

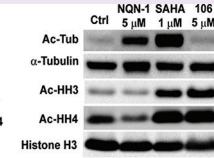
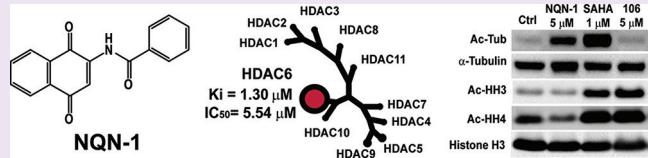
A Novel Class of Small Molecule Inhibitors of HDAC6

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Supporting Information

ABSTRACT: Histone deacetylases (HDACs) are a family of enzymes that play significant roles in numerous biological processes and diseases. HDACs are best known for their repressive influence on gene transcription through histone deacetylation. Mapping of nonhistone acetylated proteins and acetylation-modifying enzymes involved in various cellular pathways has shown protein acetylation/deacetylation also plays key roles in a variety of cellular processes including RNA splicing, nuclear transport, and cytoskeletal remodeling. Studies of HDACs have accelerated due to the availability of small molecule HDAC inhibitors, most of which contain a canonical hydroxamic acid or benzamide that chelates the metal catalytic site. To increase the pool of unique and novel HDAC inhibitor pharmacophores, a pharmacological active compound screen was performed. Several unique HDAC inhibitor pharmacophores were identified *in vitro*. One class of novel HDAC inhibitors, with a central naphthoquinone structure, displayed a selective inhibition profile against HDAC6. Here we present the results of a unique class of HDAC6 inhibitors identified using this compound library screen. In addition, we demonstrated that treatment of human acute myeloid leukemia cell line MV4-11 with the selective HDAC6 inhibitors decreases levels of mutant FLT-3 and constitutively active STAT5 and attenuates Erk phosphorylation, all of which are associated with the inhibitor's selective toxicity against leukemia.



Histone deacetylases (HDACs) regulate a broad range of cellular processes by modulating protein structure and function *via* lysine deacetylation.¹ Lysine deacetylation is a dynamic and reversible process mediated by acetyl transferases and HDACs. The balance of these dynamic post-translational modifications is theorized to play key roles in cellular signaling and homeostasis similar to those of protein phosphorylation.^{2,3} Although protein acetylation is best known for its role in transcriptional regulation *via* histone acetylation,^{4,5} recent studies have shown over 3,500 acetylation sites in approximately 1,700 acetylated proteins; in comparison to the current phosphoproteome at about 6,600 phosphorylation sites and 2,200 proteins.³ Thus, the acetylome is approaching the size of the phosphoproteome and extends the mechanistic relevance and research interests in HDACs well beyond the field of transcriptional regulation and chromatin biology.

The initial classification of human HDACs is based on sequence homology and phylogenetic relationships to yeast homologues. There are four distinct classes of HDACs: class I (HDAC1, 2, 3, and 8), class IIa (HDAC4, 5, 7, and 9), class IIb (HDAC6 and 10), and class IV (HDAC11), which represent the Zn^{2+} -dependent deacetylases.⁶ The class III sirtuins are mechanistically diverse NAD^+ -dependent deacetylases.⁷ Chemical inhibitors have always played a key role in the study of HDACs and their biological processes.^{8,9} Suberoylanilide hydroxamic acid (SAHA, Vorinostat; Merck Research Laboratories) and Romidepsin (FK-228; Gluocester Pharmaceuticals) were approved for the treatment of cutaneous T-cell lymphoma within 10 years of their discovery.^{10,11} HDAC inhibitors have also been shown to be promising therapies for

