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Kinetic and Thermodynamic Rationale for SAHA Being a Preferential Human HDAC8 Inhibitor as Compared to the Structurally Similar Ligand, TSA

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Abstract

Of the different hydroxamate-based histone deacetylase (HDAC) inhibitors, Suberoylanilide hydroxamic acid (SAHA) has been approved by the FDA for treatment of T-cell lymphoma. Interestingly, a structurally similar inhibitor, Trichostatin A (TSA), which has a higher *in vitro* inhibitory-potency against HDAC8, reportedly shows a poor efficacy in clinical settings. In order to gain the molecular insight into the above discriminatory feature, we performed transient kinetic and isothermal titration calorimetric studies for the interaction of SAHA and TSA to the recombinant form of human HDAC8. The transient kinetic data revealed that the binding of both the inhibitors to the enzyme showed the biphasic profiles, which represented an initial encounter of enzyme with the inhibitor followed by the isomerization of the transient enzyme-inhibitor complexes. The temperature-dependent transient kinetic studies with the above inhibitors revealed that the bimolecular process is primarily dominated by favorable enthalpic changes, as opposed to the isomerization step; which is solely contributed by entropic changes. The standard binding-enthalpy (ΔH^0) of SAHA, deduced from the transient kinetic as well as the isothermal titration calorimetric experiments, was 2–3 kcal/mol higher as compared to TSA. The experimental data presented herein suggests that SAHA serves as a preferential (target-specific/selective) HDAC8 inhibitor as compared to TSA. Arguments are presented that the detailed kinetic and thermodynamic studies may guide in the rational design of HDAC inhibitors as therapeutic agents.

INTRODUCTION

The acetylation and deacetylation of a specific lysine residue of the histone tail mediated, respectively, via histone acetyl transferase (HAT) and histone deacetylase (HDAC) play a pivotal role in eukaryotic gene expression.¹ Aside from histones, over 3700 distinct lysine-acetylation sites have been identified in the human proteome, which serve as targets for the above enzymes.² Various cellular processes such as cell growth and differentiation, DNA replication and recombination, metabolism, cell signaling, etc. are regulated via the acetylation and deacetylation of myriad varieties of non-histone proteins.³ Since HDACs cause deacetylation of acetylated-lysine moieties present on a wide variety of non-histone proteins, these enzymes should appropriately be called as “Lysine Deacetylases.”²

Human HDACs have been classified into four classes based on the homology of their gene sequence. Class I (HDAC1, 2, 3, and 8) and Class II (HDAC4, 5, 6, 7, 9, and 10) HDACs

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are Zn^{2+} -dependent lysine deacetylases, and are inhibited by canonical HDAC inhibitors.⁴ Unlike the above two classes, Class III HDACs (Sirtuins) are Zn^{2+} -independent lysine deacetylases which require NAD^+ as a cofactor, and are not inhibited by a canonical HDAC inhibitor.⁵ Class IV (HDAC 11) has a very poor sequence homology with other members of HDACs.⁶

HDACs are high priority drug targets for the treatment of various human diseases including cancer.^{7, 8} Different HDAC isozymes are overexpressed in various forms of human cancers.⁹ Inhibitors of HDACs are known to cause an anticancer effect both *in vitro* as well as in xenograft animal model.¹⁰ The HDAC inhibitors, namely SAHA and Romidepsin, have already been approved by the FDA for the treatment of T-cell lymphoma. Moreover, several others HDAC inhibitors are in the advanced stage of clinical trials.¹¹ However, the currently known inhibitors of HDAC produce severe side-effects on cancer patients, presumably because they indiscriminately targets several HDAC isozymes, many of which are vital for normal physiological process. Thus, there has been an ongoing effort to develop/design their alternative which would show a better *in vivo* efficacy.¹²

The inhibition constant (K_i) and the equilibrium dissociation constant (K_d) of an HDAC inhibitor often do not correlate with its *in vivo* efficacy. For instance, the *in vitro* potency of TSA against human HDACs is several fold higher than that of SAHA, but the latter inhibitor shows a better efficacy in the clinical settings.¹³ It is widely known that the physico-chemical (Lipinski parameters) as well as the ADME (absorption, distribution, metabolism and excretion) properties of a drug candidate play significant roles in defining its *in vivo* efficacy.^{14, 15} The hydroxamate-based HDAC inhibitors, such as TSA and SAHA, reportedly do not contain optimal physiochemical and ADME properties.^{16, 17} Interestingly, even the structurally similar compounds could have a marked difference in their ADME properties.¹⁷ A poor oral bioavailability of SAHA could be conceived from the fact that its linker domain contains an amide moiety, which is likely to reduce the oral bioavailability of the drug due to a strong hydrogen-bonding interaction with water molecules.¹⁸ On the other hand, a poor bioavailability of TSA could be partly correlated with the non-rotatable bonds of its linker domain. The latter feature reduce the molecular flexibility, an important parameter which has been proposed to be positively correlated with the membrane permeability and bioavailability.¹⁹

Aside from the ADME properties, the therapeutic efficacies of certain drugs have been correlated with the transient kinetic and the thermodynamic parameters of the protein-ligand complexes.^{20, 21} Markgrenn and co-workers have investigated the significance of k_{on} and k_{off} of the drug-target interaction in determining the therapeutic efficacy of HIV protease inhibitors.²² Copeland *et al.* have extensively reviewed the influence of a residence-time of the receptor- ligand complex on various biological functions.²³ Likewise, Davis and colleagues reported that the downstream biological effects of a peptide serving as an agonist/ antagonist of TCR-MHC complex are dictated by the kinetic parameters of receptor-ligand interaction.²⁴ Furthermore, Freire and other investigators suggest that the thermodynamic parameters for the drug-target interaction could be utilized as a complementary tool for lead-optimization process in order to enhance the therapeutic efficacy of a drug.^{21, 25, 26} In view of these literature precedent, it appeared logical to investigate the detailed kinetic and thermodynamic features for the binding of structurally similar ligands, SAHA and TSA, to the recombinant form of human HDAC8. Our experimental data provide the kinetic and thermodynamic rationale for SAHA being the target-specific/selective inhibitor of HDAC8 as compared to its structurally similar counterpart, TSA.

