Mechanism of N-Acylthiourea-mediated Activation of Human Histone Deacetylase 8 (HDAC8) at Molecular and Cellular Levels*

Received for publication, July 28, 2014, and in revised form, January 8, 2015. Published, JBC Papers in Press, January 20, 2015, DOI 10.1074/jbc.M114.600627

Raushan K. Singh‡, Kyongshin Cho‡, Satish K. R. Padi‡, Junru Yu, Manas Haldar‡, Tanmay Mandal‡, Changhui Yan‡, Gregory Cook‡, Bin Guo‡, Sanku Mallik‡, and D. K. Srivastava††

From the Departments of ‡Chemistry and Biochemistry, ¶Animal Science, §Pharmaceutical Sciences, and †Computer Science, North Dakota State University, Fargo, North Dakota 58108

Background: N-Acylthiourea (TM-2-51) is a selective activator for human histone deacetylase 8 (HDAC8). To probe the molecular mechanism of the enzyme activation, we performed a detailed account of the steady-state kinetics, thermodynamics, molecular modeling, and cell biology studies. The steady-state kinetic data revealed that TM-2-51 binds to HDAC8 at two sites in a positive cooperative manner, and it produces anticancer effect in neuroblastoma cells.

Results: TM-2-51 modulates the binding thermodynamics/kinetics of substrate/inhibitor to HDAC8, and it enhances the cellular expression of p53/p21.

Conclusion: TM-2-51 binds to HDAC8 at two sites in a positive cooperative manner, and it produces anticancer effect in neuroblastoma cells.

Significance: These mechanistic studies will shed light on designing HDAC-selective activators as potential therapeutic agents.

Epigenetic processes in eukaryotic cells are manifested via the covalent modification of DNA as well as histone proteins (1). The latter involves acetylation, methylation, sumoylation, and other modifications of selected residues of histones. The acetylation level of the lysine residues of the histone tails is modulated via the dynamic interplay between histone acetyltransferase and histone deacetylase (HDAC). The catalytic activities of these enzymes alter the chromatin structure and thereby regulate the vital cellular processes such as DNA replication, repair, transcription, and others (2). Because the histones were identified as the first cellular targets of human HDACs, these enzymes were initially thought to be the histone modifiers, localized in the nucleus (3). Subsequently, a myriad of non-histone targets of these enzymes were discovered, highlighting their roles in various metabolic and regulatory processes (4).

In humans, 18 different HDAC isozymes have been identified, and these are grouped, on the basis of their sequence homology to the gene/protein found in yeast, into four major classes (5). HDAC isozymes belonging to class I (HDAC1, -2, -3, and -8) and class II (HDAC4–7, -9, and -10) require the Zn$^{2+}$ ion for their catalytic activities, and these enzymes are inhibited by canonical/"pan" inhibitors, such as trichostatin A, SAHA, and romidepsin, among others (1, 2, 5, 6). Unlike the above two classes, class III HDACs (sirtuins) do not require the Zn$^{2+}$ ion for catalysis. Instead, they utilize NAD$^{+}$ as a co-substrate during the catalytic cycle (7). The class IV HDAC has a single member (HDAC11) and is unrelated to other HDAC isozymes (8). These HDAC isozymes reportedly show different target specificity/selectivity, tissue distribution, and subcellular localization, signifying their nonredundant function in the cellular system (9).

Among all the HDAC isozymes, HDAC8 possess several unique structural/functional features (10). Unlike other members of the class I HDACs, HDAC8 is known to interact with a limited number of proteins under in vivo conditions, although it is localized both in the nucleus and the cytosol (10, 11). Interestingly, despite its nuclear localization, HDAC8 reportedly does not catalyze the deacetylation of acetylated histones under in vivo conditions. Thus, HDAC8 is unlikely to be involved directly in the classical epigenetic regulation, as exhibited by other HDAC isozymes (12). On the other hand, a variety of acetylated non-histone proteins of nucleus, such as ERR-α, hEST1B (human ever-shorter telomere 1B), and SMC3,
Mechanism of HDAC8 Activation by N-Acylthiourea

are selectively deacetylated by HDAC8, resulting in the regulation of associated physiological processes (13–16). Recent studies have shown that HDAC8 regulates the expression of tumor suppressor protein p53 in a HoxA-dependent manner (17), an observation that appears to be corroborated by our cellular studies on SH-SY5Y cells (see "Results").

Gene knock-out studies suggest that the global deletion of HDAC8 in mice is perinatally lethal due to skull instability (18). Diminution in the expression level of HDAC8 and/or the impaired activity of the enzyme has been correlated with various pathophysiological conditions, such as neuroblastoma cancer, chronic obstructive pulmonary disease, Cornelia de Lange syndrome, and other metabolic disorders (14, 15, 19–21). In view of its involvement in diverse pathophysiological processes, HDAC8 has been considered a high priority drug target for the treatment of several human diseases including cancer. Depending on the disease state, both the activation (e.g. in the case of chronic obstructive pulmonary disease and Cornelia de Lange syndrome) and inhibition (e.g. in case of neuroblastoma and T-cell lymphoma cancers) of HDAC8 are desirable under the physiological condition. Although several HDAC8-selective inhibitors have been identified in various laboratories, we have been the first to discover N-acetylthiourea derivatives as the isozyme-selective "activators" of HDAC8 (22), of which TM-2-51 was found to exhibit the highest potency (Fig. 1). More recently, TM-2-51 has been reported to rescue the catalytic activity of the mutant HDAC8, found in Cornelia de Lang syndrome patients, further highlighting the therapeutic benefits of the HDAC8-selective activators (23).

In conjunction with HDAC8 activation, it should be noted that the activation of Sirtuin1 (a member of the class III HDAC family) by resveratrol and its derivatives has been controversial (24, 25). It has been widely debated whether resveratrol truly activates Sirtuin1 by directly binding to the enzyme site or whether the activation is facilitated because of its binding with the aromatic groups of the fluorogenic substrate used in the enzyme assay (25, 26). However, such controversy has been mitigated recently by the demonstration that Sirtuin1 cleaves physiological peptide substrates harboring aromatic amino acids (analogous to the coumarin moiety of the fluorogenic substrate) at the C-terminal end of the acetylated lysine moiety (26). In this context, it should be pointed out that unlike other HDACs, HDAC8 seems to have an obligatory requirement of aromatic amino acid residues, which mimic the fluorogenic coumarin moiety of the commercial Fluor-de-Lys® substrate (Fig. 1), for catalysis (27). In addition, over 400 nuclear proteins have been found to contain aromatic amino acids at the C-terminal ends of their acetylated lysine residues, and these serve as the targets for various HDAC isozymes (27).

To gain insight into the molecular mechanism of HDAC8 activation by TM-2-51, we performed detailed kinetic, thermodynamic, and molecular modeling studies for the binding of the activator to the enzyme. The experimental data revealed that the activator binds to HDAC8 at two distinct sites, and it modulates the kinetic properties of the enzyme. We further evaluated the efficacy of TM-2-51 on selected human neuroblastoma cells, where the cellular malignancy has been correlated with the overexpression of HDAC8 (20). We observed that TM-2-51 selectivity induces growth inhibition and apoptosis in SH-SY5Y but not in BE(2)-C neuroblastoma cells, a discriminatory feature that appears to be encoded in the p53 genotype of the above cells. Our mechanistic and cellular studies shed light on the therapeutic potential of the HDAC8-selective activators for the treatment cancer and other human diseases.

EXPERIMENTAL PROCEDURES

Materials

TM-2-51 and coumarin-SAHA were synthesized in our laboratory using previously published protocols (22, 29). SAHA, Fluor-de-Lys® (KI-104 (Fig. 1)), and deacetylated lysine-coumarin adduct (BML-KI142) were purchased from Enzo Life Sciences. The recombinant form of HDAC8 was expressed and purified as described previously (28). Trypsin used in the enzyme assay was purchased from Sigma. SH-SY5Y and BE(2)-C human neuroblastoma cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Anti-p53 and anti-β-tubulin monoclonal antibodies, used in the Western blot analysis, were purchased from Santa Cruz Biotechnology. Anti-p21 monoclonal antibody was purchased from Cell Signaling Technology.