neurodegenerative diseases and inflammatory disorders.^{12–14} However, how HDAC inhibitors achieve their therapeutic effects is still poorly understood. Recent studies using class-specific substrates display divergent enzymatic properties for class IIa HDACs distinguishing them from class I and IIb HDACs.^{15,16} The unique biochemistry associated with class IIa HDACs further complicates the mechanistic understanding of HDACs and their inhibitors. The commonly used pan-HDAC inhibitor, SAHA, appears to inhibit only Class I and IIb HDACs, with significantly lower activity against class IIa HDACs, and its inhibition profile is shared with other well-known HDAC inhibitors.^{17,18} Proteomic studies showing that only 10% of all acetylation sites are even sensitive to SAHA treatment further emphasize the need for novel HDAC inhibitors.³ Unique and selective HDAC inhibitors targeting specific isoforms could potentially facilitate the understanding of HDAC-related molecular mechanisms. Currently four major structurally unique classes of HDAC inhibitors exist: hydroxamic acids, benzamides, short chain fatty acids, and large cyclic peptides. All these inhibitors share a common pharmacophore composed of a zinc-metal binding motif, a linker region, and a surface recognition domain.¹⁹ These commonalities of HDAC inhibitors could potentially limit the development of novel and unique HDAC inhibitors capable of inhibiting broad or selective HDACs.

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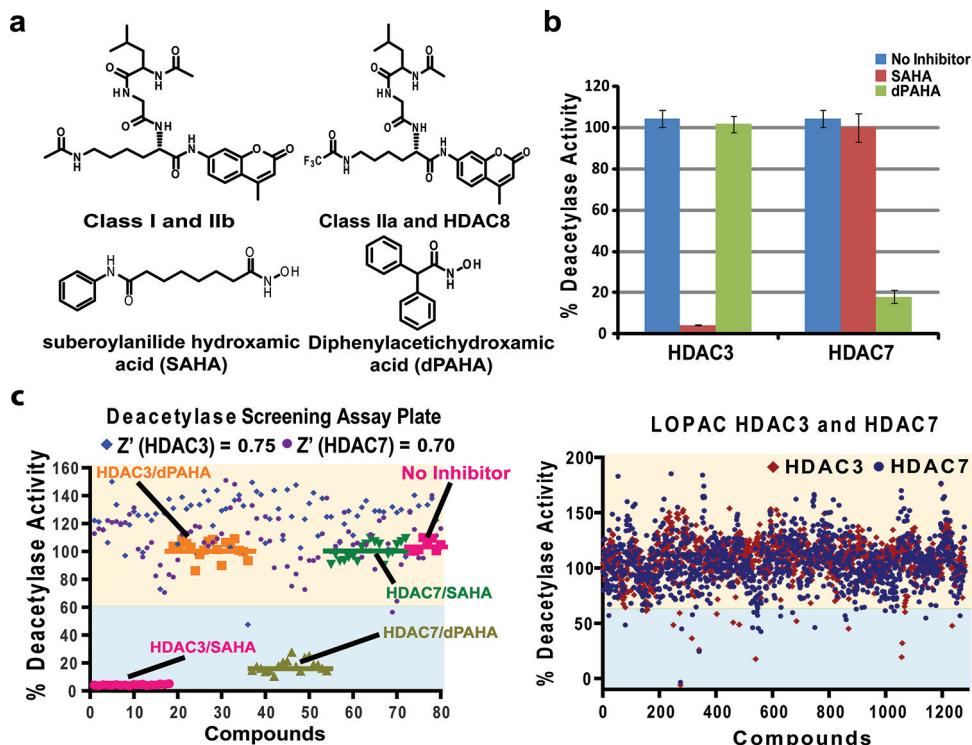


Figure 1. HDAC class-specific substrates, inhibitors, and compound screen. (a) Structures of class I and class IIa specific substrates. SAHA and dPAHA are used for positive inhibition control of class I HDAC and class IIa respectively ($10 \mu\text{M}$). (b) Orthogonal enzymatic deacetylase activities and inhibition profile of SAHA and dPAHA. (c) Typical results for a 96-well plate screen (left panel) and results of 1280 LOPAC screen hits. Compounds that exhibited greater than 40% HDAC inhibition at $10 \mu\text{M}$ are considered a positive hit from the primary screen (right panel).

