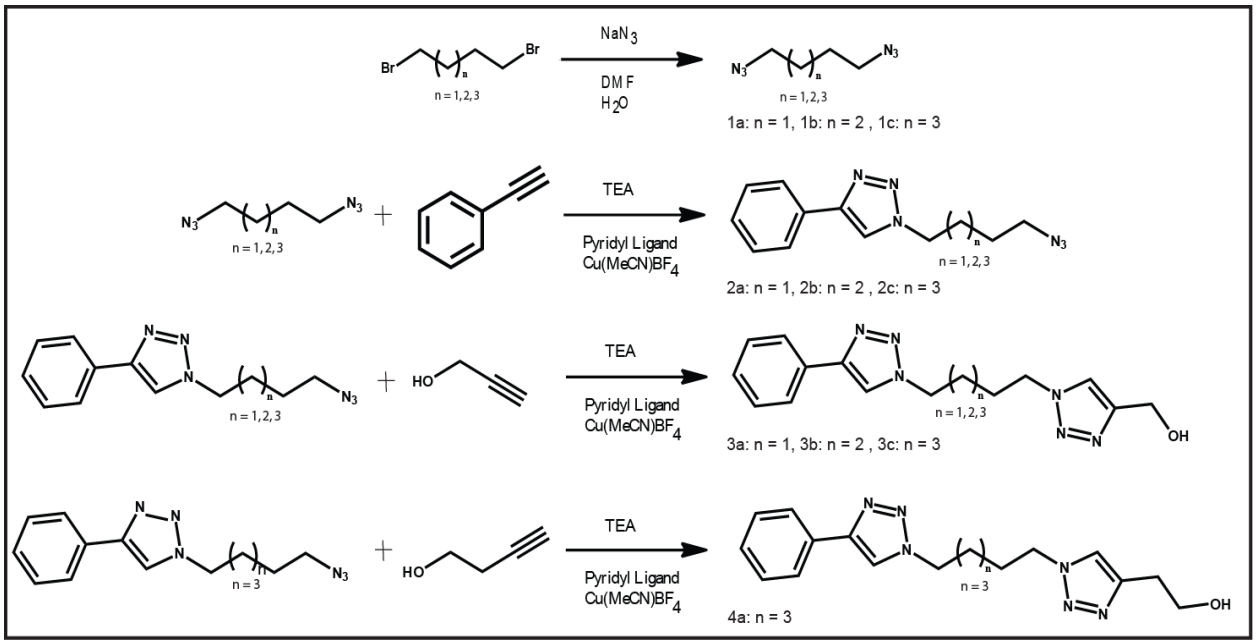


Figure 7: 1, 2, 3, - Triazole for Zinc Binding Group Synthesis

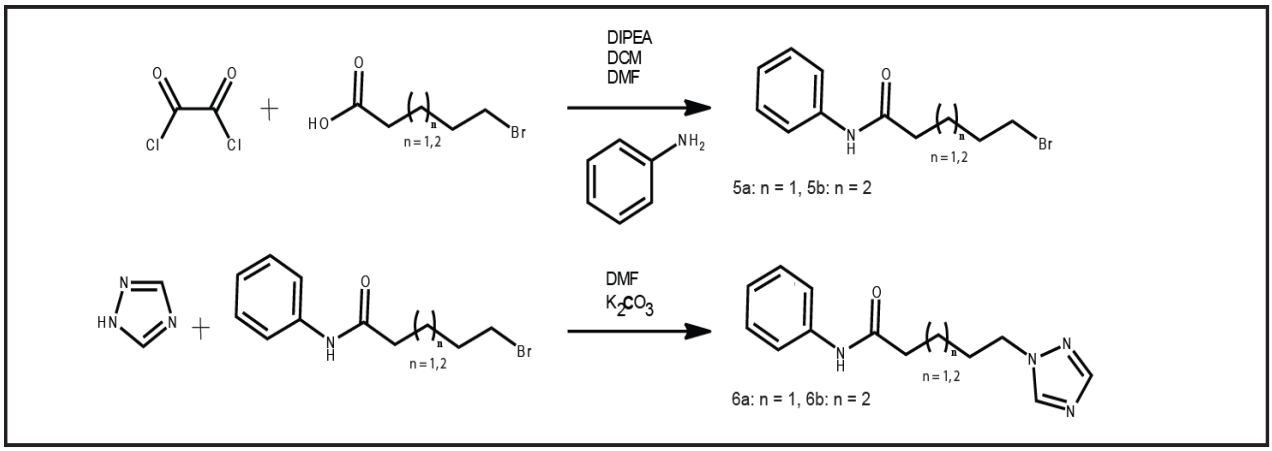


Figure 8: 1, 2, 4 - Triazole for Zinc Binding Group Synthesis

SIRT1

The compounds appear to have a slight trend with SIRT1 depending on the type of triazole (Figure 9 and Figure 13). In comparison of **3a-c** and **4a**, the smaller alkyl linker shows inhibition where as the longer linker lead to activation of SIRT1 enzyme. Compound 3a has the smallest linker is the only compound with a 1,2,3-triazole that actually inhibits SIRT1. Between compounds **3b**, **3c** and **4a** as the linker gets longer the more activating abilities the compound has. Compound **4a** not only has the longest linker (6 carbons) but it also has two carbons between the triazole and the hydroxyl group. This gives the 'side-arm' the more flexibility with the additional carbons and probably a better ability to interact with the zinc molecule. The compounds without the 'side-arms', the 1,2,4-triazoles both have inhibiting properties. As the linker gets longer with this set of compounds the more inhibition with SIRT1 is observed. The 1,2,4-triazoles appear to have an opposing trend with SIRT1 than with the 1,2,3-triazoles. Compound **6b** has the strongest inhibition properties compared to both types of

triazoles. None of the compounds are very strong activators or inhibitors compared to the controls resveratrol and suramin sodium (Figure 10 and Figure 13). Resveratrol causes a two hundred percent increase in activity of SIRT1. Suramin Sodium causes one hundred percent inhibition of SIRT1.

