THE SAGA OF SAHA: HISTONE DEACETYLASE INHIBITORS AS ANTI-CANCER AGENTS

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INTRODUCTION

Common cancer treatment techniques, such as chemotherapy, take advantage of apoptosis, to eliminate malignant cells within tumors.[1] Although this approach is effective on a wide variety of tumors, it is not highly selective and thus causes its adverse effects on healthy tissue. An improved strategy for cancer treatment would be to use small molecules to selectively differentiate cancerous cells into normal cells. This has been demonstrated using a large range of compounds on a variety of cancers.[2] The most effective compound discovered to date, suberolylanilide hydroxamic acid (SAHA), was reported the same year as isolation and characterization of histone deacetylase (HDAC) -1996.[3]

DMSO: AN HISTORICAL OVERVIEW AS A CELL DIFFERENTIATOR.

DMSO differentiates erytholeukemia cells

In 1971, Charlotte Friend made the remarkable discovery that DMSO differentiates murine virus-induced erythroleukemia cells into normal cells, as she attempted to determine the toxicity of DMSO to the leukemic cell lines.[4] It was found that cells grown in media containing 2% DMSO experienced only a slight decrease in the rate of growth within the first 48 hours, but after 5 days, Friend reported the surprising result that ~96% of the leukemia cells had either begun differentiation or had already differentiated into normal cells.

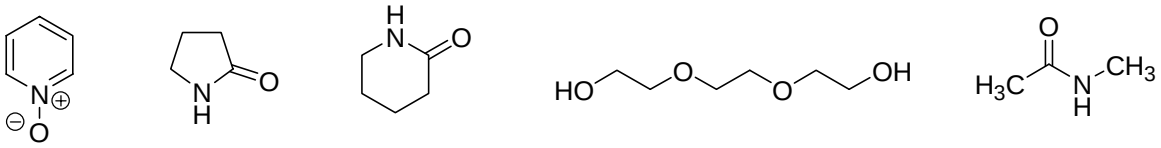
Although the exact cellular role of DMSO-mediated differentiation was not elucidated until 1996, numerous studies were carried out over the intervening 25 year period to determine the target of DMSO.[5,6,7,8] Overall, these studies found that the differentiation proceeds through a normal erythropoiesis chain of events, that include globin mRNA and hemoglobin accumulation, increase in iron uptake and heme synthesis, appearance of erythrocyte membrane antigens, cessation of cell division, hyperacetylation of histones, and characteristic morphological changes. An early observation by Friend was the apparent cellular commitment to differentiation after 1 to 24 hours of incubation in 2% DMSO, with subsequent growth in DMSO-free media. It was later found that cells should be preincubated with DMSO for at least 24-30 hours to allow the cellular concentration of DMSO to be

concentration equivalent to that of the incubation medium.

Improving on DMSO

The effectiveness of DMSO in differentiating cells was clearly demonstrated by a large number of labs, but the high concentration of DMSO (280 mM) required for treating cancer *in vivo* is not practical; a better compound was clearly needed. A variety of compounds based on dipole moment, presence or absence of methyl groups and molecular size of DMSO were synthesized and investigated.[9] Valuable information about the requirements of the differentiating agents were determined through these studies. It was found that hexamethylphosphoric triamide, urea, and ethylene carbonate, all highly polar compounds, were inactive as inducers, implying that polarity was not as important as initially thought. The highly polar, active inducing agents, pyridine-N-oxide (**1**), 2-pyrrolidinone (**2**), piperidone (**3**) and triethylene glycol (**4**), were also discovered from the original group of compounds (Figure 1).

Figure 1. Active inducing agents.