Methods

Steady-state Kinetics of the HDAC8-catalyzed Reaction—The steady-state kinetic studies for the HDAC8-catalyzed reaction were performed using Fluor-de-Lys® as the fluorogenic substrate via a trypsin-coupled assay as described previously (29). The initial rate of the HDAC8-catalyzed reaction was determined as a function of the substrate concentration in the absence and the presence of varying concentrations of the activator. The experimental data were analyzed using the Michaelis-Menten and Hill equations to determine the steady-state kinetic parameters. The concentration of the reaction product, generated during the HDAC8-catalyzed reaction, was calculated from a standard plot of fluorescence unit versus product concentration under the same experimental conditions/setting used in the steady-state kinetic experiments.
Global Analysis of the Steady-state Kinetic Data—The steady-state kinetic data for the substrate and activator concentration-dependent reactions were found to adhere to a "general" kinetic model (Scheme 1). However, because of the inherent complexity of the proposed model, we opted to "globally" fit the steady-state data using DynaFit software (30). The above software uses recursive/iterative approaches to solve complex algebraic and differential equations.

Spectrofluorometric Titration Studies—The fluorescence measurements were performed on an LS-50B (PerkinElmer Life Sciences) spectrofluorometer. The binding of TM-2-51 to HDAC8 was found to quench the intrinsic fluorescence of the enzyme. The binding isotherm for the interaction of TM-2-51 with HDAC8 was obtained by titrating 1 μM HDAC8 with increasing concentrations of TM-2-51 in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 3 mM MgCl₂, 10% glycerol, and 1 mM Tris(2-carboxyethyl)phosphine (TCEP) at 25 °C. The intrinsic fluorescence of HDAC8 was plotted as a function of TM-2-51 concentration. The data were analyzed using the following quadratic equation (Equation 1) to obtain the equilibrium dissociation constant (Kₐ) and stoichiometry (n) of the HDAC8-TM-2-51 complex.

\[ F = C(A_{tot} + K_d + nE_{tot}) \left[ \left( \frac{E_{tot}n + A_{tot} + K_d}{2} \right)^{2} - 4E_{tot}nA_{tot} \right]^{1/2} \]

(Eq. 1)

In Equation 1, F is the measured fluorescence intensity (RFU), and Eₜot and Aₜot are the total enzyme and activator concentrations, respectively. Kₐ, n, and C refer to the equilibrium dissociation constant of the enzyme-activator complex, the stoichiometry of the enzyme-activator complex, and the fluorescence coefficient, respectively.

Isothermal Titration Calorimetric Studies—Isothermal titration calorimetry (ITC) experiments were performed on a VP-ITC microcalorimeter (GE Healthcare). All experiments were performed in duplicate or triplicate using the same stock solutions of the ligands and the enzyme. The mean values of the ITC-derived thermodynamic parameters and the associated standard deviations are reported under "Results." The thermodynamic parameters for the binding of TM-2-51 to HDAC8 were obtained by titration of 10 μM HDAC8 with 45 aliquots (4 μl each) of 500 μM TM-2-51. The working solution of TM-2-51 used in the above experiment was prepared in 10% DMSO, which does not impair the enzyme activity. The DMSO concentration was kept the same in both the ITC sample cell and the injection syringe to avoid the heat of dilution of DMSO during the course of the titration. The thermodynamic parameters for the binding of SAHA to the free and the TM-2-51-bound forms of HDAC8 were obtained by titration of 10 μM HDAC8 with 45 aliquots (4 μl each) of 200 μM SAHA in the absence and the presence of 100 μM TM-2-51 in the above Tris-HCl buffer. The experimental data were analyzed by appropriate binding models using the Origin® software package, which yielded the association constant (Kₐ) and enthalpic (∆H) changes. The binding free energy (∆G) and the entropic (∆S) changes were calculated by Equations 2 and 3, respectively.

\[ ΔG° = -RT\ln K_a \]  
(Eq. 2)

\[ ΔS° = (ΔH° - ΔG°)/T \]  
(Eq. 3)

Dissociation Off-rate Measurements—The dissociation off-rate of coumarin-SAHA from HDAC8 was determined via the stopped-flow method (Applied Photophysics). The solutions of HDAC8 (1 μM) and c-SAHA (10 μM) were mixed with 200 μM SAHA via the stopped-flow syringes. The time-dependent increase in the fluorescence signal of c-SAHA due to its displacement from the enzyme site by non-fluorescent enzyme inhibitor (SAHA) was monitored using a 395-nm "cutoff" filter (λₑx = 325 nm). To determine the influence of the enzyme-bound TM-2-51 on the dissociation off-rate of c-SAHA, the above experiment was performed in the presence of 100 μM TM-2-51 (in a syringe containing 2 μM HDAC8). In each experimental setup, at least 10 kinetic traces were collected and averaged. The averaged kinetic traces were analyzed by a single exponential rate equation to obtain the dissociation off-rate of c-SAHA from HDAC8 in the absence and presence of TM-2-51.

Molecular Docking of TM-2-51 to HDAC8-Substrate Complex—The docking of TM-2-51 to the HDAC8-sustrate complex was performed using the docking method BUMBLE, developed by Kasahara et al. (31). BUMBLE models the spatial distribution of the ligand fragments around the protein fragments using a distribution function derived from the protein-ligand complexes of the structural databases. To predict the docking of a ligand to its cognate protein, BUMBLE divides the ligand into fragments of three consecutive atoms. Then, the interaction hot spots of the ligand fragments are predicted around the protein structure using the distribution function. The ligand conformations are subsequently generated by connecting the hot spots, and the resultant conformations are ranked in order of the interaction propensity scores. Unlike the commonly used molecular docking software packages, the putative binding site(s) of a ligand on its target protein predicted by the BUMBLE method is not influenced by the ligand-induced conformational changes in protein structures (31), as observed for ligand binding to HDAC8 (32).

To determine the putative binding sites of TM-2-51 on HDAC8, the Protein Data Bank structure of the HDAC8-substrate complex (code 2V5W) was downloaded from the protein data bank. The Mol2 file of the ligand (TM-2-51) was created using ChemDraw software, and it was converted to the Protein Data Bank file using PyMol software. The Protein Data Bank files of the HDAC8-substrate complex and TM-2-51 were uploaded onto the BUMBLE server. The predicted binding sites of TM-2-51 on the HDAC8-substrate complex were taken as the model of the enzyme-activator complex.
Mechanism of HDAC8 Activation by N-Acylthiourea

For the apoptosis assay, the neuroblastoma cells were cultured in a 6-well flat-bottom tissue culture plates (1 × 10^5 cells/ml) and treated with DMSO (control) or 80 μM TM-2-51 for 72 h. Apoptosis was determined by double staining of the cells with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (Sigma-Aldrich). The cells were harvested, washed with PBS, and stained in the assay buffer (Sigma-Aldrich). The cells were stained with FITC-conjugated annexin V and propidium iodide and then analyzed using an Accuri C6 flow cytometer and Cflow software (Accuri Cytometers, Ann Arbor, MI).

Western Blot Analysis—The SH-SY5Y and BE(2)-C cells were treated with DMSO (control) or 80 μM TM-2-51 for 48 h. The treated cells were lysed in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in phosphate-buffered saline). Protease inhibitor mixture (Roche Applied Science) was added to the lysis buffer before use. The protein concentration was determined using a Biophotodetector detergent-compatible protein assay system (Bio-Rad). The cell extract containing the protein samples was subjected to SDS-PAGE analysis, and the protein bands were transferred onto a nitrocellulose membrane. The membrane was blocked using 5% nonfat milk in PBS for 30 min. Thereafter, the membrane was washed three times for 10 min using PBST buffer (PBS buffer supplemented with Tween 20), incubated with primary antibody, and incubated subsequently with horseradish peroxidase-conjugated secondary antibody. Signals were developed with enhanced chemiluminescence reagent (Pierce).