HDAC6

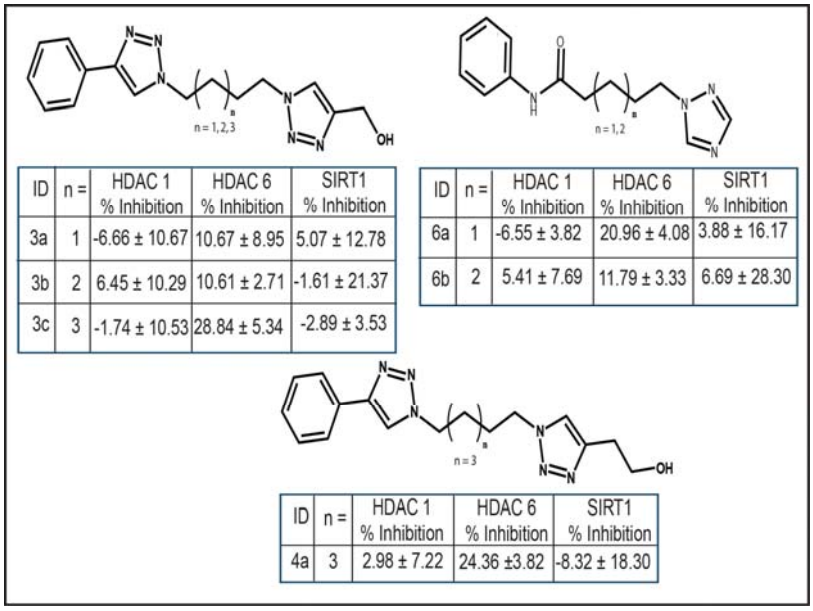
The most inhibition out of all three studies is with HDAC6 (Figure 9 and Figure 12). Again the 1,2,4-triazoles appear to have an opposing trend from the 1,2,3-triazoles. The 1,2,4-triazoles, compounds **6a-b**, show that as the linker gets longer the percent inhibition decreases. With the longer linker there is about 50 percent less inhibition than with the shorter linker. The 1,2,3-triazoles, compounds **3a-c** shows a positive correlation between linker length and percent inhibition. Between the four and five carbon linker there is not much of a difference in inhibition. The six carbon linker causes a three fold increase in inhibition. The 1,2,3-triazole compound **4a** has a six carbon linker and a two carbon spacer between the triazole and the hydroxyl group. With the above trend in compounds **3a-c** it would be expected that **4a** would be even more active due to the increase in length but the inhibition decreases slightly. This compound may be too long for the binding pocket. A selective known inhibitor for HDAC6 is tubacin (Figure 3). Tubacin is very similar to SAHA in zinc-binding moiety but has a much larger cap group region. This may help to explain why the largest percent inhibition is observed with the 1,2,3-triazoles. A 1,2,3-triaozle is present right before the phenyl group. Both of these combined form a larger cap recognition region. The 1,2,3-triazoles are also similar to the tubacin because they do not contain the amide bond that SAHA and

compounds **6a-b** have. The increase in cap group size and the lack of the amide bond is exploiting the capping group modifications more than the zinc binding group to cause a HDAC class selective inhibition.

Selectivity

Determining if a compound is a strong inhibitor or activator is only half of the goal when designing a drug target. More importantly, the molecule needs to be not only active against the desired target but also selective. If the compound stops all HDAC activity that can be just as detrimental to the disease as allowing the HDAC to be overly active. These compounds show selectivity towards being able to inhibit HDAC6 over HDAC1. There is no pattern present with HDAC1, whereas with HDAC6 there is a strong inhibition pattern. The compounds could inhibit up to twenty times more against HDAC6 than they could HDAC1. The compounds could also inhibit up to 5 times more against HDAC6 than they could with SIRT1. The compounds also show selectivity because they are able to activate SIRT1.

It is also interesting to note the selectivity between the different types of triazoles in SIRT1 and HDAC6. With SIRT1, the 1,2,4-triazoles primarily inhibited SIRT1 where as 1,2,3-triazoles activated SIRT1. The inhibition with HDAC6 can be controlled by using the linker length but the inhibition pattern is opposing with the 1,2,3-triazoles compared to the 1,2,4-triazoles. This selectivity for HDAC6 inhibition and SIRT1 activation makes these good molecules to consider optimizing to try to get the maximum amount of inhibition or activation for each class of HDACs.