MATERIALS AND METHODS

TSA was purchased from Enzo Life Sciences (Farmingdale, NY). SAHA (suberoylanilide hydroxamic acid) was custom synthesized by Biomol Laboratories (Plymouth Meeting, PA). Coumarin-SAHA (c-SAHA) was synthesized in our laboratory as described previously.²⁷ Recombinant form of human HDAC8 was purified from *E. coli* as described previously.²⁷

Equilibrium Binding Studies for HDAC8-Inhibitor Interactions

All the steady-state spectrofluorometric studies were performed in protein storage buffer (50 mM Tris, pH 7.5, containing 100 mM NaCl, 3 mM MgCl₂, 10 % glycerol and 1 mM TCEP) on a Perkin-Elmer Lambda 50-B spectrofluorometer which was equipped with a magnetic stirrer and thermostated water bath using a 4 × 4 mm² square quartz cuvette. The change in intrinsic fluorescence signal of HDAC8 upon binding of an inhibitor was used to obtain the binding isotherm of the enzyme-inhibitor complex. In order to determine the equilibrium dissociation constant of an inhibitor for HDAC8, a fixed concentration of HDAC8 (1.5 μM) was titrated with an increasing concentration of the respective inhibitor in the protein storage buffer. The fluorescence emission spectrum of HDAC8 was monitored at 340 nm after excitation at 295 nm. The resulting binding isotherms for the HDAC8-inhibitor complex were analyzed via the complete solution of the quadratic equation (Eq.1).

$$F = C(I_{\text{tot}} + K_d + nE_{\text{tot}}) - \frac{[(E_{\text{tot}}n + I_{\text{tot}} + K_d)^2 - 4E_{\text{tot}}nI_{\text{tot}}]^{1/2}}{2} \quad \text{Eq. 1}$$

In Eq. 1 F is fluorescence signal of protein/ligand after the addition of inhibitor, E_{tot} , and I_{tot} refer to the total enzyme and total inhibitor concentration, K_d is the equilibrium dissociation constant of the enzyme-inhibitor complex, n is stoichiometry of the enzyme-inhibitor complex and C is the change in the amplitude of the signal.

Transient kinetics of HDAC8-ligand interaction

To determine the rate constants of binding as well as dissociation of HDAC8 inhibitors from the enzyme's site, transient kinetic experiments were performed using an Applied Photophysics SX-18MV stopped-flow system. The above stopped-flow system, which has a dead time of 1.3 ms, was operated in fluorescence mode with an emission path length of 2 mm. The time-dependent decrease in the intrinsic HDAC8 fluorescence was monitored by exciting the reaction at 280 nm using a cut-off filter of 320 nm. All of the transient kinetic experiments were performed at least ten times in 50 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 1 mM TCEP. The resultant kinetic traces were averaged, and were analyzed by the data analysis package provided by Applied Photophysics. For association kinetics, all the experiments were performed under pseudo first order condition. The kinetic traces were analyzed using single and double exponential rate equations (Eq. 2 and Eq. 3) as follows.

$$\text{RFU} = \text{Amp} \exp(-k_{\text{obs}} \cdot t) + \text{offset} \quad \text{Eq. 2}$$

In Eq. 2 RFU is the fluorescence signal at a given time. Amp and k_{obs} are the total amplitude and observed rate constant, respectively.

$$\text{RFU} = \text{Amp}_1 \exp(-k_{\text{obs}1} \cdot t) + \text{Amp}_2 \exp(-k_{\text{obs}2} \cdot t) + \text{offset} \quad \text{Eq. 3}$$

In Eq. 3 RFU represents the fluorescence signal at a given time. Amp_1 and Amp_2 are the respective amplitudes associated with observed rate constant ($k_{\text{obs}1}$ and $k_{\text{obs}2}$). Observed rate constants were measured as a function of the ligand concentration and data were analyzed using appropriate kinetic models as described in subsequent sections.

Determination of the dissociation rate of HDC8-inhibitor complexes

The dissociation rate (k_{off}) of TSA and SAHA from the enzyme's site was determined utilizing a fluorescent analog of SAHA, coumarin-SAHA (c-SAHA), as the displacing ligand. A competitive binding of c-SAHA to HDAC8 (concomitant with displacement of the enzyme bound non-fluorescent inhibitor) results in a decrease in the fluorescence signal at 400 nm ($\lambda_{ex} = 325$ nm).²⁷ The time-dependent change in the fluorescence signal was used to measure dissociation rate of the enzyme-inhibitor. Scheme 1 outlines the principle involved in determining the dissociation rate of the HDAC8-inhibitor complex.

When the HDAC8-TSA/SAHA complex is mixed with an excessive concentration of c-SAHA, the mass action drives the overall equilibrium towards the enzyme-c-SAHA complex. It is important to note that the association kinetics of c-SAHA is several folds faster than that of SAHA (our unpublished results). Under the above conditions the rate of formation of the HDAC8-c-SAHA complex is given by the dissociation rate of the inhibitor. The dissociation rate of the inhibitor was measured by mixing the following species: [HDAC8] = 1 μ M and [I] = 20 μ M (syringe I) with 100 μ M c-SAHA (syringe II) via the stopped-flow system, and monitoring the time-dependent decrease in fluorescence signal ($\lambda_{ex} = 325$ nm, "cutoff" filter = 395 nm) due to formation of the HDAC8-c-SAHA complex. The data was analyzed using a single exponential rate equation by data analysis software package provided by Applied Photophysics.

Temperature dependence of transient kinetic measurements

The temperature-dependent transient kinetic experiments were performed to delineate the energetics of the ligand-protein interaction utilizing the stopped-flow system. The experimental temperature was maintained using a circulating water-bath. The observed rate constant and the rate constants associated with the transient kinetic of binding and dissociation of ligand were determined at different temperatures, and they were analyzed by the Arrhenius equation (Eq. 4).

$$1/k_{obs} \text{ or } 1/k = A \exp(-E_a/RT) \quad \text{Eq. 4}$$

In the above equation k_{obs} and k , respectively, are the observed rate constant and the rate constant obtained from transient kinetic experiments, A is the frequency factor, E_a is the Arrhenius activation energy and T is the temperature in Kelvin. To convert E_a into the transition state enthalpy ($\Delta H^\ddagger$), the following relationship was used (Eq. 5).

$$\Delta H^\ddagger = E_a - RT \quad \text{Eq. 5}$$

The activation free energy ($\Delta G^\ddagger$) was calculated using the Eyring equation (Eq. 6).

$$\Delta G^\ddagger = -RT \ln(kh/k_B T) \quad \text{Eq. 6}$$

where R is the gas constant (1.986 cal K⁻¹ mol⁻¹), T is the absolute temperature, h is Plank's constant (1.58 $\times 10^{-34}$ cal s), and k_B is Boltzmann's constant (3.3 $\times 10^{-24}$ cal K⁻¹).