1 **2** **3** **4** **5**

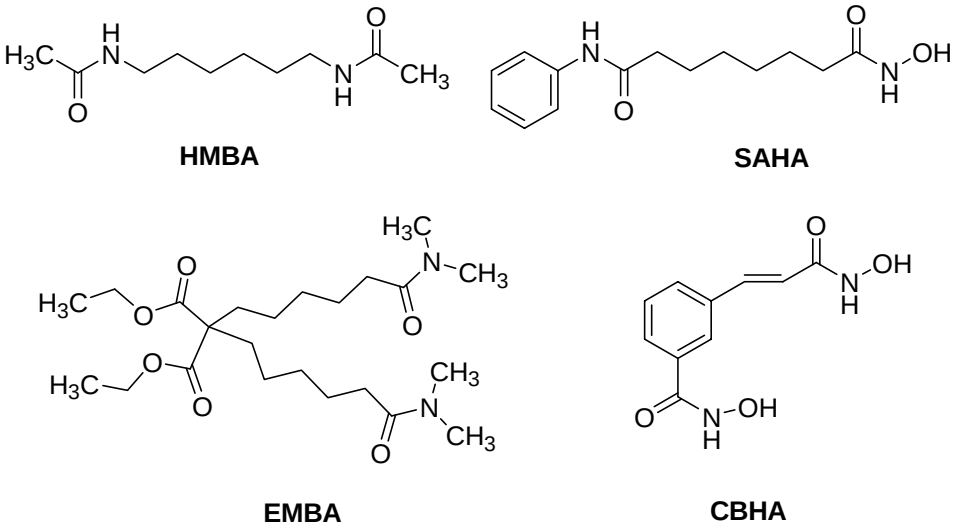
The results with these compounds imply that no methyl groups were required for active differentiating inducers. One compound from this group that showed promise was N-methylacetamide (**5**), with an optimal cellular concentration of 30 mM. In an attempt to increase the local effective concentration of the inducer, N-methylacetamide was dimerized by linkage of the nitrogen through a varying number of methylene groups. The most promising number of methylenes in the linker was six, and the best analog, hexamethylene bisacetamide (HMBA) had an optimal concentration of 5 mM (Figure 2).[10] The increased potency of this best synthetic inducer of differentiation prompted workers to start clinical studies. In phase II clinical studies, HMBA showed efficient action in combating cancers. Of the 28 patients, 9 achieved complete or partial remission lasting from 1 to 16 months. However, these results were not without side effects. Due to HMBAs short half-life *in vivo* (about 1.5 hours), a constant amount had to be administered to maintain effective levels within the body. HMBA also

caused severe thrombocytopenia in the patients administered the drug, which limited the amount that could be administered.

In attempts to find more potent inducers, Breslow and coworkers developed a large variety of new compounds with varying functionality. Three of the developed compounds, which showed great promise in differentiation, were diethyl bis(pentamethylene-N,N-dimethylcarboxamide)malonate (EMBA), suberoylanilide hydroxamic acid (SAHA) and *m*-carboxycinnamic acid bis-hydroxamide (CBHA) (Figure 2). Of these three compounds, SAHA showed the most promise having an optimal concentration of 2 μM, being 2500 times more potent than HMBA.[11]

An interesting observation made by many independent research groups was the accumulation of acetylated histones after treatment with these differentiation-inducing agents. This biological phenomenon implied that the histones were being acetylated but not deacetylated, as normally happens. This observation implied that these treatments either stimulated histone acetyltransferase (HAT), the enzyme responsible for acetylation of histones, or inhibited histone deacetylase (HDAC), the enzyme responsible for deacetylating histones.

Figure 2. High affinity inducers.



HISTONE DEACTYLASE

HDAC Function

Eukaryotic cells contain DNA wrapped around proteins called histones; this complex is referred to as the nucleosome. Histones allow DNA to tightly wrap into a coil ca. 30 nm in diameter, which further builds upon itself, eventually forming chromosomes.[12] There are five distinct types of histones associated with eukaryotic DNA: H1, H2A, H2B, H3 and H4; each have distinct numbers of amino acids and hence differing molecular weights. Association of histones with DNA to form nucleosomes has been shown to entail an octamer of histones consisting of two tetrameric subunits containing H2A, H2B, H3 and H4 in each subunit. The two combined subunits form a cylinder around which DNA wraps and is held in place by an association of its negatively charged backbone with the positively charged lysines, and to a small extent positively charged arginines, and an association of H1 on the outside of the cylinder.[13]

Acetylation of histones by HAT effectively neutralizes the positive charge on lysine, which decreases the interaction between histone and DNA which loosens the nucleosome structure. This conformational change activates transcription by promoting the access of RNA polymerase, transcription factors, regulatory complexes, and other transcriptional machinery to the DNA template.[14] This process can be regulated by the presence of HDACs, which control the amount of acetylation by opposing the action of HATS, and thereby control the extent to which transcription can occur.