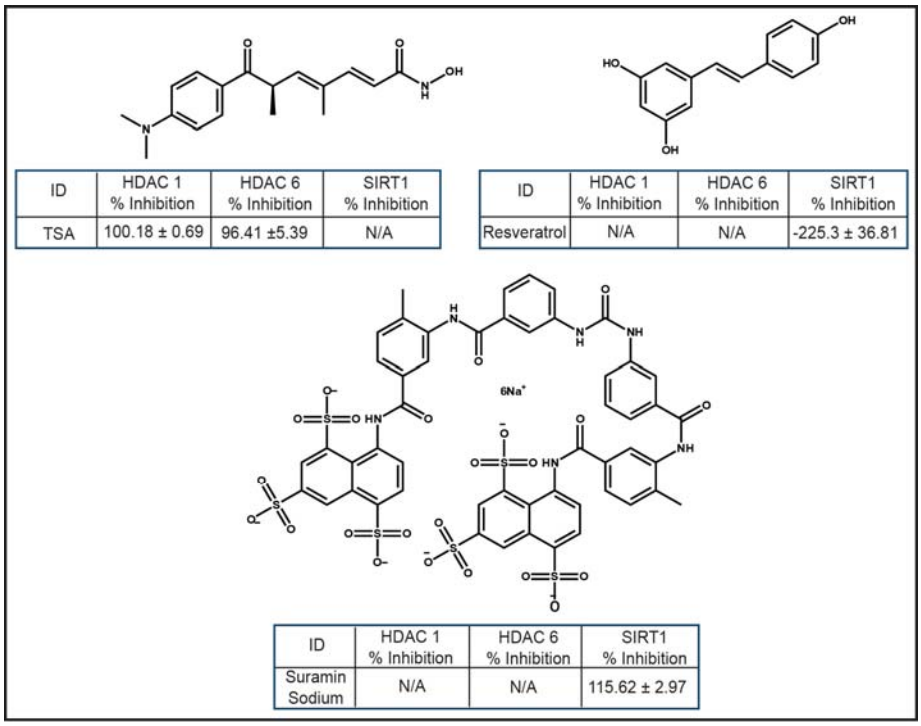


ID	n =	HDAC 1 % Inhibition	HDAC 6 % Inhibition	SIRT1 % Inhibition
3a	1	-6.66 ± 10.67	10.67 ± 8.95	5.07 ± 12.78
3b	2	6.45 ± 10.29	10.61 ± 2.71	-1.61 ± 21.37
3c	3	-1.74 ± 10.53	28.84 ± 5.34	-2.89 ± 3.53

ID	n =	HDAC 1 % Inhibition	HDAC 6 % Inhibition	SIRT1 % Inhibition
6a	1	-6.55 ± 3.82	20.96 ± 4.08	3.88 ± 16.17
6b	2	5.41 ± 7.69	11.79 ± 3.33	6.69 ± 28.30

ID	n =	HDAC 1 % Inhibition	HDAC 6 % Inhibition	SIRT1 % Inhibition
4a	3	2.98 ± 7.22	24.36 ±3.82	-8.32 ± 18.30

Figure 9: HDAC1, HDAC6, and SIRT1 Assay Results: Results for 3a-c, 4a, 6a-b at a final concentration of 25 uM. The results are represented with the standard deviation of 3 independent trials.



ID	HDAC 1 % Inhibition	HDAC 6 % Inhibition	SIRT1 % Inhibition
TSA	100.18 ± 0.69	96.41 ±5.39	N/A

ID	HDAC 1 % Inhibition	HDAC 6 % Inhibition	SIRT1 % Inhibition
Resveratrol	N/A	N/A	-225.3 ± 36.81

ID	HDAC 1 % Inhibition	HDAC 6 % Inhibition	SIRT1 % Inhibition
Suramin Sodium	N/A	N/A	115.62 ± 2.97

Figure 10: HDAC1, HDAC6, and SIRT1 Assay Results: Results for Control Compounds at a final concentration of 25 uM. The results are represented with the standard deviation of 3 independent trials.