The Library of Pharmacologically Active Compounds (LOPAC, 1280 compounds) was screened for potential inhibitory activity against class I and IIa HDACs. We identified five potential novel HDAC inhibitors displaying different potencies and preferential selectivity against HDAC isozymes *in vitro*. In cells, one inhibitor induced selective hyperacetylation of tubulin indicating HDAC6 inhibition matching its *in vitro* profile.^{20,21} Several analogues were further investigated, and one inhibitor, NQN-1, displayed a highly selective inhibition profile of HDAC6 *in vitro* and induced hyperacetylated tubulin in cells. Moreover, previous studies have shown HDAC6 controls Hsp90 chaperone activity through deacetylation of Hsp90.^{22,23} We have shown that NQN-1 induces Hsp90 acetylation and causes a decrease in Hsp90 client protein FLT-3, STAT5, and phosphorylated-Erk levels in the human acute myeloid leukemia cell line MV4-11, with associated cell death. Interestingly, hydroxamic acid selective HDAC6 inhibitors, tubacin and tubastatin A, did not induce acetylated Hsp90. In addition, the HDAC6 inhibitor caused an increase in ubiquinated protein levels in cells and promoted degradation of mutant FLT-3 through proteasome dependent pathways. It has great potential to be used as a therapeutic agent against AML or combined synergistically with other anticancer agents such as Hsp90 ATPase inhibitor, 17-AAG, or proteasome inhibitor, Bortezomib.

RESULTS AND DISCUSSION

Hit Identification Using Class-Specific HDAC Substrate Screening Assay. Unique small molecule inhibitors for different classes of HDACs are valuable as chemical tools or potential therapies against a variety of diseases. We have developed a high-throughput-compatible HDAC screen assay

using HDAC class-specific substrates. SAHA and diphenyl acetic hydroxamic acid (dPAHA) are used as positive controls for class I and class IIa, respectively (Figure 1a–c). We have demonstrated that the positive inhibitor controls are orthogonal to each class of HDACs at $10 \mu\text{M}$ as shown in Figure 1b, which is consistent with previous studies.^{17,24} HDAC3 and HDAC7 have the highest activity *in vitro* and are used as the recombinant enzymes of choice to identify potential novel HDAC class I and II inhibitors. Although HDAC3 and HDAC7 are not identical to all other isozymes, in this study we demonstrate their utility in identifying new HDAC inhibitors that display different inhibition preferences *in vitro* (Figure 2).

The LOPAC library was screened at $10 \mu\text{M}$ concentration for each compound (Figure 1d). The average Z' factor value for assays in both formats is 0.75 for HDAC3 plates and 0.7 for HDAC7 plates (Figure 1c). Initially, 24 compounds were selected from the primary hits for HDAC3 and HDAC7 to have $>40\%$ inhibition (standard deviations are 17.0% for HDAC3 plates and 21.7% for HDAC7 plates). Two point titrations were subsequently performed for each hit compound, and inhibitor candidates were chosen by eliminating compounds containing heavy metals or DNA chelators/intercalators and those with poor Log P and water solubility issues. Only five compounds remained as positive hits (Figure 2). In addition, none of the five positive hits were identified as nonspecific inhibitors due to *in vitro* aggregation verified by an aggregate formation assay against the same LOPAC compounds by assaying with/without 0.01% detergent Triton X-100 against activity of AmpC β -lactamase (a completely unrelated protein target).²⁵

Confirmation of *In Vitro* HDAC Inhibition and Selectivity Using HDAC Deacetylase Biochemical Assay. The five compounds identified with the LOPAC