RESULTS

Fig. 2 shows the initial rate of the enzyme-catalyzed reaction as a function of the substrate concentration in the presence of 0, 2, and 20 μM TM-2-51. Note that at all these activator concentrations, the enzyme catalysis exhibits hyperbolic dependence on the substrate concentration. The solid, smooth lines (Fig. 2) indicate the best fit of the data according to the Michaelis-Menten equation, which yielded K_M and V_max values of 650 ± 68 μM and 0.82 ± 0.04 RFU/s, respectively, in the absence of the activator. At 2 and 20 μM activator concentrations, the above parameters were determined to be 467 ± 43 μM and 1.88 ± 0.07 RFU/s and 184 ± 18 μM and 3.9 ± 0.1 RFU/s, respectively. We transformed the V_max values (RFU/s) to turnover rates (k_cat) using the standard plot of RFU versus the product concentration. The k_cat values of HDAC8 in the absence and presence of 2 and 20 μM activator concentrations were found to be 0.0072 ± 0.0004, 0.018 ± 0.002, and 0.035 ± 0.003 s⁻¹, respectively. These data clearly suggest that the activator modulates both the K_M and k_cat values of the enzyme.

To determine the activation parameters of the HDAC8-catalyzed reaction, we performed the steady-state kinetic experiment at two arbitrarily chosen and fixed (60 μM and 1.5 mM) concentrations of the substrate and increasing concentrations of the activator. Fig. 3A shows the plot of the initial rates of the HDAC8-catalyzed reaction as a function of increasing concentration of the activator at 60 μM substrate. Given the K_M for the substrate being 650 μM, only about 8% of the enzyme was expected to be saturated in the presence of the above substrate concentration. To our surprise, we noted that under the latter condition, the activator concentration-dependent rate of the HDAC8-catalyzed reaction was sigmoidal in nature (Fig. 3A). This feature was clearly evident at the lower concentration regime of the activator (Fig. 3A, inset). Hence, we analyzed the data of Fig. 3A by using the Hill equation, which yielded the magnitude of the activation constant (K_app) and the Hill coefficient (n_Hill) as being equal to 6.1 ± 0.7 μM and 1.7 ± 0.1, respectively. When we performed the above experiment in the presence of a higher (1.5 mM) substrate concentration, where the enzyme was expected to be ~72% saturated, the K_app and n_Hill values were found to be 4.0 ± 0.3 μM and 1.4 ± 0.1, respectively. Note that both the K_app and the n_Hill values decreased upon an increase in the relative saturation of the enzyme (from 8 to 72%) by the substrate. As discussed below, the above feature arises due to binding of TM-2-51 at two distinct sites on the enzyme surface, and such binding gives rise to the phenomenon of positive cooperativity during the catalytic cycle of the enzyme; the
extent of cooperativity is dependent on the relative saturation of the enzyme by the substrate.

Direct Binding Studies of TM-2-51 to HDAC8 via Spectrofluorometric Titration—To eliminate the possibility that the TM-2-51-mediated activation of HDAC8 was not due to some kinetic artifact, we explored various methods to probe the direct binding of the activator to the enzyme. In this endeavor, we observed that TM-2-51 quenches the intrinsic fluorescence of the enzyme ($\lambda_{\text{em}} = 295 \text{ nm}$, $\lambda_{\text{ex}} = 340 \text{ nm}$) at a concentration of TM-2-51, resulting in the decrease in fluorescence signal at 340 nm as a function of the activator concentration. The solid line is the best fit of the experimental data according to the quadratic equation (Equation 1 under "Methods") for the dissociation constant ($K_d$), stoichiometry ($n$), and the maximum fluorescence change for binding of the activator to the enzyme as being equal to $0.28 \pm 0.04 \text{ M}$, $0.97 \pm 0.02$, and 74.8 RFU, respectively.

Because the concentrations of HDAC8 and TM-2-51 were comparable, the binding isotherm of Fig. 4 was analyzed by a complete solution of the quadratic equation (Equation 1) for the enzyme-activator interaction. The solid smooth line shown on Fig. 4 is the best fit of the experimental data, yielding the dissociation constant ($K_d$), stoichiometry ($n$), and the maximum fluorescence change for binding of the activator to the enzyme as being equal to $0.28 \pm 0.04 \text{ M}$, $0.97 \pm 0.02$, and 74.8 RFU, respectively. It should be pointed out that due to scattering of the light as well as the inner filter effect at high concentrations of TM-2-51, we could not detect the second binding site of the activator by the fluorescence titration experiment. The prevalence of the two binding sites of the activator for HDAC8 and the associated binding affinities are evident from the isothermal titration calorimetric data.

Isothermal Titration Calorimetric Studies for the Binding of TM-2-51 to Free HDAC8—To further determine the binding affinity of TM-2-51 to HDAC8, we performed ITC studies as...
Mechanism of HDAC8 Activation by N-Acylthiourea

As described under “Methods,” Fig. 5 shows a representative ITC profile for the binding of TM-2-51 to HDAC8. The raw calorimetric data and the ITC-derived binding isotherm for the interaction of TM-2-51 to HDAC8 are shown in the top and bottom panels of Fig. 5, respectively. As there was no reason to use a lower concentration of TM-2-51 (as opposed to that noted in the fluorescence titration experiment (Fig. 4). Of the remaining two binding sites, the weakest binding site (Table 1) is similar to the KA value (0.28 μM; KA = 3.6 × 10^6 M^{-1}) of the enzyme-activator complex, obtained from the fluorescence titration experiment (Fig. 4). Of the remaining two binding sites, the weakest binding site (K_A = 1.97 × 10^4 M^{-1}) is either due to nonspecific binding of the activator to the enzyme or the DMSO-mediated perturbation of the enzyme structure (34, 35). Hence, we did not consider its functional role in formulating the overall kinetic mechanism of enzyme activation. Because the overall ITC profile of Fig. 5 could be analyzed only by the sequential (and not by the independent binding site model, it appeared evident that the observed binding affinity of the second site (K_A = 1.5 × 10^6 M^{-1}) was influenced by the binding of the activator at the first binding site. The existence of two binding sites of the activator on the enzyme surface is consistent with both our modeling as well as the global analysis of our cumulative steady-state kinetic data. However, unlike the steady-state kinetic data for the activation of the enzyme (showing positive cooperativity (Fig. 3)), the binding of the activator to the free enzyme (K_A:Site 1 = 2.1 × 10^6 M^{-1} > K_A:Site 2 = 1.5 × 10^5 M^{-1} (Fig. 5 and Table 1)) is consistent with the phenomenon of negative cooperativity. The binding affinities of the activator to Site 1 (determined both by fluorescence titration as well as by ITC) and Site 2 (determined by ITC) are comparable with the kinetically derived (from the global fit of the steady-state kinetic data) K_A values of the free enzyme-activator complexes.

Effect of TM-2-51 on the Binding of SAHA to HDAC8 —In view of our observation that TM-2-51 decreases the K_m value of the enzyme substrate (Fig. 2), it was of interest to determine whether the activator truly enhances the binding affinities of non-hydrolyzable substrate analogs. We realized that one of the canonical inhibitors of the enzyme, namely SAHA, exhibits structural and binding features similar to those of the substrate and it serves as a competitive inhibitor against the substrate during the enzyme catalysis (29, 32). Hence, we used SAHA as a putative substrate analog to probe the influence of TM-2-51 on its binding affinity to HDAC8 by performing the ITC studies.

Fig. 6 shows the ITC profiles for the binding of SAHA to HDAC8 in the absence (left panel) and presence (right panel) of TM-2-51. Note that unlike the complex ITC profile for the binding of TM-2-51 to HDAC8 (Fig. 5), the binding of SAHA to free (Fig. 6, left panel) and TM-2-51-bound HDAC8 (right panel) conform to the single-site binding model. Hence, the ITC profiles of Fig. 6 were analyzed by the single binding site model, and the derived thermodynamic parameters are summarized in Table 2. Note that although the presence of TM-2-51 increases the association constant (K_A) for the binding of SAHA to HDAC8 by ~2-fold, it markedly changes the associated enthalpic (ΔH) and entropic (ΔS) parameters. Whereas the ΔH value for the binding of SAHA to HDAC8 (in the presence of TM-2-51) becomes less favorable, the corresponding ΔS value becomes more favorable, resulting in a favorable free energy gain of ~0.4 kcal/mol.