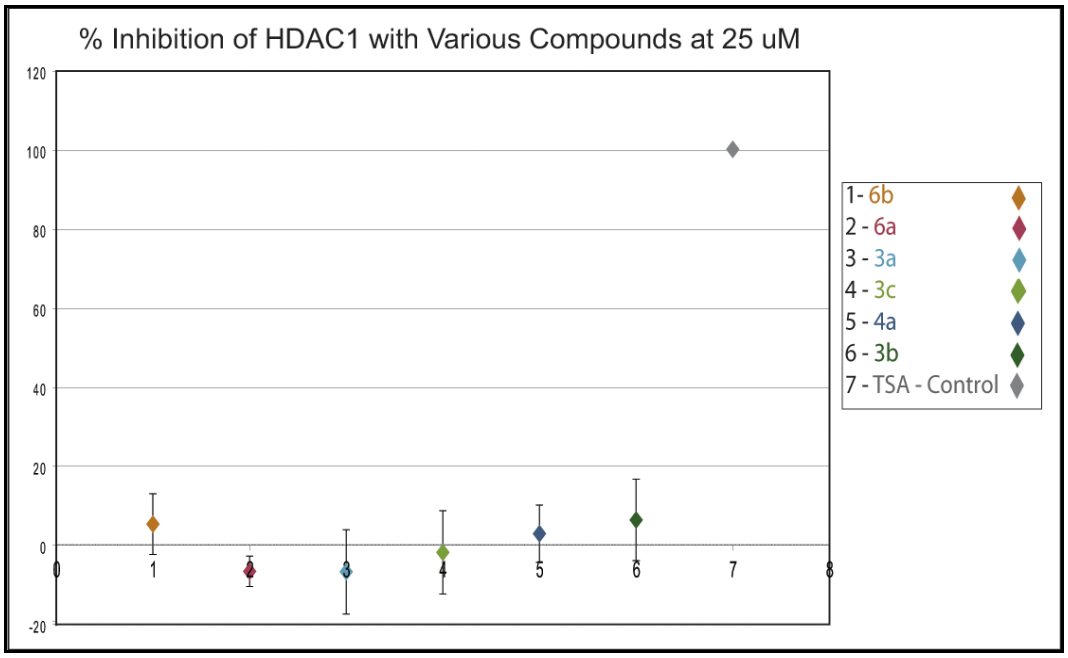


Figure 11: HDAC1 Inhibition Graph with Various Compounds at 25 uM

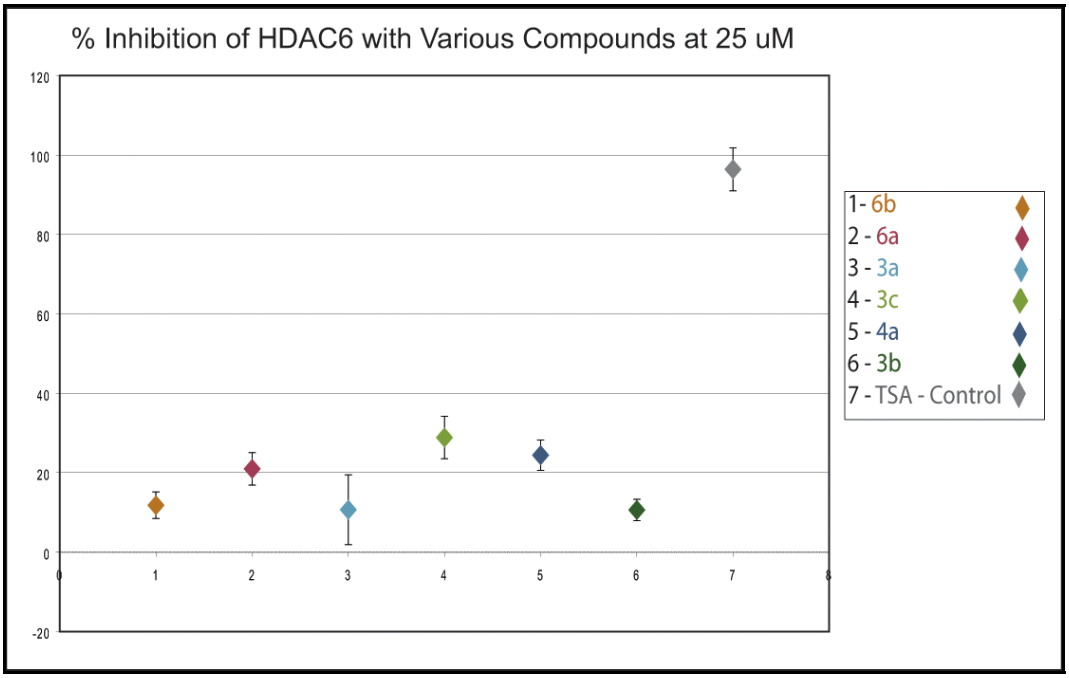


Figure 12: HDAC6 Inhibition Graph with Various Compounds at 25 uM

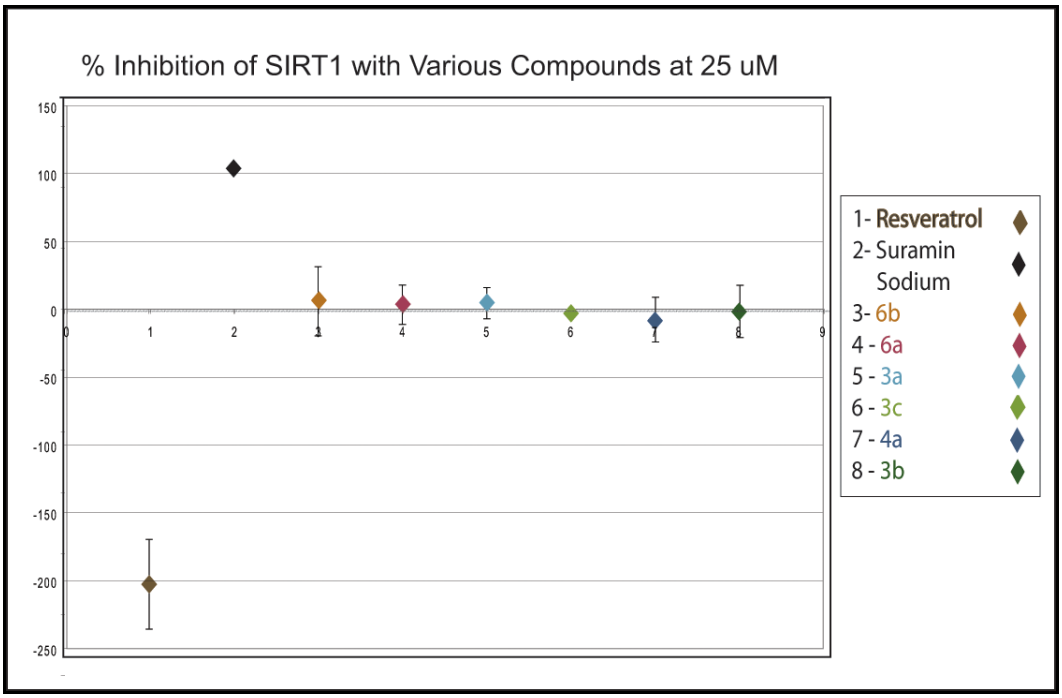


Figure 13: SIRT1 Inhibition Graph with Various Compounds at 25 uM

Conclusion

Though none of the synthesized compounds are strong activators or inhibitors of any of the classes of HDACs there are definitely some observed trends. The 1,2,3-triazoles and 1,2,4-triazoles do not have a specific pattern with HDAC1. There was not much activity with the 1,2,3-triazoles or the 1,2,4-triazoles against SIRT1 but there was a definite pattern within the molecules. As the linker increased in the 1,2,3-triazoles the activation of SIRT1 also increased. The 1,2,4-triazoles showed an opposing pattern, as the linker length increased the inhibition of SIRT1 increased. The best inhibition was observed with HDAC6. The compounds appear to be selective at inhibiting HDAC6 as compared to HDAC1. The compounds activated SIRT1 more than inhibited, thus making the compounds more selective in favor of inhibiting HDAC6.