HDAC discovery and isolation

Acetylation of lysine residues located at the highly basic N-terminal domains of core histones has been found to be a reversible. This process can be followed by incubation of isolated nuclei in the presence of sodium acetate-2-^{14}C and observing the subsequent uptake.[15] With knowledge that histones are acetylated and deacetylated, many researchers began to evaluate the use of small molecules to try to find an inhibitor of this so called histone deacetylase (HDAC) activity, even though the enzyme responsible was still not isolated. The first major finding came when n-butyrate was found to cause a reversible accumulation of hyperacetylated histones within the nuclei of cells.[16] Following this, Yoshida *et al.* found that R-trichostatin A (TSA) (**5**) and trapoxin A (TPX) (**6**) were potent inhibitors of HDAC activity (Figure 3).[17,18] The compound TSA, which was first isolated from *Streptomyces hygroscopicus*, was found to be a reversible inhibitor of HDAC (75 nM), whereas TPX, first isolated from fungal metabolites, was an irreversible inhibitor of HDAC (<10 nM).[19] This irreversible activity was found to depend on the terminal epoxide of TPX. Schreiber *et al.* devised a novel approach for isolating HDACs utilized the covalent binding characteristic TBX and that relied on the aliphatic epoxyketone of TPX being isosteric with N-acetyl lysine (Figure 4).[20]

Figure 3. Natural product HDAC inhibitors.

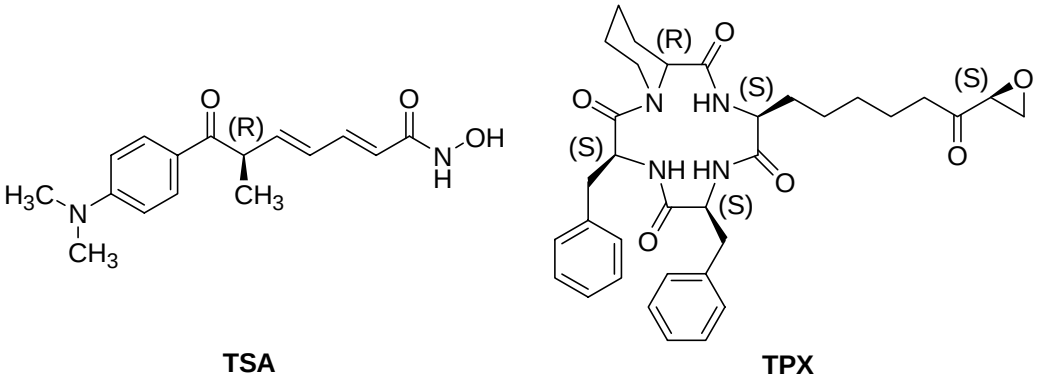
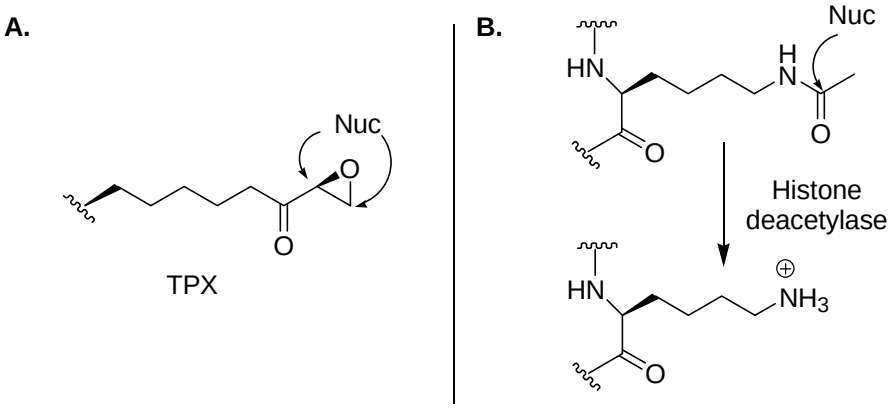
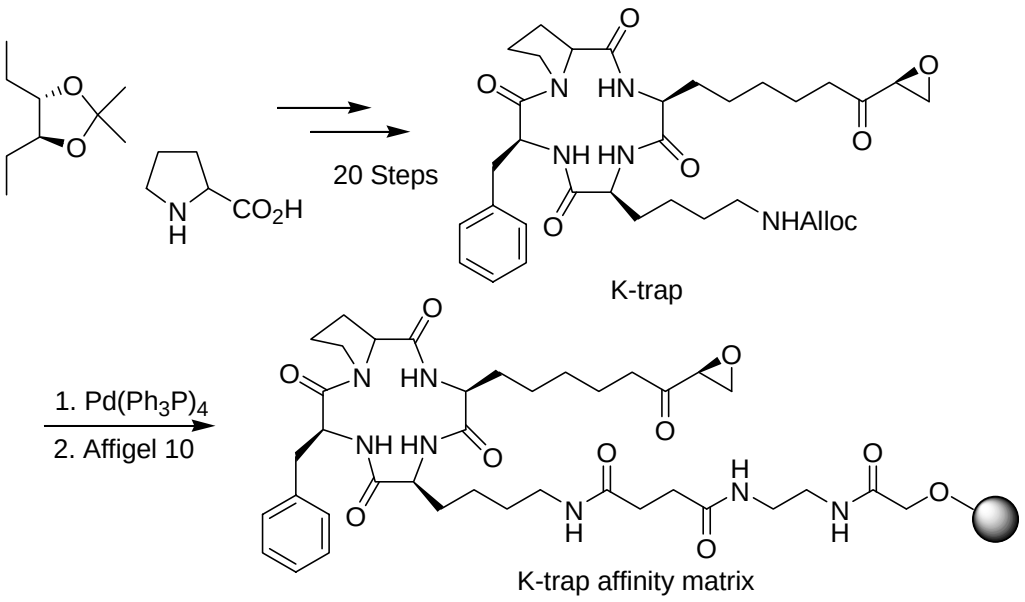