### TABLE 1

| Thermodynamic parameters | Site 1 | Site 2 | Site 3 |
|--------------------------|--------|--------|--------|
| K_A (M^{-1})             | 2.09 × 10^5 ± 1.05 × 10^8 | 1.5 × 10^5 ± 7.1 × 10^3 | 1.97 × 10^5 ± 6.8 × 10^7 |
| ΔH (kcal/mol)            | -17.8 ± 0.6 | -12.6 ± 0.7 | -10.4 ± 0.4 |
| ΔS (cal/mol/deg)         | -31.0 ± 1.5 | -18.6 ± 2.3 | -15.3 ± 2.0 |

The thermodynamic parameters were derived from the best fit of ITC data of Fig. 5 by three-site sequential binding model.
Effect of TM-2-51 on the Dissociation Off-rate of a Substrate Analog from HDAC8—To gain further mechanistic insight as to how the binding of TM-2-51 enhances the catalytic turnover rate ($k_{\text{cat}}$) of the HDAC8-catalyzed reaction, we measured the dissociation off-rate of a fluorescent substrate analog, coumarin-SAHA (previously synthesized in our laboratory (28)). Fig. 7 shows representative stopped-flow kinetic traces for the displacement of c-SAHA from free and the HDAC8-TM-2-51 complex. These kinetic traces were analyzed using the single exponential rate equation, yielding the dissociation off-rate of a fluorescent substrate analog, coumarin-SAHA from the above enzyme species as equal to 0.77 ± 0.03 and 0.39 ± 0.02 $s^{-1}$, respectively. Evidently, the dissociation off-rate of c-SAHA from the enzyme site was impaired by about 2-fold in the presence of TM-2-51, which is in agreement with a 2-fold increase in the association constant ($K_a$) of SAHA for HDAC8 in the presence of TM-2-51 (Table 2). Based on the above activator-mediated decrease in the dissociation off-rate of c-SAHA (serving as a substrate analog), we posit that an increased residence time of the substrate within the catalytic pocket of the enzyme is one of the contributing factors for enhancing the catalytic efficiency of the enzyme.

Molecular Modeling of TM-2-51 to HDAC8-Substate Complex—As our attempt to crystalize HDAC8 in the presence of TM-2-51 (in collaboration with Dr. David Christianson at the University of Pennsylvania) had not yet been successful, to gain insight into the mode of binding of the activator to HDAC8, we resorted to performing molecular docking studies via the fragment-based BUMBLE method (31). The molecular docking of TM-2-51 to the HDAC8-substrate complex (Protein Data Bank code 2V5W) revealed that the activator specifically binds to HDAC8 at two distinct sites. This was evident by a cluster of multiple docked conformers of TM-2-51 at two specific sites. In addition, we observed a single conformer of bound TM-2-51 at the third site on the HDAC8 surface (data not shown). We ascribed the third binding site as being nonspecific; this is presumably the site (Site 3) that yielded the weakest binding affinity during the ITC studies shown in Fig. 5. Fig. 8 shows the top-ranked conformers of TM-2-51 bound at two distinct sites on the enzyme surface. The first-ranked conformer of TM-2-51 (Fig. 8, shown in purple) is wrapped around the acetylsine (primarily hydrophobic) moiety of the substrate. The two phenyl moieties of TM-2-51 are juxtaposed parallel to each other to make π-π contact, and furthermore,}

### TABLE 2
Thermodynamic parameters for the binding of SAHA to free and activator-bound forms of HDAC8

| Condition                   | Stoichiometry | $K_a$ ($M^{-1}$) | $\Delta G$° (kcal/mol) | $\Delta H$° (kcal/mol) | $T\Delta S$° (kcal/mol) |
|-----------------------------|---------------|------------------|------------------------|------------------------|-------------------------|
| Free HDAC8                  |               | $0.89 \pm 0.15$  | $9.22 \times 10^3 \pm 2.5 \times 10^4$ | $-8.13 \pm 0.05$ | $-10.73 \pm 0.15$ | $-2.6 \pm 0.1$ |
| HDAC8-TM-2-51 complex       |               | $0.85 \pm 0.11$  | $1.96 \times 10^3 \pm 3.1 \times 10^3$ | $-8.58 \pm 0.04$ | $-7.08 \pm 0.32$ | $1.5 \pm 0.1$ |

**FIGURE 6.** ITC profiles for the binding of SAHA to the free (left) and the activator-bound form (right) of HDAC8. The top panels show the raw ITC data generated by titration of 1.45 ml of 10 μM free and TM-2-51-bound HDAC8 by 45 injections (4 μl each) of 200 μM SAHA. The area under each peak was integrated and plotted against the molar ratio of SAHA to HDAC8 or HDAC8-TM-2-51 complex. The solid smooth lines represent the best fit of the experimental data according to the single-site binding model. The resultant thermodynamic parameters are summarized in Table 2.

**FIGURE 7.** Dissociation off-rates of coumarin-SAHA from free (A) and activator-bound (B) HDAC8. The stopped-flow kinetic traces for the increase in fluorescence intensity with a cutoff filter at 395 nm ($λ_{\text{ex}} = 325$ nm) due to the dissociation of coumarin-SAHA from free (A) and activator-bound (B) HDAC8 upon mixing with SAHA. The premixing concentrations of the individual species in the stopped-flow syringes and other experimental conditions are described under “Methods.” The solid smooth lines are the best fit of the experimental data according to the single exponential rate equation, yielding the dissociation off-rate constants of c-SAHA from free and TM-2-51-bound forms of HDAC8 as being equal to 0.77 ± 0.03 s⁻¹ and 0.39 ± 0.02 s⁻¹, respectively.
Mechanism of HDAC8 Activation by N-Acylthiourea

FIGURE 8. Molecular docking of TM-2-51 to HDAC8-substrate complex. The enzyme-bound TM-2-51 bound at the first and second sites are shown in purple and yellow, respectively. The fluorogenic peptide substrate containing the acetylated lysine moiety is shown in green.

the anilino moiety of TM-2-51 makes a T-shaped aromatic dimer with the imidazole ring of the catalytic His-180 residue (Fig. 8). An ab initio calculation has shown that the maximum interaction energy contained in the parallel and T-shaped benzene dimers due to the π-π interactions are −1.46 and −2.48 kcal/mol, respectively (36). Because the above energetic changes have been derived for the formation of the benzene dimers, they are not directly applicable in predicting the interaction energy contained in the parallel and T-shaped benzene dimers due to the π-π interactions are −1.46 and −2.48 kcal/mol, respectively (36). Because the above energetic changes have been derived for the formation of the benzene dimers, they are not directly applicable in predicting the interaction energy contained in the parallel and T-shaped benzene dimers due to the π-π interactions. The modeling data further suggest that the activator would not preclude the binding of the substrate as observed experimentally.

The aforementioned binding mode of TM-2-51 further provides a rationale for activator-mediated enhancement in the kcat value of HDAC8. As noted with urea, the thiourea moiety of TM-2-51 is expected to disrupt the structure of both ordered and disordered water molecules in the catalytic pocket of HDAC8 (37), which is presumably reflected in about a 4-kcal/mol favorable binding entropy of SAHA (used as a substrate analog) with the enzyme in the presence of TM-2-51 (Table 2). Such a feature may cause a differential solvation of the substrate in the ground versus the corresponding transition states, leading to the diminution of the free energy barrier of HDAC8 catalysis in the presence of the activator. Hence, it is not surprising that aside from decreasing the Km value of the substrate, TM-2-51 increases the kcat value of the enzyme.