Future Work

To further the study of HDAC1, HDAC6, and SIRT1 using triazoles as zinc binding groups several changes can be studied with this class of molecules. The substituents on both 1,2,3- and 1,2,4-triazoles need to be altered to determine if the zinc binding can be increased or decreased (Figure 14). The studies with the 1,2,3-triazoles that have the 'side-arms' appear to allow some inhibition control. The length of the linkers in both the 1,2,3- and 1,2,4-triazoles can be changed to determine the optimal length for each class of HDAC. The subsituents on the cap groups can also be changed to increase the cap recognition region. Especially, for HDAC6 increasing the size of the cap recognition region may further help it the compounds to become more selective. The rigidity of the linker chain can be changed to determine if making the molecules more like TSA rather than SAHA could increase the activity and maintain the selectivity. Also, control compounds that mimic SAHA and previously synthesized compounds found in literature that contain the desired linker length also need to be tested (Figure 14).

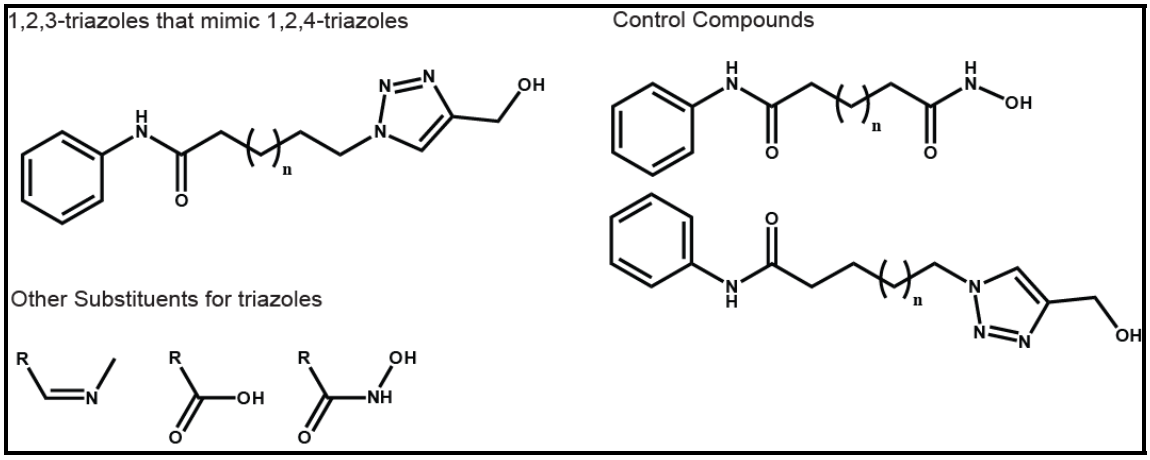
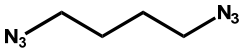


Figure 14: Future Compounds

Experimental

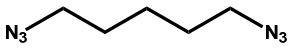
Materials: ^{1}H NMR and ^{13}C NMR spectra were recorded using a Varian Inova instrument operating at 600 MHz. Chemical shifts were reported in parts per million (ppm) relative to deuterated solvent signal. High-resolution ESI mass spectra were recorded using a JEOL AccuTOF MS (orthogonal TOF) instrument with DART source calibrated with a PEG positive standard. Absorption spectra were collected on a Thermo Scientific Evolution 600. Fluorescence Spectrometry was performed using a Perking Elmer LS 55 instrument and/or a BioTek Synergy multimode plate reader. Flash column chromatography was performed using standard grade silica 230 x 400 mesh from sorbent technologies. Analytical thin-layer chromatography (TLC) was performed using aluminum backed silica gel TLC plates with UV indicator from Sorbent Technologies. The HDAC fluorimetric drug discovery kits for HDAC 1 and Sirtuin were purchased from Biomol International. Recombinant HDAC 6 was also purchased from Biomol International. Anhydrous solvents were purified using a Grubbs solvent system. Reagent grade chemicals were used unless otherwise indicated.

WARNING: **Low molecular weight azides are potentially explosive. Appropriate safety emasures should always be taken when handling these compounds.**

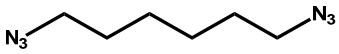


1a: 1,4-Diazidobutane: 1,4-Dibromobutane (2.00 g, 9.26 mmol) was dissolved in dimethylformamide (20.6 mL). Sodium Azide (23.2 mmol) was dissolved in

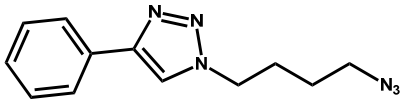
water (2.57 mL) and added to the dibromide solution. The reaction stirred at room temperature overnight (12-18 hours). Reaction progress was monitored by GC-MS. Upon consumption of starting material, water was added to the reaction mixture. The water was extracted with diethyl ether 3 times. The organic layers were combined and rinsed with water 3 times, saturated copper (II) sulfate 1 time, and brine 1 time. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. This material was used without further purification.



1b: 1,5-Diazidopentane: 1,5-Diazidopentane was prepared from 1,5-dibromopentane as listed above for 1,4-Diazidobutane.

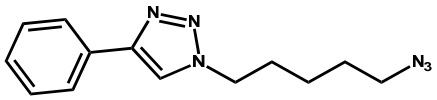


1c: 1,6-Diazidohexane: 1,6-Diazidohexane was prepared from 1,6-dibromohexane as listed above for 1,4-Diazidobutane.