Figure 4. Nucleophile within active site of HDAC (A) proposed to react with the epoxide of TPX and (B) to deacetylate a histone lysine



To take advantage of the reactive epoxide in target identification studies by affinity trapping, TPX had to be attached to an affinity matrix. Because, TPX itself is not amendable to direct modification, Schreiber replaced one of the phenylalanine residues with a lysine residue, through which it could be covalently linked to a solid support. This new compound, which was synthesized in 20-steps from commercially available (R)-proline and (S,S)-threitol acetonide, was named K-trap, and it is structurally similar to TPX B, which differs from TPX A only by having a pyrrolidine in the backbone rather than a piperidine. Linkage of K-trap to the solid support, Affigel 10, afforded the K-trap affinity matrix needed to purify HDAC (Figure 5).[21]

Figure 5. K-trap synthesis and attachment to the solid support.



Use of this gel, in combination with anionic exchange resins, allowed them to isolate the HDAC protein in a denatured state. Subsequent protein microsequencing provided the amino acid sequence and confirmed the identity of HDAC.

HDACs and cancer treatment

It has been shown that the level of histone acetylation directly correlates to a wide variety of biological activities. Specifically, inhibition of HDAC can cause over expression of a variety of genes.[22] The over expression of these genes and their protein products causes growth arrest, differentiation, or apoptosis in a variety of cells. HDACs are typically over expressed in tumor cells, thus inhibition of HDACs can be a selective means for inducing differentiation of tumor cells, converting them from a malignant to a normal phenotype. This makes inhibition of HDAC a promising approach for the treatment of various cancers.[23]