Isothermal Titration Calorimetric (ITC) studies

The enthalpy (ΔH^0) of binding of TSA and SAHA to HDAC8 was determined by performing isothermal titration calorimetric experiments on a VP-ITC (Microcal Inc., Northampton, MA). The enzyme and inhibitor solutions were properly degassed under vacuum. The ITC sample cell was filled with 1.8 mL (effective volume = 1.4 mL) of 10 μ M HDAC8 in 50 mM Tris, pH 7.5, containing 100 mM NaCl, 3 mM MgCl₂, 10 % glycerol and 1 mM TCEP. HDAC8 was titrated with 45 aliquots (4 μ L each) of TSA/SAHA prepared in

the HDAC8 dialysis buffer. The reaction mixture was continuously stirred at 250 rpm during the calorimetric titration. Raw experimental data were obtained as the amount of heat produced per second upon the addition of the ligand into the sample cell. The magnitude of heat produced per injection was calculated by the integration of area under individual peaks using Origin Software, provided with the ITC instrument. The observed heat signal in each injection was due to the heat associated with the binding and the background signal which was mainly due to the heat of dilution. The heats of dilution determined in the control experiments were essentially similar to the heat signal obtained at the end of the titration, so the average signal of the last five injections was used as the background heat signal. The final data were presented as the amount of heat produced per injection as a function of the molar ratio of the ligand. The data were analyzed using Origin Software (available from the Microcal), as described previously by Wiseman *et al.*,²⁸ yielding the value of stoichiometry (n), association constant (K_a), and the standard enthalpy change (ΔH°) for the binding of an inhibitor to HDAC8.

RESULTS

In a cursory manner, we observed that intrinsic fluorescence of HDAC8 is quenched upon binding to various enzyme inhibitors including TSA and SAHA. Figure 1 shows the steady state emission spectrum of 1.5 μM HDAC8 in 50 mM Tris-Cl, pH 7.5, containing 100 mM NaCl, 3 mM MgCl_2 , 10 % glycerol, and 1 mM TCEP upon excitation at 295 nm. HDAC8 showed the fluorescence emission maxima (λ_{max}) of 340 nm. Binding of an inhibitor (TSA/SAHA) led to the quenching of HDAC8's intrinsic fluorescence without any shift in the emission maxima (Figure 1). In order to probe the mechanism of the fluorescence quenching in the above cases, we performed the excited-state life-time measurement of HDAC8's tryptophan residues. The experimental data revealed that fluorescence life-time of the enzyme's tryptophan did not change upon binding of TSA/SAHA, suggesting the fluorescence quenching is "static" (rather than "dynamic") in nature (data not shown).

The equilibrium binding study of an inhibitor to HDAC8 was pursued utilizing the change in HDAC8's fluorescence signal upon ligand-binding. Figure 2 shows the titration of a fixed concentration of HDAC8 (1.5 μM) with an increasing concentration of the inhibitor. The fluorescence intensity of HDAC8 at 340 nm, which is dependent on its fractional occupancy of an inhibitor, is hyperbolically dependent on the inhibitor concentration. During the course of titration, the concentration of HDAC8 was comparable to that of the inhibitor; hence, the binding isotherms were analyzed by a complete solution of the quadratic equation (Eq. 1), yielding the equilibrium dissociation constant (K_d) for the binding of TSA and SAHA being equal to $0.39 \pm 0.08 \mu\text{M}$ and $1.2 \pm 0.2 \mu\text{M}$, respectively. We further determined the K_i (inhibition constant) values of TSA and SAHA using the protocol as described previously by our group,²⁷ and found their magnitudes to be $0.15 \pm 0.02 \mu\text{M}$ and $0.45 \pm 0.11 \mu\text{M}$, respectively. Note the similarity between the K_d and K_i values for both the ligands, suggesting that the inhibition or the quenching of the intrinsic HDAC8's fluorescence is due to the binding of the ligands to the enzyme active site. Furthermore, our experimental data suggests that HDAC8 has only one binding site for TSA, and the second TSA molecule bound to HDAC8, which has been previously observed in crystallographic studies, is presumably due to the crystal lattice forces.²⁹

Transient kinetics studies of inhibitor binding

To determine the kinetic mechanism of the binding of an inhibitor to HDAC8, we performed transient kinetic experiments using the stopped-flow system. Figure 3 (left panel) shows the representative stopped-flow kinetic trace for the association of TSA to HDAC8 obtained upon mixing 1 μM HDAC8 with 10 μM TSA via the stopped-flow syringes. The change in

the intrinsic fluorescence of HDAC8 due to the binding of an inhibitor was monitored upon exciting the mixture at 280 nm and using the cut-off filter of 330 nm. The kinetic trace was analyzed with a double-exponential rate equation yielding the fast (k_{obs1}) and the slow observed rate constant (k_{obs2}) as $5.58 \pm 0.09 \text{ s}^{-1}$ and $0.21 \pm 0.01 \text{ s}^{-1}$, respectively. We performed a similar stopped-flow transient kinetic experiment for the binding of SAHA to HDAC8. Figure 3 (right panel) shows the representative stopped flow kinetic trace for the association of SAHA to HDAC8 obtained upon mixing 1 μM HDAC8 with 20 μM SAHA via the stopped-flow syringes. The kinetic trace was analyzed with a double-exponential rate equation, yielding the fast (k_{obs1}) and slow observed rate constant (k_{obs2}) as $2.78 \pm 0.31 \text{ s}^{-1}$ and $0.087 \pm 0.043 \text{ s}^{-1}$, respectively. Note that the magnitude of both the fast and slow observed rate constants for the binding of TSA to HDAC8 is higher than those of SAHA, suggesting that the association kinetics of the former ligand is faster.

To delineate the microscopic pathways for the binding of an inhibitor to HDAC8, we measured the observed rate constants as a function of the inhibitor concentration. Figure 4 shows the concentration dependence of the observed rate constant (k_{obs}) for the binding of TSA and SAHA to HDAC8 (left and right panel, respectively). Note that while the magnitude of the fast observed rate constant (k_{obs1}) is linearly dependent on the ligand concentration, the slow observed rate constant (k_{obs2}) essentially remains constant. This observation is similar to what has been observed for the binding of TA-calmodulin to myosin muscle myosin light chain kinase by Trentham and collaborators.³⁰ The data were analyzed in the light of a two-step binding mechanism (Scheme 2).