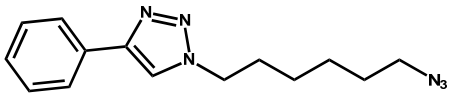


2a: 1-(4-azidobuty)-4-phenyl-1H-1,2,3-triazole: The 1,4-diazidobutane (1.00 g, 7.14 mmol), phenylacetylene (0.290 g, 2.86 mmol), and triethylamine (398 uL, 2.86 mmol) were dissolved in acetonitrile (35.8 mL). 5,6-diphenyl-3-(pyridine-2-yl)-1,2,4-

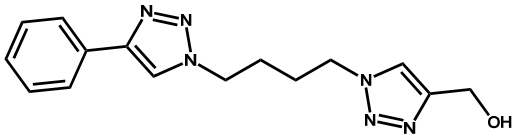
triazine (8.68 mg, 0.028 mmol) and tetrakis(acetonitrile-N)copper(I) tetrafluoroborate (8.88 mg, 0.0280 mmol) were added to the reaction solution. The reaction was stirred at room temperature overnight (12-18 hours). After concentration *in vacuo* the crude product was purified via silica gel chromatography with a gradient elution using ethyl acetate and hexanes.



2b: 1,5-(5-azidopentyl)-4-phenyl-1H-1,2,3-triazole: 1,5-(5-azidopentyl)-4-phenyl-1*H*-1,2,3-triazole was prepared as listed above for 1-(4-azidobuty)-4-phenyl-1*H*-1,2,3-triazole.



2c: 1,6-(6-azidohexyl)-4-phenyl-1H-1,2,3-triazole: 1,6-(6-azidohexyl)-4-phenyl-1*H*-1,2,3-triazole was prepared as listed above for 1-(4-azidobuty)-4-phenyl-1*H*-1,2,3-triazole.



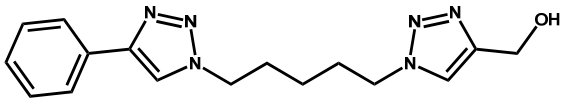
3a: (1-(4-(4-phenyl-1H-1,2,3-triazole-1-yl)butyl)-1H-1,2,3-triazol-4-yl)methanol: 1-(4-azidobuty)-4-phenyl-1*H*-1,2,3-triazole (0.400 g, 0.830 mmol), propargyl alcohol (50.6 uL, 0.870 mmol),37and triethylamine (115 uL,0.830 mmol)

were dissolved in acetonitrile (5.53 mL). 5,6-diphenyl-3-(pyridine-2-yl)-1,2,4-triazine (26.1 mg, 0.083 mmol) and tetrakis(acetonitrile-N)copper(I) tetrafluoroborate (25.7 mg, 0.0830 mmol) were added to the reaction solution. The reaction was stirred at room temperature overnight (12-18 hours). After concentration *in vacuo* the crude product was purified via column chromatography with a gradient elution using ethyl acetate and hexanes.

^{1}H NMR (d_6-DMSO, 600 MHz) δ 7.72 (s, 1H), 7.12 (s, 1H), 7.00 (d, 2H, J= 12), 6.60 (t, 2H, J= 6), 6.49 (t, 1 H, J= 6), 4.31 (t, 1H, J= 6), 3.65 (d, 2H, J= 6), 3.59 (t, 3H, J= 6), 3.54 (t, 3H, J= 6), 3.25 (m, 2H), 2.32 (d, 3H, J= 6), 1.66 (m, 2H), 0.98 (m, 4H).

^{13}C NMR (DMSO, 151 MHz) δ147.9, 146.3, 130.7, 128.8, 127.8, 125.1, 122.6, 121.3, 55.0, 48.8, 48.6, 48.5, 26.8, 26.7.

HRMS (ESI) m/z calculated 299.16203 ($C_{15}H_{19}N_6O_1$, $[M+H]^+$), m/z observed 299.16058($C_{15}H_{19}N_6O_1$, $[M+H]^+$. Mass Difference (ppm) -4.87.



3b: (1-(5-(4-phenyl-1H-1,2,3-triazole-1-yl)pentyl)-1H-1,2,3-triazol-4-yl)methanol: (1-(5-(4-phenyl-1*H*-1,2,3-triazole-1-yl)pentyl)-1*H*-1,2,3-triazol-4-yl)methanol was prepared as listed above for (1-(4-(4-phenyl-1*H*-1,2,3-triazole-1-yl)butyl)-1*H*-1,2,3-triazol-4-yl)methanol.

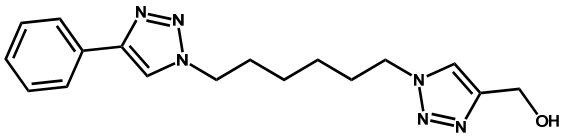
^{1}H NMR (d_6-DMSO, 600 MHz) δ 7.55 (s, 1H), 7.47 (d, 2H, J= 12), 7.25 (s, 1H),

7.06 (t, 2H, J= 6), 6.97 (t, 1H, J= 6), 4.35 (s, 2H), 4.04 (t, 2 H, J= 6), 3.99 (t, 2H, J=

6), 3.02 (s, 1H), 1.61 (m, 4H), 0.99 (m, 2H).

^{13}C NMR (DMSO, 151 MHz) δ148.7, 147.5, 130.6, 128.8, 125.6, 121.9, 120.0, 56.2, 49.8, 49.7, 29.5, 29.4, 23.2.

HRMS (ESI) m/z calculated 313.17768 ($C_{16}H_{21}N_6O_1$, $[M+H]^+$), m/z observed 313.17728 ($C_{16}H_{21}N_6O_1$, $[M+H]^+$. Mass Difference (ppm) -1.28.