HDAC TARGETING

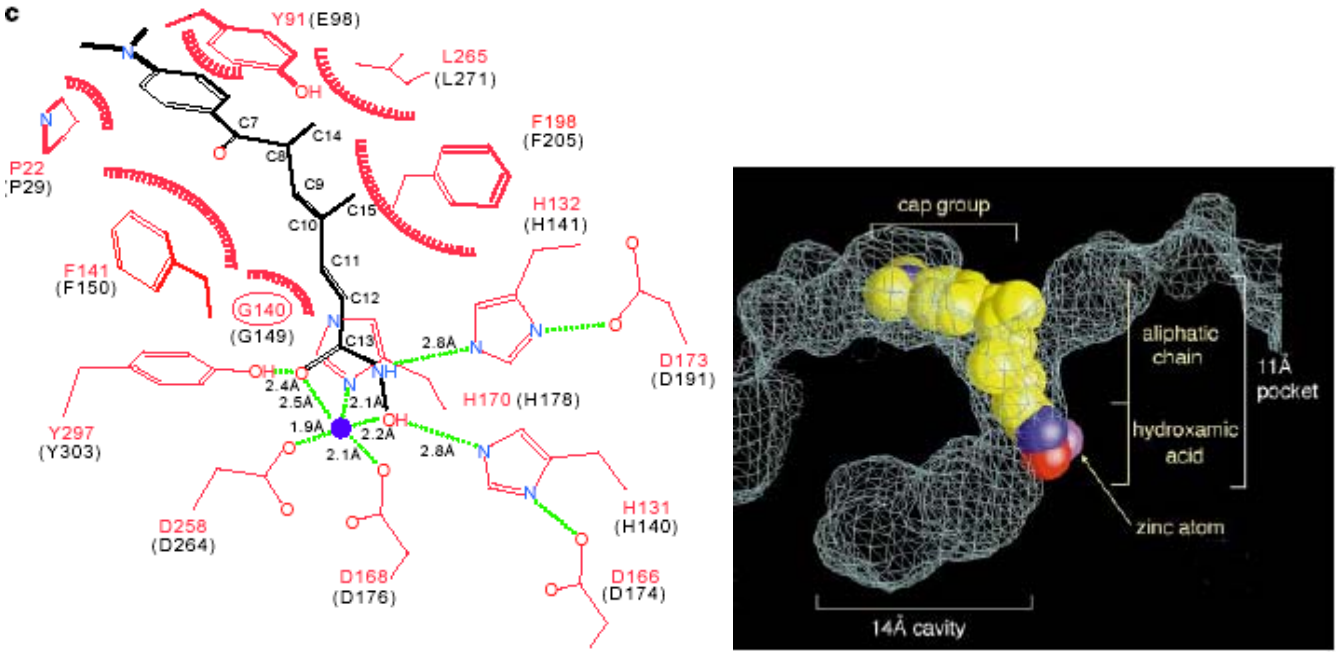
Role of HDACs

With the finding that HDAC inhibition is the cause of the selective differentiation malignant cells, new experimentation began to flourish and previous results were rationalized. It was at this time that the

link between SAHA activity and HDAC inhibition was discovered, and the molecular pathway of SAHA action could be studied directly.

To determine how SAHA and other inhibitors interact with HDAC, and possibly to maximize these interactions, a crystal structure of an inhibitor bound to HDAC was elucidated. Finding the proper inhibitor is often crucial to the process of obtaining a good crystal structure, and because of its high potency (optimal concentration 75 nM) and its defined structure, TSA proved to be the best choice.[24] Taking advantage of TSA, Breslow and coworkers first showed that HDAC deacetylates histones *in vitro* only after being incubated in the presence of zinc chloride. This was consistent with earlier suggestions that HDAC is a metalloprotein and therefore requires a metal cofactor.[25] Crystal structures were then obtained with TSA bound in the active pocket of an HDAC homologue. Clearly, it can be seen that the hydroxamic acid of TSA is chelating to the active zinc atom (Figure 7).[26]

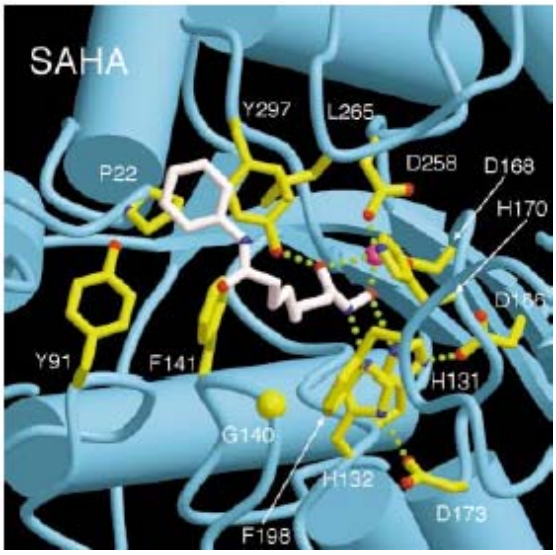
Figure 7. Close-up view of TSA-Zn^{2+} complex (red labels represent homolog residues, black labels represent corresponding HDAC residues) and space fill of TSA in active site.[20]



To see whether the same type of interactions exist between SAHA and HDAC, a crystal structure of SAHA bound to the HDAC homologue was also elucidated (Figure 8). As expected, the hydroxamic acid moiety in SAHA acts in the same fashion as in TSA. Differences in binding can be attributed to the

aliphatic chain of SAHA, which is more flexible than that of TSA. This flexibility limits the amount of hydrophobic interaction that can take place once SAHA is bound within the active site.

Figure 8. Crystal structure of SAHA bound to HDAC homologue.[20]



Beyond SAHA

Now that the crystal structure of an inhibitor bound to HDAC has been determined, work on finding even more potent inhibitors has been stimulated. Emphasis has been placed on varying the zinc chelating functionality, and changing the backbone structure of the molecule to allow more non-covalent interactions to take place between the inhibitor and the binding pocket.[27,28,29,30]

Conclusion

Overall, the field of HDAC inhibitors is relatively new and unexplored. Early successes such as SAHA have set the bar high for possible future drugs, and have revealed many aspects of HDACs activity. Currently SAHA, has passed phase I clinical trials with apparently flying colors, and it has entered into higher order clinical trials, being carried out by Atom Pharma.[31] Research into other possible drugs should continue to produce many new viable candidates and help to broaden the field.

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