In the above binding mechanism, the fast phase (bimolecular step) is likely to be due to the initial binding/encounter of the inhibitor (I) to the enzyme site, whereas the slow phase (isomerization) could be attributed to the isomerization of the transient encounter-complex (HDAC8-I*) to produce the final reaction product (HDAC8-I**). The constants k_{+1} , k_{-1} , k_{+2} , and k_{-2} are the rate constants of the association kinetics. The linear regression analysis of the data for k_{obs1} with the gradient and intercept, respectively, provides the values of k_{+1} and k_{-1} , and which were found to be $2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and 1.2 s^{-1} , respectively, for the TSA-binding. The corresponding values of the above kinetic parameters for the SAHA-binding were $6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and 1.4 s^{-1} . Evidently, the second order rate constant for the bimolecular (k_{+1}) step is one order of magnitude greater for the TSA-binding to HDAC8 as compared to SAHA. The value of k_{obs2} , which is representative of the isomerization step, is independent of the ligand concentration, and it is comprised of the sum of k_{+2} and k_{-2} .

Dissociation rate (k_{off}) of HDAC8 inhibitors

We determined the dissociation rates (k_{off}) of TSA and SAHA from the enzyme's site utilizing coumarin-SAHA (c-SAHA) as a fluorescent probe. The c-SAHA serves as a competitive ligand which triggers the dissociation of the non-fluorescent inhibitors (e.g., TSA and SAHA), when utilized in an excessively high concentration. Figure 5 shows the stopped-flow kinetic trace for the dissociation of TSA from the enzyme's site, which was obtained by mixing a solution containing 1 μM HDAC8 and 20 μM TSA with 100 μM c-SAHA via the stopped-flow syringes. The solid line is the best fit of the experimental data using the single exponential rate equation, yielding the value of k_{off} of TSA as 0.11 s^{-1} . The k_{off} of SAHA has been previously reported by our group as 0.41 s^{-1} , which is 4-fold higher than that of TSA.²⁷ For the kinetic model of the ligand-protein interaction described in Scheme 2, k_{off} of the ligand is given by the Eq. 7.³⁰

$$k_{off} = (k_{-1}k_{-2})/k_{+2} \quad \text{Eq. 7}$$

Taking into account the magnitudes of the dissociation rate (k_{off}) of TSA and SAHA as well as the observed rate constants (k_{obs}) of their association kinetics, we could deduce the values of all the four constants (k_{+1} , k_{-1} , k_{+2} , and k_{-2}), and thereby predict the value of equilibrium dissociation constant (K_d) for the binding of the above inhibitors. The equilibrium dissociation constants of TSA and SAHA deduced from the transient kinetic experiments were 0.49 μ M and 6.8 μ M, respectively. Note that the above values are in close agreement (within experimental errors) with the directly measured K_d values determined via a direct titration of the enzyme with the inhibitors determined from the steady-state experiments (Table 1). The similarity between the directly determined and the kinetically calculated K_d values validates the authenticity of the two-step binding mechanism of TSA and SAHA with HDAC8.

Temperature dependence of association kinetics

To determine the energetic contributions for the binding of TSA and SAHA to HDAC8 both in ground and putative transition states, we performed the temperature-dependent stopped-flow experiments. Figure 6 shows the temperature dependence of the observed rate constants for the association of TSA and SAHA. The fast observed rate constant (k_{obs1}) increases as a function of temperature, whereas k_{obs2} essentially remained constant for the binding of both inhibitors. The activation energy of the bimolecular step of the ligand-protein interaction was calculated using the Arrhenius Equation (Eq. 4). The activation energy (E_a) for the bimolecular step (fast phase) of the TSA and SAHA-binding to HDAC8 were 12 kcal/mol and 15 kcal/mol, respectively. Note that the activation energy for the association of SAHA is 3 kcal/mol higher than that of TSA.

We further investigated the effect of temperature on the forward and the reverse rate constants associated with the bimolecular step, i.e., k_{+1} and k_{-1} . Energy of activation was calculated from the temperature dependence of k_{+1} and k_{-1} for the binding of TSA and SAHA. The energy of activation (E_a) for the forward and the reverse steps of the bimolecular step for the binding of TSA were 9.3 kcal/mol and 13.5 kcal/mol, respectively. The corresponding values of the above activation parameters for the SAHA-binding were 11.4 kcal/mol and 18.5 kcal/mol. Evidently, the activation energy (E_a) for both the forward and the reverse reactions of the bimolecular step for the SAHA-binding is higher than that of TSA, which accounts for the fact that the association kinetics of SAHA to HDAC8 is slower.

Temperature dependence of dissociation rate (k_{off}) of TSA and SAHA from HDAC8's site

In view of delineating the energetics of dissociation of TSA and SAHA from the HDAC8's active site, we measured the dissociation rates at different temperatures ranging from 5 to 25 °C. Figure 7 shows the Arrhenius plots for the k_{off} of TSA and SAHA. The solid lines are the best fit of the experimental data according to the Arrhenius Equation (Eq. 4) with the activation free energy (E_a) of 6.4 kcal/mol and 15 kcal/mol, respectively, for the dissociation of TSA and SAHA from the enzyme's site. Note that the activation energy (E_a) for the dissociation of SAHA is about 2-fold higher than that of TSA. Although, the activation energy (E_a) for the dissociation of TSA is about 2-fold lower than that of SAHA, its dissociation rate (k_{off}) at 25 °C is 4-fold lower. Evidently, enthalpic and entropic changes associated with the formation of the ground as well as the putative transition states of the enzyme-inhibitor complex prior to the dissociation of the above inhibitors from the HDAC8's site are significantly different.

Isothermal Titration Calorimetric (ITC) studies for the binding of TSA and SAHA

In order to measure the calorimetric enthalpy for the binding of TSA and SAHA to HDAC8, we performed isothermal titration calorimetric experiments (Methods section). Figure 8

shows the titration of 10 μM HDAC8 with 45 injections (4 μl each) of 200 μM of inhibitor (TSA/SAHA) in 50 mM Tris buffer pH 7.5, containing 100 mM NaCl, 3 mM MgCl_2 , 10 % glycerol and 1 mM TCEP at 25 $^\circ\text{C}$. The panel A of Figure 8 shows the raw calorimetric data, representing the amount of heat produced (negative exothermic peaks) following each injection of the inhibitor. The area under each peak represents the amount of heat produced upon the binding of the inhibitor to HDAC8. The panel B of Figure 8 shows the plot of the amount of heat generated per injection as a function of the molar ratio of the ligand. The smooth lines in the above figure represent the best fit of the experimental data according to the equation described by Wiseman *et al.*,²⁸ yielding the thermodynamic parameters for binding of TSA and SAHA to HDAC8 (see Table 2). The ΔH^0 for the binding of SAHA and TSA to HDAC8 were -10.9 ± 0.15 and -8.9 ± 0.12 kcal/mol, respectively. Evidently, ΔH^0 for the binding of SAHA is 2 kcal/mol higher than that of the TSA-binding to HDAC8. Furthermore, the entropic penalty for the binding of TSA measured at 25 $^\circ\text{C}$ is 10-fold lower than that of SAHA.