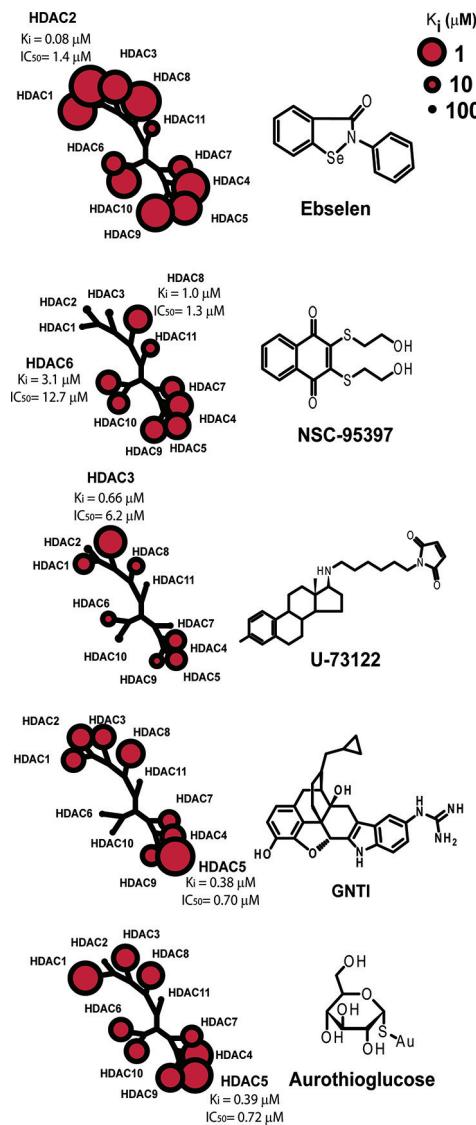


Figure 2. Identification of five positive hit HDAC inhibitors *in vitro*. The five positive compounds from the screen were further tested with full panel of HDACs. The inhibition profiles are represented according to the HDAC phylogenetic classification. IC₅₀ and K_i are labeled for the most inhibited HDAC for each compound.

library screen were further characterized with a HDAC deacetylase homogeneous assay with class-specific substrates (acetylated substrate: HDAC1, 2, 3, 6, and 10; trifluoroacetylated substrate: HDAC4, 5, 7, 8, 9, and 11).¹⁷ The median inhibition concentration (IC₅₀) was measured through a 12 point titration of inhibitor concentrations using a full panel of human recombinant HDAC enzymes. Each compound displayed a different inhibition potency and selectivity toward different HDAC isozymes (Figure 2, Table 1, and Supporting Information). *In vitro*, ebselen (2-phenyl-1,2-benziselenazol-3(2H)-one), an antioxidant, and aurothioglucose (1-thio-D-glucopyranose gold salt), an anti-inflammatory compound, displayed inhibitory activity for both class I and IIa HDACs. U-73122 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]-amion]hexyl]-1*H*-pyrrole-2,5-dione), an aminosteroid, showed an isozyme-selective preference toward HDAC3. NSC-95397 (2,3-bis[(2-hydroxyethyl)thio]-1,4-naphthoquinone), a dual-specificity phosphatase inhibitor, displayed preferences toward HDAC6 and class IIa HDACs. GNTI (guanidinyl-naltrindole trifluoroacetate), an opioid antagonist used in research that is highly selective for the κ opioid receptor, displayed inhibition preference toward HDAC5 (Figure 2 and Table 1).

All five compounds have unique structural motifs and display differential inhibition activity against different HDAC isozymes *in vitro*. The IC₅₀'s for the five compounds are in the micromolar to high nanomolar range, and apparent K_i values were calculated using the Cheng–Prusoff equation, which is consistent with the measurements from the initial library screens.²⁶ Further structure–activity relationship analysis could potentially yield novel and selective HDAC inhibitors for different HDAC isozymes and increase the collection of HDAC inhibitor pharmacophores.