The second to top-ranked conformer of TM-2-51 (shown in yellow) bound to the second site on HDAC8 is proximal to the first binding site of the activator. The benzoyl moiety of the activator at the second site makes a close contact with the indole moiety of Trp-141, which is located at the bottom end of the active site pocket of the enzyme (6). Hence, the activator bound at the second site has the potential to modulate the geometry of the catalytic pocket of the enzyme via a long-range allosteric interaction. The latter feature is likely to be manifested as a positive cooperative profile in the HDAC8-catalyzed reaction as a function of TM-2-51 concentration (Fig. 3). The above structural/functional feature is further supported by the fact that the active site of HDAC8 is constituted by several pocket-forming loops, which are reportedly highly flexible in nature (6, 10). Furthermore, since Trp-141 is a unique residue found only in the HDAC8 isozyme (10), it is not surprising that TM-2-51 serves as an isozyme-selective activator (22).

Efficacy of TM-2-51 in Human Neuroblastoma Cells—Of all the HDAC isozymes, the overexpression of HDAC8 is highly pronounced in neuroblastoma tumors, and the HDAC8-selective inhibitors have been shown to have an anti-proliferative effect in BE(2)-C human neuroblastoma cells, forcing them to differentiate (20). In view of the above facts, we wished to evaluate the efficacy of TM-2-51 in two human neuroblastoma cells, namely SH-SY5Y and BE(2)-C. We measured cell proliferation and apoptosis in both of these cell lines in the presence of TM-2-51. To our surprise, we observed that although TM-2-51 induced growth inhibition and apoptosis in SH-SY5Y cells, it did not exhibit any effect on BE(2)-C cells. The growth inhibition of SH-SY5Y cells was found to be dependent on the concentration of the activator as well as the incubation time (Fig. 9, left panel). We observed an ∼22% decrease in proliferation of SH-SY5Y cells in the presence of 80 μM TM-2-51 for 72 h as compared with the control. However, under the above experimental condition, TM-2-51-mediated apoptosis in SH-SY5Y cells was about ∼45% as compared with the DMSO-treated cells used as control (Fig. 9, right panel). We believe the origin of the above difference lies in the relatively rapid changes in the molecular processes responsible for yielding the apoptotic signals prior to the impairment of energy metabolism and cell death.

Yan et al. (17) recently demonstrated that the expression of both the wild-type and mutant tumor suppressor protein, p53, in various cancer cell lines are regulated by HDAC8. In this regard, we noted that SH-SY5Y and BE(2)-C cells express wild-type and mutant forms of p53, respectively (38). Given these facts, it appeared plausible that the p53 genotype was responsible for the above noted differential effects in two types of neuroblastoma cells. To test this hypothesis, we performed a Western blot analysis of the cell extracts of both TM-2-51- and DMSO-treated SH-SY5Y and BE(2)-C cells. The experimental data revealed that the expression level of p53 was moderately enhanced in the activator-treated SH-SY5Y cells but not in BE(2)-C cells (Fig. 10). To ascertain whether the above effect is relayed in the downstream cellular process, we evaluated the expression level of p21 (a cell cycle inhibitor), a well-characterized target of p53 (38). We observed that the protein expression level of p21 was noticeably enhanced in the TM-2-51-treated SH-SY5Y cells but not in BE(2)-C cells (Fig. 10). We believe the above difference in the cell-specific efficacy of TM-2-51 lies in
the differential susceptibility of the wild-type versus mutant p53 toward proteasomal degradation.

DISCUSSION

The experimental data presented herein provide a detailed mechanistic account of the isozyme-selective activation of HDAC8 by an N-acylthiourea derivative (TM-2-51), which was discovered previously in our laboratory (22). The key findings of this study are as follows. (i) The activator binds to HDAC8 at two distinct sites, and the steady-state kinetic profiles of the enzyme-catalyzed reaction as a function of the activator concentration conform to the phenomenon of positive cooperativity. (ii) The magnitude of the positive cooperativity (Hill coefficient) depends on the concentration of the enzyme substrate (Fig. 3). (iii) Whereas the activator slightly decreases the $K_m$ value of the substrate, it markedly increases the catalytic turnover rate ($k_{cat}$) of the enzyme (Fig. 2), resulting in an increase in the specificity constant ($k_{cat}/K_m$ value) of the enzyme. (iv) Due to quenching of the intrinsic fluorescence signal of the enzyme by the activator (Fig. 4), as well as generation of the heat signal upon enzyme-activator interaction (Fig. 5), we could directly determine the binding affinities of the activator at different sites on the enzyme surface. Independently determined binding affinities of TM-2-51 to the enzyme (Figs. 4 and 5) are in close agreement with their corresponding binding constants derived from the global analysis of the steady-state kinetic data (Table 3). (v) The activator modulates the kinetic and thermodynamic parameters for the binding of SAHA (used as a putative substrate analog) with the enzyme (Figs. 6 and 7). (vi) The activator selectively induces growth inhibition and apoptosis in SH-SY5Y neuroblastoma cells but not in BE(2)-C neuroblastoma cells (Fig. 9). The molecular origin of the above selectivity lies in the p53-specific genotype of the above cells (38).

All of the structural, kinetic, and thermodynamic data suggest that SAHA binds to the active site pocket of HDAC8 (28, 29, 32, 39). Because TM-2-51 does not obliterate the binding of SAHA to the enzyme (Fig. 6), it implies that both the activator and the inhibitor (used to serve as a substrate analog) can simultaneously bind to the enzyme. This is not surprising in view of the marked malleability/flexibility of the active site pocket of HDAC8, as evident from its ability to interact with structurally diverse ligands with modest binding affinities (6, 10, 32, 39). The kinetic (Fig. 3) and thermodynamic (Fig. 5) data presented herein clearly suggest that the activator binds to the enzyme site in a cooperative manner. Whereas the binding of the activator to the enzyme-substrate complex conforms to the positive cooperative behavior (Fig. 3), that to the free enzyme is consis-

![Figure 9](image1.png)

**FIGURE 9.** Effect of TM-2-51 on cell growth (left) and apoptosis (right) on human SH-SY5Y and BE(2)-C human neuroblastoma cells as measured by the MTS assay and annexin V cell apoptosis kit, respectively. Prior to evaluating apoptosis, the cells were treated with DMSO (control) or $80 \mu M$ TM-2-51 for 72 h.

![Figure 10](image2.png)

**FIGURE 10.** Western blot analysis of whole cell extract of SH-SY5Y and BE(2)-C cells treated with DMSO (control) or $80 \mu M$ TM-2-51 for 72 h to detect the expression levels of p53 and p21 proteins. Note that unlike BE(2)-C cells, the expression of both p53 and p21 was enhanced in the activator-treated SH-SY5Y cells as compared with the control.

| $K_m$ (M) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$) | $K_a$ (M) | $K_a$ (s$^{-1}$) |
|----------|-----------------|-----------------|--------|-----------------|
| 650 ± 20 | 0.007 ± 0.001 s$^{-1}$ | 10.8 M$^{-1}$ s$^{-1}$ | 0.28 ± 0.04 M$^{-1}$ s$^{-1}$ |
| 580 ± 26 | 0.022 ± 0.014 s$^{-1}$ | 124 M$^{-1}$ s$^{-1}$ | 19.75 ± 0.64 M$^{-1}$ s$^{-1}$ |
| 277 ± 13 | 0.052 ± 0.003 s$^{-1}$ | 152 M$^{-1}$ s$^{-1}$ | 3.06 ± 0.28 M$^{-1}$ s$^{-1}$ |

**TABLE 3**

Kinetic parameters for the HDAC8-catalyzed reaction in the presence of TM-2-51

The kinetic parameters of the HDAC8-catalyzed reaction were obtained from the global analysis of the steady-state enzyme kinetic data using DynaFit (29) as per Scheme 1.
Mechanism of HDAC8 Activation by N-Acylthiourea

tent (Fig. 5) with the negative cooperative phenomenon. Given that HDAC8 is a monomeric enzyme, it is conceivable that the origin of the observed cooperativity is not due to the classical site-site interaction as originally observed with oligomeric enzymes (40–42). Instead, the origin of the observed cooperativity in the binding of TM-2-51 with HDAC8 must lie in the “long-range” (allosteric) interaction between the activator binding sites (Fig. 8). In view of our experimental as well as theoretical considerations, we proposed a minimal kinetic model for the HDAC8-catalyzed reaction in the presence of both the substrate and the activator (Scheme 1). It should be emphasized that our model (Scheme 1) has no bearing on the classical allosteric (e.g. MWC, KNF, Adair’s) models (40–42), and neither does it have any preconceived bias for the cooperative binding of the activator. In this regard, our model falls in the category of a general kinetic model of the HDAC8-catalyzed reaction, and as elaborated below, it explains all of our kinetic and thermodynamic data presented under “Results.”