DISCUSSION

With our observation that the intrinsic fluorescence of HDAC8 is quenched upon binding of an inhibitor, we could investigate the detailed transient kinetic mechanism for the interaction of two structurally similar inhibitors, TSA and SAHA, with the recombinant form of human HDAC8. The experimental data presented herein revealed the following salient features of the association and dissociation kinetics of the above ligands. The association of both TSA and SAHA to HDAC8 conformed to two kinetically resolvable steps i.e., the first (bimolecular) step leading to the formation of the transient enzyme-inhibitor complex followed by the second isomerization step (Scheme 2). The first (bimolecular) step is faster than the isomerization in both of the above cases. Even though TSA and SAHA are structurally similar ligands, their association and dissociation kinetics are markedly different. The kinetics of the binding of TSA to HDAC8 is faster than SAHA, whereas the dissociation rate of TSA from the enzyme's site is 4-times slower. Whereas the first (bimolecular) step for the binding of both the inhibitors is primarily contributed by enthalpic changes, the isomerization step is solely given by entropic changes. The standard binding enthalpy (ΔH^0) of SAHA derived from the transient kinetic as well as the isothermal titration experiments is about 2–3 kcal/mol higher than that given by TSA.

HDAC8 contains four tryptophan residues, of which two are partially exposed to the bulk solvent.²⁹ The ligand-induced conformational change in HDAC8 due to the binding of an inhibitor would alter the microenvironment of above tryptophan residues, resulting into quenching of the intrinsic protein fluorescence. Furthermore, the excited-state life-time of the HDAC's tryptophan remained the same upon the binding to TSA or SAHA, suggesting the fact that the inhibitor forms a ground-state complex with tryptophan, which is non-fluorescent in nature.

It is important to note that the equilibrium dissociation constants (K_d) for the binding of TSA and SAHA to HDAC8 deduced from the transient kinetic experiment are in the close agreement (within experimental error) with the values obtained via the equilibrium binding experiments (Table 1), validating the authenticity of the proposed kinetic model (Scheme 2). The forward rate constants, k_{+1} and k_{+2} , for the interaction TSA to HDAC8 are an order of magnitude higher than those of SAHA. We believe that the origin of the above feature lies in the structural differences in SAHA and TSA (Figure 9) and their mode of interaction with HDAC8. Note that the dimethyl aniline moiety of TSA makes a π - π interaction with Y100 residue of HDAC8 (see Figure 9, left panel).²⁹ The above feature would facilitate the proper orientation of the binding partners, thus enhancing the magnitude of the forward rate constants (k_{+1} and k_{+2}) for the TSA-binding to HDAC8. On the other hand, the rate reverse

constants for the initial binding (k_{-1}) and the isomerization of the enzyme-inhibitor complex (k_{-2}) are of similar magnitudes for both the ligands. A comparative account of the experimentally determined K_d and the microscopic rate constants for the binding of these ligands to the enzyme reveals that the isomerization step plays an important role in enhancing the binding affinity of the both TSA and SAHA to HDAC8; albeit it is more pronounced in the former case. We measured the dissociation rate of TSA and SAHA utilizing a fluorescent analog of SAHA, coumarin-SAHA. The magnitude of dissociation rate (k_{off}) of the inhibitor from the enzyme's site is dependent on the value of three rate constants, k_{-1} , k_{-2} and k_{+2} , ($k_{off} = k_{-1} k_{-2} / k_{+2}$; Eq. 7). Since, the forward isomerization rate constant (k_{+2}) for the interaction of TSA with HDAC8 is an order of magnitude higher than SAHA, the former ligand dissociates slower (4-fold) from the enzyme's site. The fast dissociability of SAHA from HDAC8 can be argued to decrease the resident time of the enzyme-SAHA complex in physiological system, and such feature has a potential to obviate untoward side-effects associated with an extended downstream cell signaling process.

We further delineated the energetics profiles of the enzyme-inhibitor interactions by performing the temperature-dependent transient kinetic studies (see Figure 10). The microscopic rate constants for the binding of both TSA and SAHA were determined, and they were translated into the energy of individual components (see Table 3). The data in Table 3 was utilized to construct the energy diagrams for the binding of TSA and SAHA to HDAC8 (Figure 10). The free energy diagrams shown in Figure 10 (left panel) reveal that ΔG^0 for the formation of the enzyme-inhibitor encounter-complex and the subsequent isomerization step are about 0.9 kcal/mol and 0.7 kcal/mol more favorable for TSA than for SAHA. In addition, the putative transition states ($\Delta G^\ddagger$) for the formation of the encounter and the isomerized-complexes are 0.8 kcal/mol and 1.1 kcal/mol more favorable for TSA than for SAHA. Clearly, as compared to TSA, SAHA requires more structural adjustments with the enzyme's active site pocket to attain the encounter and the isomerized complexes, implying to the fact that latter ligand (SAHA) makes more specific interactions with the enzyme. Note that the linker moiety of TSA is shorter, bulkier, and rotationally constrained as comparison to SAHA (Figure 9). We believe that rotational flexibility and hydrophobicity of SAHA (vis a vis TSA), as well as the entropic penalty associated with the HDAC8-SAHA interaction, are responsible for energetic unfavorability. The above thermodynamic signature (ΔG^0 and ΔH^0) for the SAHA-binding is likely to enhance the specificity/selectivity of the SAHA-binding to HDAC8. However, the above energetic constrains is more favorable for the dissociation of SAHA from the enzyme's site because of the high entropic favorability. It is important to note that the Arrhenius activation energy (E_a) for the observed dissociation (k_{off}) of SAHA from the enzyme's site is about 2-fold higher than that of TSA (Figure 7), suggesting the fact that the HDAC8-SAHA complex requires a greater amount of heat energy ($\Delta H/E_a$) to reach the putative transition state prior to the complete dissociation of the ligand from the target site. Thus, a higher value of E_a for the observed dissociation of SAHA from the enzyme site further attests the fact that it makes more specific interactions with the enzyme as compared to TSA.