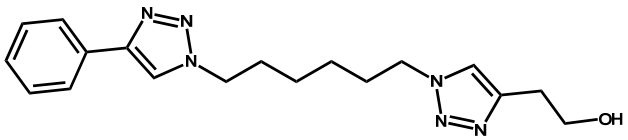


3c: *(1-(4-(6-phenyl-1H-1,2,3-triazole-1-yl)hexyl)-1H-1,2,3-triazol-4-yl)methanol:* (1-(4-(6-phenyl-1*H*-1,2,3-triazole-1-yl)hexyl)-1*H*-1,2,3-triazol-4-yl)methanol was also prepared as listed above for (1-(4-(4-phenyl-1*H*-1,2,3-triazole-1-yl)butyl)-1*H*-1,2,3-triazol-4-yl)methanol.

^{1}H NMR (d_6-DMSO, 600 MHz) δ 7.61 (s, 1H), 7.54 (d, 2H, J= 2), 7.30 (s, 1H), 7.14 (t, 2H, J= 6), 7.05 (t, 1H, J= 6), 4.46 (d, 2H, J= 6) , 4.11 (t, 2H, J= 6), 4.05 (t, 2H, J= 6), 3.12 (s, 1H), 1.67 (m, 2H), 1.62 (m, 2H), 1.10 (m, 4H).

^{13}C NMR (DMSO, 151 MHz) δ147.6, 130.7, 128.9, 128.1, 125.6, 121.8, 119.9, 56.3, 50.1, 49.9, 30.0, 25.8, 25.8.

HRMS (ESI) m/z calculated 327.1933 ($C_{17}H_{23}N_6O_1$, $[M+H]^+$), m/z observed 327.19202 ($C_{17}H_{23}N_6O_1$, $[M+H]^+$. Mass Difference (ppm) -4.03.

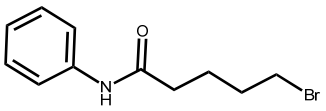


4a: ***2-(1-(6-(4-phenyl-1H-1,2,3-triazol-1-yl)hexyl)-1H-1,2,3-triazol-4-yl)ethanol:*** 2-(1-(6-(4-phenyl-1*H*-1,2,3-triazole-1-yl)hexyl)-1*H*-1,2,3-triazol-4-yl)ethanol (0.400 g, 1.18 mmol), propargyl alcohol (89.3 uL, 1.18 mmol), and trieithylamine (164 uL, 1.18 mmol) were dissolved in acetonitrile (7.86 mL). 5,6-diphenyl-3-(pyridine-2-yl)-1,2,4-triazine (5.58 mg, 0.0180 mmol) and tetrakis(acetonitrile-N)copper(I) tetrafluoroborate (5.65 mg, 0.0180 mmol) were added to the reaction solution. The reaction was stirred at room temperature overnight (12-18 hours). After concentration *in vacuo* the crude product was purified via column chromatography with a gradient elution using ethyl acetate and methanol.

^{1}H NMR (d_6-DMSO, 600 MHz) δ7.62 (s, 1H), 7.56 (d, 2H, J= 12), 7.19 (d, 1H, J= 12), 7.16 (t, 2H, J= 6), 7.05 (t, 1H, J= 12), 4.14 (t, 2H, J= 12), 4.06 (t, 2H, J= 6), 3.69 (s, 2H), 3.13 (s, 1H), 2.67 (t, 2H, J= 12), 1.69 (t, 2H, J= 6), 1.63 (t, 2H, J= 6), 1.12 (m, 4H).

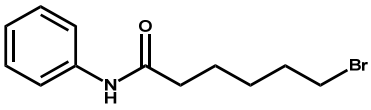
^{13}C NMR (DMSO, 151 MHz) δ 147.6, 130.7, 128.9, 128.1, 125.6, 119.9, 61.3, 50.1, 49.9, 30.0, 30.0, 29.1, 25.8.

HRMS (ESI) m/z calculated 341.20898 ($C_{18}H_{25}N_6O_1$, $[M+H]^+$), m/z observed 341.20949 ($C_{18}H_{25}N_6O_1$, $[M+H]^+$. Mass Difference (ppm) 1.48.

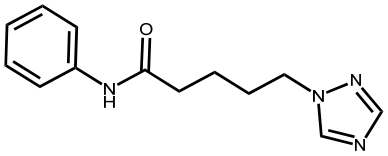


5a: *5-bromo-N-phenylpentanamide:* 5-bromopentanoic acid (1.00 g, 5.52 mmol) was dissolved in DCM (15.5 mL) with a catalytic amount of DMF (56.0 uL). The reaction mixture was cooled to -5 ^{o}C. A solution of 2 M oxalyl chloride (532 uL, 6.09 mmol) in DCM (3.04 mL) was added drop wise to the reaction with vigorous stirring over 15 minutes. The suspension was warmed to room temperature and stirred for 1 hour. The volatiles were evaporated under a stream of nitrogen. The residue was dissolved in DCM (25.6 mL) and cooled to zero ^{o}C. DIEA (2.021 mL, 11.6 mmol) was added drop wise. The aniline (504 uL, 5.52 mmol) was added drop wise over 1 hour. The reaction mixture was warmed to room temperature after the addition of the aniline and the reaction stirred for 2 hours.

The reaction was quenched by addition of water (5.00 mL). The solution was extracted with DCM 2 times. The DCM layer was washed with saturated sodium bicarbonate 2 times, water 1 time, 1M hydrochloric acid 2 times, and sodium chloride 1 time. The organic layer was dried over sodium sulfate, filtered and concentrated down *in vacuo*.[28]



5b: 6-bromo-N-phenylhexanamide: 6-bromohexanoic acid was prepared as listed above for 5-bromo-N-phenylpentanamide.[28]

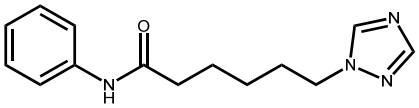


6a: N-phenyl-5-(1H-1,2,4-triazol-1-yl)pentanamide: 5-bromo-N-phenylpentamide (60.0 mg, 0.234 mmol), 1,2,4-1*H*-triazole (18.0 mg, 0.258 mmol), and freshly ground potassium carbonate (36.0 mg, 0.258 mmol) were dissolved in DMF (669.0 uL). The reaction stirred at room temperature overnight (12-14 hours). The reaction was quenched with water (5.0 mL). The water was extracted with ethyl acetate 3 times. The organic layers were combined and washed with brine 1 time. The organic layer was dried over sodium sulfate, filtered and concentrated *in vacuo*.