According to the model presented in Scheme 1, the activator (A) binds to the enzyme (E) forming EA and EAA complexes, and all of the enzyme species bind to the substrate (S) to form the corresponding E(A)S complexes, which are broken down to the reaction products. The equilibrium dissociation and catalytic rate constants of individual steps are shown on the corresponding arrows of Scheme 1. Prior to testing the validity of our model shown in Scheme 1, we expanded our steady-state kinetic studies in Figs. 2 and 3 at different concentrations of the substrate and the activator (Fig. 11). These steady-state kinetic data were collected in a matrix format such that they could either be plotted as: 1) V (velocity) versus [S] at different concentrations of TM-2-51 (Fig. 11, left panel) or (2) V versus [TM-2-51] at different concentrations of substrate (Fig. 11, right panel). We performed a global analysis of the steady-state kinetic data of Fig. 11 using DynaFit software (30). While performing the global analysis, we fixed the magnitudes of $k_{cat1}$, $k_{cat2}$, $K_{m1}$, and the enzyme (E) concentration, as their values were precisely determined by independent experiments. The solid smooth lines of Fig. 11 are the best global fit of the data, yielding the kinetic parameters summarized in Table 3. Note a marked correspondence between the fitted results (Fig. 11, solid smooth lines) and the experimental data points in both plots (i.e. V versus [S] at different concentrations of TM-2-51 and V versus [TM-2-51] at different concentrations of substrate), attesting to the validity/robustness of our kinetic model.

A thorough examination of the kinetic parameters, derived from the global analysis of the steady-state enzyme kinetic data of the HDAC8-catalyzed reaction (Table 3), unraveled the following facts. (i) Because $K_{a2}$ (0.37 μM) is nearly 1 order of magnitude lower than $K_{a1}$ (3.07 μM), it implies that the binding affinity of the second activator molecule (in the presence of the substrate) is higher than that of the first activator molecule, a feature that can give rise to the sigmoidal (positive cooperative) profile during the steady-state enzyme catalyzed reaction as a function of the activator concentration observed experimentally (Fig. 3). (ii) Because $K_{a2}$ (19.75 μM) is nearly 2 orders of magnitude higher than $K_{a1}$ (0.28 μM), it implies that in the absence of the substrate, the activator (bound to the first site) impairs its own binding at the second site. This represents a classic case of the negative cooperative phenomenon in binding of the activator (TM-2-51) to the “free” enzyme; this feature is in agreement with our ITC profile for the sequential binding of the activator to free enzyme (Fig. 5 and Table 1). It should be emphasized that our ITC data shown in Fig. 5 could only be analyzed by the “sequential” binding model and not by the “independent” binding site model. (iii) On comparing the vel-
ues of $K_{a1}$ (0.28 $\mu$M) and $K_{a1}$ (3.06 $\mu$M), it is evident that the presence of the substrate at the enzyme site impairs the binding of the activator for Site 1. On the other hand, comparing the value of $K_{a2}$ (19.75 $\mu$M) with $K_{a2}$ (0.37 $\mu$M), it is evident that the substrate assists the binding of the activator at the second site.

(iv) The decrease in the $K_m$ value of the substrate (from 650 to 580/277 $\mu$M (Table 3)) in the presence of the activator is also qualitatively consistent with the TM-2-51-mediated increase in the $K_4$ value of SAHA (from $9.22 \times 10^5$ to $1.96 \times 10^6$ M$^{-1}$ (Table 2)) derived from our ITC titration data of Fig. 6. (v)

Although the binding of the activator to the first site does not significantly alter the $K_m$ value of the substrate ($K_{m1} = 650 \mu$M; $K_{m2} = 580 \mu$M (Table 3)), it significantly enhances the catalytic turnover rate ($k_{cat1} = 0.007 \text{ s}^{-1}$; $k_{cat2} = 0.072 \text{ s}^{-1}$) of the enzyme. On the other hand, the binding of the activator at the second site decreases both the $K_m$ value of the substrate (from 580 to 277 $\mu$M) and the $k_{cat}$ value of the enzyme (from 0.072 to 0.042 $\text{ s}^{-1}$) by nearly the same magnitude. Hence, from the viewpoint of the “specificity constant” ($k_{cat}/K_m$ value (Table 3)), the binding of the first molecule of the activator exhibits the maximal advantage in enhancing the catalytic efficiency of the enzyme; practically no catalytic advantage is realized upon the binding of the second molecule of the activator to the enzyme. In view of the above consideration, we are prompted to conclude that the cooperative binding of the activator to the enzyme has no functional advantage. However, because the binding of the second molecule of the activator (which is unavoidable due to the positive cooperative binding feature) does not impair the catalytic efficiency of the enzyme, the overall functional role of the activator in enhancing the catalytic efficiency of HDAC8 is justifiable.

We attempted to formulate a relationship between the phenomenological activation constants, $K_{app}$ (obtained by fitting the sigmoidal activation profiles of Fig. 3 by the Hill equation) and the microscopic parameters derived from the global analysis of our cumulative steady-state kinetic data (Fig. 11) by our general model (Scheme 1). We realized that whereas the magnitudes of $K_{app}$ at low (60 $\mu$M; $K_{app} = 6.09 \mu$M) and high (1.5 mm; $K_{app} = 3.07 \mu$M) substrate concentrations are significantly higher than the microscopic constant for the binding of the first molecule of the activator to Site 1 of free HDAC8 ($K_{a1} = 0.28 \mu$M), they are similar to the binding affinity of the first molecule of the activator to the enzyme-substrate complex ($K_{a1} = 3.06 \mu$M). Our further attempt to derive the quantitative relationships between $K_{app}$ (Fig. 3) and the microscopic parameters ($k_{a1}$, $k_{a2}$; $k_{a12}$, and $K_{a22}$ (Table 3)) were unsuccessful. In this regard, we note that Dahlquist (33) derives the relationships between the phenomenological parameters for both positive and negative cooperative binding of ligands to their cognate proteins and the underlying microscopic constants for the two-step Adair binding model. Because our kinetic model (exhibiting both positive and negative cooperative binding phenomena) is far more complex than the two-step Adair model, the Dahlquist relationships (33) could not predict the origin of the steady-state derived $K_{app}$ and $n_H$ values on the basis of the microscopic constants of Table 3.

The question arises as to why the magnitude of the observed cooperativity ($n_H$) for the activation of the enzyme is related reciprocally to the substrate concentration. In other words, although the $n_H$ value is higher at a lower concentration of the substrate, it is lower at a higher concentration of the substrate (Fig. 3). This feature is not intuitively obvious given the fact that the $K_{a2}$ is an order of magnitude lower than $K_{a1}$ (Table 3). However, it is evident from the data in Table 3 that the binding of the activator to the free enzyme conforms to an apparent negative cooperative phenomenon ($K_{a1} = 0.28 \mu$M $< K_{a2} = 19.75 \mu$M). This is in marked contrast to the positive cooperative behavior in binding of the activator to the ES complex ($K_{a1} = 0.36 \mu$M $> K_{a2} = 0.37 \mu$M). Hence, at a low (subsaturation) concentration of the substrate, a fraction of the total enzyme is expected to exist in the free (E) form, facilitating the formation of both EA and EAA complexes at increasing concentrations of the activator. On the other hand, at high (saturation) concentration of the substrate, the relative abundance of both EA and EAA complexes is expected to be relatively lower. Hence, under the former condition (i.e. at a subsaturating concentration of the substrate), the increase in the concentration of the activator will not only form the expected EAS and EAAAs complexes, but it will also form the EA and EAA complexes, and the latter will subsequently interact with the substrate to form the corresponding complexes before undergoing catalysis. Therefore, under the above situation, the activator concentration-mediated drive to form the catalytically competent species will be influenced by the relative magnitudes of $K_{a2}$ (19.75 $\mu$M) and $K_{a2}$ (0.37 $\mu$M). We believe this is the most plausible explanation of the higher Hill coefficient ($n_H$) at the lower substrate concentration vis-à-vis that obtained at the higher concentration of the substrate.