By determining the temperature-dependent microscopic parameters for the binding of TSA and SAHA to HDAC8, we could dissect the enthalpic contributions from the overall free energy changes in both the ground and the putative transition states for the respective complexes. Since the values of k_{+2} and k_{-2} for the binding of TSA and SAHA are independent of temperature, it is tempting to propose that the isomerization step is entropically driven. Hence, the enthalpic contribution in both the ground and the putative transition states is realized up to the formation of the enzyme-ligand encounter complexes. The enthalpy diagram (see Figure 10, right panel) clearly shows that whereas SAHA (vis a vis TSA) has to cross a higher enthalpic barrier in both the forward and the reverse directions; it is enthalpically more stable in the ground state of the enzyme-inhibitor

encounter complex. A qualitatively similar conclusion is derived from the isothermal titration calorimetric studies for the binding of SAHA versus TSA (Figure 8). It is important to note the ΔH^0 for the binding of TSA as well as SAHA to HDAC8 directly measured via ITC is about 2–3 kcal/mol higher than the kinetically derived ΔH^0 (see Table 2 and Table 3). We believe the origin of this discrepancy lies in the fact that kinetically derived ΔH^0 is essentially the measure of van't Hoff enthalpy, which in many cases differs from the directly measured calorimetric enthalpy.³³

It is important to note that the molecular origin as well as the structural parameterization of ΔH^0 of a biomolecular interaction is often complex.³⁴ However, the thermodynamic studies for the binding of structurally similar ligands to a common target in conjunction with their crystallographic studies suggest that a higher (negative) binding-enthalpy (ΔH^0) is primarily linked with the specific interactions (such as hydrogen bonding) between the binding partners. For instance, the HIV protease and HMG CoA inhibitors containing a high binding-enthalpy for the target have been found to be more target-specific/selective, and reportedly serve as better drugs.³⁵ In view of the above fact, we became interested in rationalizing enthalpic favorability for the binding of SAHA to HDAC8 as compared to its structural analog (TSA) in the light of the structural features of the HDAC8-TSA and SAHA complexes. The structural data revealed that, unlike TSA, the carbonyl moiety of anilino group of SAHA makes a specific hydrogen bond with Asp101 of HDAC8 (Figure 9),²⁹ which is similar to what has been observed for the binding of 3-(1-Methyl-4-phenylacetyl-1H-2-pyrrolyl)-N-hydroxy-2-propenamide (APHA) to HDAC8.³⁶ Such hydrogen bond could easily yield an enthalpic gain of 3–4 kcal/mol in stabilization of SAHA (vis a vis TSA) to the active site pocket of HDAC8. However, we do observe that the activation enthalpy for the formation of the putative transition state from the free form of SAHA and HDAC8 is about 2 kcal/mol higher than that for TSA, which could be attributed to an enthalpic penalty associated with the desolvation of polar moieties prior to the formation of the above hydrogen bond.³⁷ A high enthalpic penalty for the attainment of the transition state for the HDAC8-SAHA interaction would further enhance the selectivity/specificity of the target-inhibitor interaction. The enthalpic gain obtained due to the formation of a specific hydrogen bond between SAHA and Asp101 is not translated into a gain in the binding affinity of the ligand. An entropic penalty of 2.0 kcal/mol is introduced presumably due to the reduction in the conformational flexibility of protein and/or ligand upon the hydrogen bonding. Unlike SAHA, the linker region of TSA does not make hydrogen bond with Asp101, yielding a lower binding enthalpy.²⁹ Furthermore, the change in entropy (ΔS^0) associated with the HDAC8-TSA encounter-complex is entropically favored by 1.8 kcal/mol, which is translated into an enhanced binding affinity of the ligand. A higher entropic gain in the case of the TSA-binding to HDAC8 could largely be due to hydrophobicity/non-polar interactions. Hydrophobic interactions, in general, promote non-specific interactions, and hence it is not surprising to see that TSA would show considerable side-effects in cellular system due to its non-specific binding to multiple off-target proteins. A cumulative account of these features clearly attests to the suitability of SAHA (over TSA) for inhibition of HDAC8 not only at the enzymatic level but presumably also under the physiological condition. This, added with the fact that SAHA exhibits better ADEM profile than TSA, it is not surprising that it has been chosen as therapeutic agent for the treatment of T-Cell lymphoma.

In conclusion, we have thoroughly elucidated the transient kinetics and the thermodynamics of the interaction of two structurally similar inhibitors (TSA and SAHA) with the recombinant form of human HDAC8. The experimental data provide the kinetic as well as the thermodynamic rationale for SAHA being a more target-specific/selective HDAC8 inhibitor as compared to TSA, and thus SAHA can elicit more selective inhibitory feature under the physiological condition. However, it should be clarified that our experimental data

alone are not adequate to substantiate or refute the potential efficacy of SAHA over TSA under the clinical settings.