^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.29 (s, 1H), 8.09 (s, 1H), 7.93 (s, 1H), 7.48 (d, 1H, J= 6), 7.27 (t, 2H, J= 6), 7.07 (t, 2H, J= 6), 4.16 (t, 2H, J= 6), 2.36 (t, 2H, J= 6), 1.94 (m, 2H), 1.69 (m, 2H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 171.1, 151.7, 143.1, 137.9, 129.0, 124.5, 120.1, 49.3, 36.4, 29.2, 22.3.

HRMS (ESI) m/z calculated 245.14024 ($C_{13}H_{17}N_4O_1$, $[M+H]^+$), m/z observed 245.13918 ($C_{13}H_{17}N_4O_1$, $[M+H]^+$. Mass Difference (ppm) -4.31.

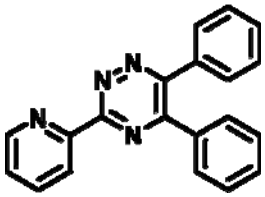


6b: N-phenyl-6-(1H-1,2,4-triazol-1-yl)hexanamide: 6-bromo-N-phenylhexanamide was prepared as listed above for N-phenyl-5-(1*H*-1,2,4-triazol-1-yl)pentanamide.

^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.16 (s, 1H), 8.05 (s, 1H), 7.40 (s, 1H), 7.50 (d, 1H, J= 6), 7.29 (t, 2H, J= 12), 7.08 (t, 2H, J= 6), 4.17 (t, 2H, J= 6), 2.35 (t, 2H, J= 12), 1.94 (m, 2H), 1.76 (m, 2H), 1.36 (m, 2H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ166.2, 147.3, 138.4, 133.3, 124.4, 119.7, 115.2, 44.8, 32.6, 24.9, 21.4, 20.1.

HRMS (ESI) m/z calculated 259.15589 ($C_{14}H_{19}N_4O_1$, $[M+H]^+$), m/z observed 259.15473 ($C_{14}H_{19}N_4O_1$, $[M+H]^+$. Mass Difference (ppm) -4.48.



7a: 5,6-diphenyl-3-(pyridin-2-yl)-1,2,4-triazine. (Z)-picolinohydrazonamide (500.0 mg, 3.67 mmol) and benzil (772 mg, 3.67 mmol) were added to 10 mL of ethanol. The slurry was heated at 80 °C for 6 h with complete dissolution. Progress of the reaction was monitored by TLC. The reaction mixture was cooled to room temperature and the resulting precipitate was filtered and washed with ethanol and ether resulting in a 76% yield.

^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.91 (d, 1H, J = 4.2 Hz), 8.70 (d, 1H, J = 7.8 Hz), 7.91 (t, 1H, J = 7.8 Hz), 7.68 (d, 2H, J = 7.8 Hz), 7.64 (d, 2H, J = 7.8 Hz), 7.46 (t, 1H, J = 6.6 Hz), 7.43-7.31 (m, 6H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 160.7, 156.4, 156.3, 152.8, 150.4, 137.0, 135.6, 135.2, 130.6, 129.9, 129.7, 129.5, 128.5, 128.5, 125.3, 124.1.

HRMS (ESI) m/z calculated 311.12967 ($C_{20}H_{15}N_4$, $[M+H]^+$), m/z

observed 311.12831 ($C_{20}H_{15}N_4$, $[M+H]^+$)

Fluorimetric HDAC Assay: The procedure was followed for HDAC 1, 6 and Sirtuin as listed by Biomol with the following specifications. HDAC 1 and 6: To the appropriate wells, 4 uL of the test inhibitor was added to obtain a 25 uM final concentration. HDAC assay buffer II was warmed for 10 minutes. The chilled enzyme was diluted with the warm buffer and added to the wells to have a final concentration of 0.2 ug/well. The substrate was diluted in the warm assay buffer and added to appropriate wells to give a final concentration of 20 uM. The reaction was incubated at 37 ^oC for 30 minutes. After the 37^oC incubation, 20 uL of the 5x developer solution with 1% TSA was added to all of the wells and incubated in the dark for 45 minutes at room temperature. The samples were diluted appropriately (any where from 1 to 10 fold with nano-pure water) to be read with an excitation of 360 nm and an emission of 460 nm on the fluorometer. If the samples were read on the plate reader, a dichroic mirror was used with 80% sensitivity. The plates were white and were read with an excitation of 360/40 and had an emission of 440/30. The data were processed using Microsoft Excel.

SIRT 1: The protocol for the sirtuins is very similar to the HDAC 6 and 1. To the appropriate wells, 4 uL of compound was added to have a 25 uM final concentration. Followed by 6 uL of 20 % NAD^+ in substrate was added to have a final concentration of 125 uM and 500 uM respectively. HDAC assay buffer II and the above assay mix was warmed for 10 minutes. The enzyme was added to the warm buffer and added to the wells to have a final concentration of 0.04 U/well. The reaction incubated at 37 ^oC for 30 minutes. After the 37 ^oC incubation, 20 uL of the 5x developer solution with 1% TSA

was added to all of the wells and incubated in the dark for 45 minutes at room temperature. The samples were diluted appropriately and read as listed for HDAC 1 and 6.

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