Our cellular studies of TM-2-51 suggest its therapeutic potential for the treatment of several human diseases that are caused by impaired HDAC8 activity, such as Cornelia de Lange Syndrome, chronic obstructive pulmonary disease, and other metabolic disorders (14, 15, 19, 21). In particular, our cell biology studies provide insight into the effectiveness of TM-2-51 on selectively inducing growth inhibition as well as apoptosis in SH-SY5Y but not BE(2)-C neuroblastoma cells (Fig. 9). Such a discriminatory feature appears to be encoded in the expression of the wild-type versus mutant forms of the tumor suppressor protein p53 in the above cell lines, respectively. Yan et al. (17) recently demonstrated that the transcription of both the wild-type and the mutant p53 genes are regulated via HDAC8 in various human cancer cells (17). In addition, when the cancer cells express the mutant forms of HDAC8 (exhibiting lower catalytic activity), the up-regulation of p53 is obviated. Interestingly, we observed a modest change in p53 protein expression level in the TM-2-51-treated SH-SY5Y cells but not in BE(2)-C cells under the same experimental condition (Fig. 10). This finding could be rationalized, at least in part, by the fact that the mutant form of p53 is unstable and presumably undergoes rapid proteasomal degradation under physiological condition (43) as compared with the wild-type p53. Thus, even though the activator has the potential to up-regulate the transcription of mutant p53 in BE(2)-C cells, the protein expression level remains unchanged. The influence of TM-2-51 on p53 expression (in SH-SY5Y cells) is relayed in the increased expression of p21, a bona fide downstream target of p53 (44). A cumulative
Mechanism of HDAC8 Activation by N-Acylthiourea

account of our cell biology data lead to the suggestion that TM-2-51 has potential to inhibit cell proliferation and enhance apoptosis in neuroblastoma cells, expressing wild-type as opposed to the mutant form of p53. We are currently investigating the generality of the above feature with other cell types, and we will report these findings subsequently. However, we note that because many cancer cells express the mutant forms of p53, the therapeutic potential of our activator would be limited only to the cancer cells expressing the wild-type p53.

The pan-HDAC inhibitors, such as SAHA, romidepsin, etc., are widely known to produce anticancer effects in various cancer cell lines as well as in xenograft animal models of cancers (45, 46). Considering the fact that HDAC8 enhances the expression of p53 (17), the treatment of cancers by HDAC inhibitors should be used with caution. This is primarily because the inhibition of HDAC8 (by SAHA and other pan-HDAC inhibitors) would suppress the up-regulation of the wild-type p53 gene, obviating the tumor-protective effects of p53 and p21. Because TM-2-51 enhances the binding affinity of SAHA (and possibly other inhibitors) to HDAC8, we surmise that our activator has the potential to function as an “adjuvant” during the SAHA (or other inhibitors) to HDAC8, we surmise that our activator has.

In summary, our mechanistic and cell biology studies presented herein will invigorate interest in designing HDAC activators as potential therapeutic agents for the treatment of cancer and possibly other human diseases.

Acknowledgment—We thank the members of our research group for their kind assistance and valuable discussion.