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References

- Berger SL. Histone modifications in transcriptional regulation. *Curr. Opin. Genet. Dev.* 2002; 12:142–148. [PubMed: 11893486]
- Choudhary C, Kumar C, Gnäd F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science*. 2009; 325:834–840. [PubMed: 19608861]
- Reichert N, Choukrallah M-A, Matthias P. Multiple roles of class I HDACs in proliferation, differentiation, and development. *Cell. Mol. Life Sci. Cmls.* 2012; 69:2173–2187.
- Gregoret IV, Lee Y-M, Goodson HV. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *J. Mol. Biol.* 2004; 338:17–31. [PubMed: 15050820]
- Sauve AA, Youn DY. Sirtuins: NAD(+)-dependent deacetylase mechanism and regulation. *Curr. Opin. Chem. Biol.* 2012; 16:535–543. [PubMed: 23102634]
- Gao L, Cueto MA, Asselbergs F, Atadja P. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. *J. Biol. Chem.* 2002; 277:25748–25755. [PubMed: 11948178]
- Kazantsev AG, Thompson LM. Therapeutic application of histone deacetylase inhibitors for central nervous system disorders. *Nat. Rev. Drug Discov.* 2008; 7:854–868. [PubMed: 18827828]
- McKinsey TA. Therapeutic potential for HDAC inhibitors in the heart. *Annu. Rev. Pharmacol. Toxicol.* 2012; 52:303–319. [PubMed: 21942627]
- Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. *Mol. Oncol.* 2007; 1:19–25. [PubMed: 19383284]
- Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat. Rev. Drug Discov.* 2006; 5:769–784. [PubMed: 16955068]
- Wagner JM, Hackanson B, Lübbert M, Jung M. Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy. *Clin. Epigenetics.* 2010; 1:117–136. [PubMed: 21258646]
- Subramanian S, Bates SE, Wright JJ, Espinoza-Delgado I, Piekarczyk RL. Clinical Toxicities of Histone Deacetylase Inhibitors. *Pharmaceuticals.* 2010; 3:275. Markgren, P.-O., Schaal, W., Hämäläinen, M., Karlén, A., Hallberg, A., Samuelsson, B., and Danielson, U. H. (2002).
- Dokmanovic M, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. *Mol. Cancer Res. MCR.* 2007; 5:981–989.
- Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 1997; 23:3–25.
- Li AP. Screening for human ADME/Tox drug properties in drug discovery. *Drug Discov. Today.* 2001; 6:357–366. [PubMed: 11267922]
- Kelly WK, O'Connor OA, Krug LM, Chiao JH, Heaney M, Curley T, MacGregore-Cortelli B, Tong W, Secrist JP, Schwartz L, Richardson S, Chu E, Olgac S, Marks PA, Scher H, Richon VM. Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. *J. Clin. Oncol.* 2005; 23:3923–3931. [PubMed: 15897550]
- Elaut G, Laus G, Alexandre E, Richert L, Bachellier P, Tourwé D, Rogiers V, Vanhaecke T. A metabolic screening study of trichostatin A (TSA) and TSA-like histone deacetylase inhibitors in rat and human primary hepatocyte cultures. *J. Pharmacol. Exp. Ther.* 2007; 321:400–408. [PubMed: 17218485]

18. Veber DF, Johnson SR, Cheng H-Y, Smith BR, Ward KW, Kopple KD. Molecular Properties That Influence the Oral Bioavailability of Drug Candidates. *J. Med. Chem.* 2002; 45:2615–2623. [PubMed: 12036371]
19. Navia MA, Chaturvedi PR. Design principles for orally bioavailable drugs. *Drug Discov. Today.* 1996; 1:179–189.
20. Lu H, Tonge PJ. Drug-target residence time: critical information for lead optimization. *Curr. Opin. Chem. Biol.* 2010; 14:467–474. [PubMed: 20663707]
21. Borea PA, Dalpiaz A, Varani K, Gilli P, Gilli G. Can thermodynamic measurements of receptor binding yield information on drug affinity and efficacy? *Biochem. Pharmacol.* 2000; 60:1549–1556. [PubMed: 11077036]
22. Markgren P-O, Schaal W, Hämäläinen M, Karlén A, Hallberg A, Samuelsson B, Danielson UH. Relationships between structure and interaction kinetics for HIV-1 protease inhibitors. *J. Med. Chem.* 2002; 45:5430–5439. [PubMed: 12459011]
23. Tummino PJ, Copeland RA. Residence time of receptor-ligand complexes and its effect on biological function. *Biochemistry.* 2008; 47:5481–5492. [PubMed: 18412369]
24. Lyons DS, Lieberman SA, Hampl J, Boniface JJ, Chien Y, Berg LJ, Davis MM. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity.* 1996; 5:53–61. [PubMed: 8758894]
25. Freire E. Do enthalpy and entropy distinguish first in class from best in class? *Drug Discov. Today.* 2008; 13:869–874. [PubMed: 18703160]
26. Ladbury JE, Klebe G, Freire E. Adding calorimetric data to decision making in lead discovery: a hot tip. *Nat. Rev. Drug Discov.* 2010; 9:23–27. [PubMed: 19960014]
27. Singh RK, Mandal T, Balasubramanian N, Cook G, Srivastava DK. Coumarin-suberoylanilide hydroxamic acid as a fluorescent probe for determining binding affinities and off-rates of histone deacetylase inhibitors. *Anal. Biochem.* 2011; 408:309–315. [PubMed: 20816742]
28. Wiseman T, Williston S, Brandts JF, Lin LN. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem.* 1989; 179:131–137. [PubMed: 2757186]
29. Somoza JR, Skene RJ, Katz BA, Mol C, Ho JD, Jennings AJ, Luong C, Arvai A, Buggy JJ, Chi E, Tang J, Sang B-C, Verner E, Wynands R, Leahy EM, Dougan DR, Snell G, Navre M, Knuth MW, Swanson RV, McRee DE, Tari LW. Structural snapshots of human HDAC8 provide insights into the class I histone deacetylases. *Structure* 1993. 2004; 12:1325–1334.
30. Török K, Trentham DR. Mechanism of 2-chloro-(epsilon-amino-Lys75)-[6-[4-(N,N-diethylamino)phenyl]-1,3,5-triazin-4-yl]calmodulin interactions with smooth muscle myosin light chain kinase and derived peptides. *Biochemistry.* 1994; 33:12807–12820. [PubMed: 7947686]
31. Dierynck I, Wit MD, Gustin E, Keuleers I, Vandersmissen J, Hallenberger S, Hertogs K. Binding Kinetics of Darunavir to Human Immunodeficiency Virus Type 1 Protease Explain the Potent Antiviral Activity and High Genetic Barrier. *J. Virol.* 2007; 81:13845–13851. [PubMed: 17928344]
32. Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. *Nat. Biotechnol.* 2007; 25:84–90. [PubMed: 17211407]
33. Naghibi H, Tamura A, Sturtevant JM. Significant discrepancies between van't Hoff and calorimetric enthalpies. *Proc. Natl. Acad. Sci.* 1995; 92:5597–5599. [PubMed: 7777555]
34. Leavitt S, Freire E. Direct measurement of protein binding energetics by isothermal titration calorimetry. *Curr. Opin. Struct. Biol.* 2001; 11:560–566. [PubMed: 11785756]
35. Kawasaki Y, Freire E. Finding a better path to drug selectivity. *Drug Discov. Today.* 2011; 16:985–990. [PubMed: 21839183]
36. Dowling DP, Gantt SL, Gattis SG, Fierke CA, Christianson DW. Structural Studies of Human Histone Deacetylase 8 and Its Site-Specific Variants Complexed with Substrate and Inhibitor. *Biochemistry.* 2008; 47:13554–13563. [PubMed: 19053282]
37. Cabani S, Gianni P, Mollica V, Lepori L. Group contributions to the thermodynamic properties of non-ionic organic solutes in dilute aqueous solution. *J. Solut. Chem.* 1981; 10:563–595.