REFERENCES

1. Ellis, L., Atadja, P. W., and Johnstone, R. W. (2009) Epigenetics in cancer: targeting chromatin modifications. Mol. Cancer Ther. 8, 1409–1420
2. Rothbart, S. B., and Strahl, B. D. (2014) Interpreting the language of histone and DNA modifications. Biochim. Biophys. Acta 1839, 627–643
3. Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996) A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 272, 408–411
4. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325, 834–840
5. Gregoretti, I. V., Lee, Y. M., and Goodson, H. V. (2004) Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J. Mol. Biol. 338, 17–31
6. Lombardi, P. M., Cole, K. E., Dowling, D. P., and Christianson, D. W. (2011) Structure, mechanism, and inhibition of histone deacetylases and related metalloenzymes. Curr. Opin. Struct. Biol. 21, 735–743
7. Marmorstein, R. (2004) Structure and chemistry of the Sir2 family of NAD+-dependent histone/protein deacetylases. Biochem. Soc. Trans. 32, 904–909
8. Gao, L., Cueto, M. A., Asselbergs, F., and Atadja, P. (2002) Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J. Biol. Chem. 277, 25748–25755
9. Reichert, N., Choukrallah, M. A., and Matthias, P. (2012) Multiple roles of class I HDACs in proliferation, differentiation, and development. Cell. Mol. Life Sci. 69, 2173–2187
10. de Ruiter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S., and van Kuilenburg, A. B. (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem. J. 370, 737–749
11. Wolfsen, N. A., Pitcairn, C. A., and Fierke, C. A. (2013) HDAC8 substrates: histones and beyond. Biopolymers 99, 112–126
12. Krennhrubec, K., Marshall, B. L., Hedglin, M., Verdin, E., and Ulrich, S. M. (2007) Design and evaluation of “linkerless” hydroxamic acids as selective HDAC8 inhibitors. Bioorg. Med. Chem. Lett. 17, 2874–2878
13. Waltregny, D., Glénisson, W., Tran, S. L., North, B. J., Verdin, E., Colige, A., and Castronovo, V. (2005) Histone deacetylase HDAC8 associates with smooth muscle α-actin and is essential for smooth muscle cell contractility. FASEB J. 19, 966–968
14. Wilson, B. J., Tremblay, A. M., Deblois, G., Sylvain-Drolet, G., and Giguère, V. (2010) An acetylation switch modulates the transcriptional activity of estrogen-related receptor α. Mol. Endocrinol. 24, 1349–1358
15. Deardorff, M. A., Bando, M., Nakato, R., Watrin, E., Itoh, T., Minamin, M., Saitoh, K., Komata, M., Katou, Y., Clark, D., Cole, K. E., De Baere, E., Decroos, C., Di Donato, N., Ernst, S., Franchise, L. J., Gyftodimou, Y., Hishinuma, K., Hultings, M., Ishikawa, Y., Jaulin, C., Kaur, M., Kiyono, T., Lombardi, P. M., Magnaghi-Jaulin, L., Mortier, G. R., Nokazi, N., Petersen, M. B., Seimiya, H., Stu, Y. M., Suzuki, Y., Takagaki, K., Wilde, J. I., Willems, P. J., Prigent, C., Gillessen-Kaesebach, G., Christianson, D. W., Kaiser, F. J., Jackson, L. G., Hirota, T., Krantz, I. D., and Shirahige, K. (2012) HDAC8 mutations in Cornelia de Lange syndrome affect the cohesin acetylation cycle. Nature 489, 313–317
16. Lee, H., Jungja, N., Villagra, A., Rezaizadeh, S., and Sato, E. (2006) Histone deacetylase 8 safeguards the human ever-shorter telomeres 1B (hEST1B) protein from histone-mediated degradation. Mol. Cell Biol. 26, 5259–5269
17. Yang, W., Liu, S., Xu, E., Zhang, J., Zhang, Y., Chen, X., and Chen, X. (2013) Histone deacetylase inhibitors suppress mutant p53 transcription via histone deacetylase 8. Oncogene 32, 599–609
18. Haberland, M., Mokalled, M. H., Montgomery, R. L., and Olson, E. N. (2009) Epigenetic control of skull morphogenesis by histone deacetylase 8. Genes Dev. 23, 1625–1630
19. Ito, K., Ito, M., Elliott, W. M., Cosio, B., Caramori, G., Kon, O. M., Barczyk, A., Hayashi, S., Adcock, I. M., Hogg, J. C., and Barnes, P. J. (2005) Decreased histone deacetylase activity in chronic obstructive pulmonary disease. N. Engl. J. Med. 352, 1967–1976
20. Oehme, I., Deubzer, H. E., Wegener, D., Pickert, D., Linke, J. P., Hero, B., Kopp-Schneider, A., Westermann, F., Ulrich, S. M., von Deimling, A., Fischer, M., and Witt, O. (2009) Histone deacetylase 8 in neuroblastoma tumorigenesis. Clin. Cancer Res. 15, 91–99
21. Balasubramanian, S., Ramos, J., Luo, W., Sirisawad, M., Verner, E., and Buggy, J. J. (2008) A novel histone deacetylase 8 (HDAC8)-specific inhibitor PCI-34051 induces apoptosis in T-cell lymphomas. Mol. Cancer Ther. 7, 2157–2164
22. Singh, R. K., Mandal, T., Balasubramanian, N., Viaine, T., Leedahl, T., Sule, C., Gröck, K., and Srivastava, D. K. (2011) Histone deacetylase activators: N-acetylthiourea serves as highly potent and isoform selective activators for human histone deacetylase-8 on a fluorescent substrate. Bioorg. Med. Chem. Lett. 21, 5920–5923
23. Decroos, C., Bowman, C. M., Moser, J. A., Christianson, K. E., Deardorff, M. A., and Christianson, D. W. (2014) Compromised structure and function of HDAC8 mutants identified in Cornelia de Lange syndrome spectrum disorders. ACS Chem. Biol. 9, 2157–2164
24. Borra, M. T., Smith, B. C., and Denu, J. M. (2005) Mechanism of human SIRT1 activation by resveratrol. J. Biol. Chem. 280, 17178–17195
25. Pacholec, M., Pleasdale, J. E., Chrunyk, B., Cunningham, D., Flynn, D., Garofalo, R. S., Griffith, D., Griffor, M., Louilakis, P., Pabst, B., Qiu, X., Stockman, B., Thanabal, V., Varghese, A., Ward, J., Withka, J., and Ahn, K. (2010) SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. J. Biol. Chem. 285, 8340–8351
26. Hubbard, B. P., Gomes, A. P., Dai, H., Li, J., Case, A. W., Considine, T., Riera, T. V., Lee, J. E. E., S. Y., Lamming, D. W., Pentelute, B. L., Schuman, E. R., Stevens, L. A., Ling, A. J., Armour, S. M., Michan, S., Zhao, H., Jiang, Y., Sweitzer, S. M., Blum, C. A., Disch, J. S., Ng P., Howitz, K. T., Rolo, A. P., Hamuro, Y., Moss, J., Perini, R. B., Ellis, J. L., Vlasuk, G. P., and Sinclair, D. A. (2013) Evidence for a common mechanism of SIRT1 regu-
loration by allosteric activators. Science 339, 1216–1219
27. Gurard-Levin, Z. A., Kim, J., and Mrksich, M. (2009) Combining mass spectrometry and peptide arrays to profile the specificities of histone deacetylases. ChemBiochem 10, 2159–2161
28. Singh, R. K., Mandal, T., Balasubramanian, N., Cook, G., and Srivastava, D. K. (2011) Coumarin-suberylanilide hydroxamic acid as a fluorescent probe for determining binding affinities and off-rates of histone deacetylase inhibitors. Anal. Biochem. 408, 309–315
29. Schultz, B. E., Misialek, S., Wu, J., Tang, J., Conn, M. T., Tahilramani, R., and Wong, L. (2004) Kinetics and comparative reactivity of human class I and class IIb histone deacetylases. Biochemistry 43, 11083–11091
30. Kuzmic, P. (1996) Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV protease. Anal. Biochem. 237, 260–273
31. Kasahara, K., Kinoshita, K., and Takagi, T. (2010) Ligand-binding site prediction of proteins based on known fragment-fragment interactions. Bioinformatics 26, 1493–1499
32. Somoza, J. R., Skene, R. J., Katz, B. A., Mol, C., Ho, J. D., Jennings, A. J., Luong, C., Arvai, A., Buggy, J. J., Chi, E., Tang, J., Sang, B. C., Verner, E., Wynands, R., Leahy, E. M., Dougan, D. R., Snell, G., Navre, M., Knuth, M. W., Swanson, R. V., McRee, D. E., and Tari, L. W. (2004) Structural snapshots of human HDAC8 provide insights into the class I histone deacetylases. Structure 12, 1325–1334
33. Dahlquist, F. W. (1978) The meaning of Scatchard and Hill plots, in Methods in Enzymology (Hirs, C. H. W., and Timasheff, S. N., eds) Vol. 48, pp. 270–299, New York
34. Byerly, D. W., McElroy, C. A., and Foster, M. P. (2002) Mapping the surface of Escherichia coli peptide deformylase by NMR with organic solvents. Protein Sci. 11, 1850–1853
35. Ziarek, J. I., Peterson, F. C., Lytle, B. L., and Volkman, B. F. (2011) Binding site identification and structure determination of protein-ligand complexes by NMR a semiautomated approach. Methods Enzymol. 493, 241–275
36. Tsuzuki, S., Honda, K., Uchimaru, T., Mikami, M., and Tanabe, K. (2002) Origin of attraction and directionality of the pi/pi interaction: model chemistry calculations of benzene dimer interaction. J. Am. Chem. Soc. 124, 104–112
37. Frank, H. S., and Franks, F. (1968) Structural approach to the solvent power of water for hydrocarbons: urea as a structure breaker. J. Chem. Phys. 48, 4746–4757
38. Van Maerken, T., Rihani, A., Dreibax, D., De Clercq, S., Yigit, N., Marine, J. C., Westermann, F., De Paeppe, A., Vandesompele, J., and Spleeman, F. (2011) Functional analysis of the p53 pathway in neuroblastoma cells using the small-molecule MDM2 antagonist nutlin-3. Mol. Cancer Ther. 10, 983–993
39. Singh, R. K., Suzuki, T., Mandal, T., Balasubramanian, N., Haldar, M., Muller, D. J., Strode, J. A., Cook, G., Mallik, S., and Srivastava, D. K. (2014) Thermodynamics of binding of structurally similar ligands to histone deacetylase 8 sheds light on challenges in the rational design of potent and isozyme-selective inhibitors of the enzyme. Biochemistry 53, 7445–7458
40. Adair, G. S., with the collaboration of Bock, A. V., and Field, H., Jr. (1925) The hemoglobin system. VI: The oxygen dissociation curve of hemoglobin. J. Biol. Chem. 63, 529–545
41. Monod, J., Wyman, J., and Changeux, J. P. (1965) On the nature of allosteric transitions: a plausible model. J. Mol. Biol. 12, 88–118
42. Koshland, D. E., Jr., Némethy, G., and Filmer, D. (1966) Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry 5, 365–385
43. Terzian, T., Suh, Y. A., Iwakuma, T., Post, S. M., Neumann, M., Lang, G. A., Van Pelt, C. S., and Lozano, G. (2008) The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. Genes Dev. 22, 1337–1344
44. Laptenko, O., Beckerman, R., Freulich, E., and Prives, C. (2011) p53 binding to nucleosomes within the p21 promoter in vivo leads to nucleosome loss and transcriptional activation. Proc. Natl. Acad. Sci. U.S.A. 108, 10385–10390
45. Bolden, J. E., Peart, M. J., and Johnstone, R. W. (2006) Anticancer activities of histone deacetylase inhibitors. Nat. Rev. Drug Discov. 5, 769–784
46. Rodríguez-Paredes, M., and Esteller, M. (2011) Cancer epigenetics reaches mainstream oncology. Nat. Med. 17, 330–339