Figure 1. The steady state fluorescence emission spectra (black traces) of 1.5 μ M HDAC8 upon excitation at 295 nm. The intrinsic fluorescence of HDAC8 is quenched (red trace) upon binding to TSA.

Figure 2. Binding isotherms for the interaction of TSA (left Panel) and SAHA (right panel) with HDAC8. The decrease in intrinsic fluorescence of HDAC8 at 340 nm ($\lambda_{ex}=295$ nm) has been plotted as a function of an increasing concentration of the respective ligand. The solid lines are the best fit of the experimental data, yielding the K_d values of 0.39 ± 0.08 μ M and 1.2 ± 0.2 μ M for TSA and SAHA, respectively.

Figure 3.

Representative stopped-flow kinetic traces for the binding of TSA (left panel) and SAHA (right panel) to HDAC8. The solid smooth lines represent the best fit of the data for the inhibitor-binding according to the double exponential rate equation yielding the fast and the slow observed rate constants which were found to be $5.58 \pm 0.09 \text{ s}^{-1}$ and $0.21 \pm 0.01 \text{ s}^{-1}$ for the TSA-binding. The corresponding values for the SAHA-binding were $2.78 \pm 0.31 \text{ s}^{-1}$ and $0.087 \pm 0.043 \text{ s}^{-1}$, respectively.

Figure 4.

Concentration dependence of the observed rate constants for the binding of TSA (left panel) and SAHA (right panel) to HDAC8. A linear regression line to the data ($\bullet$) for the TSA-binding provides the gradient (k_{+1}) and the intercept (k_{-1}), respectively, as $2.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and 1.2 s^{-1} , whereas the horizontal line shows the best fit of the data ($\circ$) providing the magnitude of the k_{obs2} ($= k_{+2} + k_{-2}$) as 0.37 s^{-1} . A linear regression line to the data ($\bullet$) for the SAHA-binding provides the gradient (k_{+1}) and the intercept (k_{-1}), respectively, as $6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and 1.4 s^{-1} . The best fit of the data ($\circ$) showing the horizontal line determines the magnitude of the k_{obs2} ($= k_{+2} + k_{-2}$) as 0.089 s^{-1} .

Figure 5.

The representative stopped-flow trace for the dissociation of TSA from HDAC8's active site. The dissociation of the enzyme-bound TSA was triggered by the mixing of the HDAC8-TSA complex with the high concentration of c-SAHA. The solid smooth line is the best fit of the experimental data according to the single exponential rate equation, yielding the dissociation rate of TSA as $0.11 \pm 0.02 \text{ s}^{-1}$.

Figure 6.

Temperature dependence of the observed rate constants for the bimolecular and the isomerization step of the HDAC8 interaction with TSA and SAHA. The Arrhenius activation energy (E_a) for the bimolecular step for TSA (left panel) and SAHA (right panel) were calculated from the best fit of experimental data using the Arrhenius equation as 12 and 15 kcal/mol, respectively. The isomerization step has zero activation energy.

Figure 7. Arrhenius plots for the observed dissociation rate of TSA and SAHA measured using c-SAHA. The solid lines are the best fit of the experimental data using the Arrhenius equation with the activation energy as 5.6 kcal/mole and 12.8 kcal/mole for TSA (left panel) and SAHA (right panel), respectively.

Figure 8.

ITC profile for the binding of TSA (left panel) and SAHA (right panel). Panel A shows the calorimetric data. The solid smooth lines in panel B represent the best fit of the experimental data yielding the thermodynamic parameters of ligand binding listed in Table 2.

Figure 9.

Closer view of the active site pocket of HDAC8 bound with TSA and SAHA. The dimethyl aniline moiety of TSA makes a π - π interaction Y100 residue of HDAC8 (pdb 1T64). The Asp 101 residue of HDAC8 makes a specific hydrogen bond with the connector group of SAHA (pdb 1T69).

Figure 10.

Free energy (left panel) and enthalpy (right panel) profiles for the binding of TSA and SAHA. The free energies and the enthalpies of the enzyme and the ligand were taken as being equal to zero.

Scheme 1.

Measurement of dissociation rate of HDAC8 inhibitor

$$k_{obs1} = k_{+1} [\text{I}] + k_{-1}$$

$$k_{obs2} = k_{+2} + k_{-2}$$

Scheme 2.

Two step binding mechanism of HDAC8 inhibitors to enzyme

Table 1

Comparison of K_d calculated from transient kinetic method with spectrofluorometric titration.

Ligand	k_{+1} ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	k_{+2} (s^{-1})	k_{-2} (s^{-1})	K_d (kinetic method)	K_d (spectrofluorometric titration method)
TSA	2.3×10^5	1.2	0.34	0.03	0.49 μM	0.39 $\pm$ 0.08 μM
SAHA	6.0×10^4	1.4	0.069	0.02	6.8 μM	1.2 $\pm$ 0.2 μM

Table 2

Summary of the thermodynamic parameters for the binding of TSA and SAHA to HDAC8 at 25 °C

Inhibitor	ΔG^0 (kcal/mol)	ΔH^0 (kcal/mol)	$T\Delta S^0$ (kcal/mol)	K_a (M^{-1})	Stoichiometry
SAHA	-8.4	-10.9 ± 0.15	-2.5	8.1×10^5	0.71
TSA	-8.6	-8.9 ± 0.12	-0.3	1.67×10^6	0.82

Table 3

Transient kinetic parameters for the interaction of TSA and SAHA with HDAC8

Parameter (Energy in kcal/mol)	TSA	SAHA
ΔG^0 (overall)	-8.5	-7.0
$\Delta G^0 (k_{-1}/k_{+1})$	-7.2	-6.3
$\Delta G^\ddagger_{k_{+1}}$	10.1	10.9
$\Delta H^\ddagger_{k_{+1}}$	8.6	10.8
$\Delta G^\ddagger_{k_{-1}}$	17.3	17.2
$\Delta H^\ddagger_{k_{-1}}$	14.0	19.1
$\Delta H(k_{-1}/k_{+1})$	-5.4	-8.3
$T\Delta S^0 (k_{-1}/k_{+1})$	1.8	-2.0
$\Delta G (k_{-2}/k_{+2})$	-1.4	-0.7
$\Delta G^\ddagger_{k_{+2}}$	18.1	19.0
$\Delta G^\ddagger_{k_{-2}}$	19.5	19.7