In Vivo Activity of Selected Compounds. Activity *in vitro* does not necessarily represent how these compounds will act *in vivo*. HDACs are commonly known to function in large complexes with other functional proteins including other HDACs, and inhibitors affecting recombinant HDAC enzymes *in vitro* might not affect the HDAC complex activity.²⁷ Furthermore, the special biochemistry of class IIa HDACs and the lack of an identified class IIa cellular substrate necessitate further investigation in regards to the relevance of class IIa HDAC enzymatic activity. Regardless, the five compounds were examined for their ability to induce histone

Table 1. HDAC Inhibition IC₅₀ and K_i for the Five LOPAC Hit Compounds^a

	ebselen		NSC-95397		U-73122		GNTI		aurothioglucose	
	IC ₅₀	K _i								
HDAC1	1.5 ± 0.4	0.15	>180	N/A	38.4 ± 15.5	4.11	30.2 ± 7.3	3.24	4.4 ± 1.1	0.47
HDAC2	1.4 ± 0.3	0.08	>100	N/A	>100	N/A	26.2 ± 7.6	1.48	>180	N/A
HDAC3	2.1 ± 1.5	0.23	>100	N/A	6.2 ± 4.0	0.66	22.7 ± 3.1	2.43	11.5 ± 3.2	1.23
HDAC4	1.2 ± 0.2	0.20	7.2 ± 2.8	1.23	31.2 ± 0.2	5.33	13.4 ± 1.6	2.29	4.1 ± 2.1	0.7
HDAC5	0.78 ± 0.1	0.42	3.1 ± 0.2	1.68	10.5 ± 7.4	5.68	0.70 ± 0.1	0.38	0.72 ± 0.5	0.39
HDAC6	12.8 ± 2.6	3.10	12.7 ± 2.5	3.07	45.2 ± 12.6	10.96	>180	N/A	14.4 ± 5.8	3.49
HDAC7	10.9 ± 2.2	3.09	13.1 ± 4.3	3.72	>180	N/A	18.9 ± 1.0	5.36	22.4 ± 4.4	6.35
HDAC8	0.35 ± 0.2	0.11	1.3 ± 0.1	1.03	10.9 ± 3.1	8.63	2.0 ± 0.4	1.58	1.4 ± 0.3	1.11
HDAC9	0.97 ± 0.5	0.20	4.8 ± 1.4	2.04	29.8 ± 5.6	12.67	12.3 ± 1.7	5.23	2.8 ± 0.1	1.19
HDAC10	0.95 ± 0.4	0.42	14.5 ± 1.5	6.40	>100	N/A	>180	N/A	4.9 ± 1.5	2.16
HDAC11	22.5 ± 2.4	4.41	37.8 ± 1.4	7.41	10	N/A	10	N/A	>180	N/A

^aIC₅₀ values lower than 100 μM were determined from at least two independent titrations (Supporting Information). The IC₅₀ values were converted to K_i using Cheng–Prusoff equation assuming classical inhibitory mechanism.

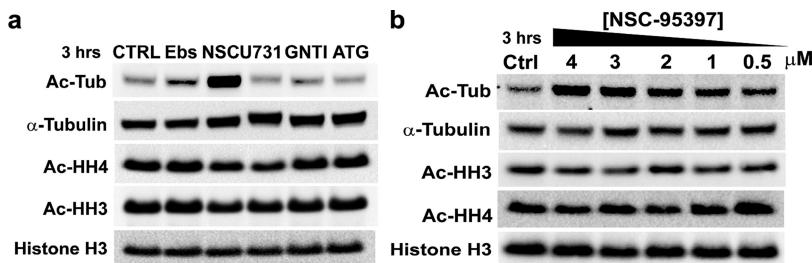


Figure 3. Induction of acetylated protein in more complex cellular context. (a) AML cells, MV4-11, were treated with 10 μ M concentration of each compound. The acetylation levels were assessed for acetylated tubulin (Ac-Tub), acetylated histone H3 (Ac-HH3), and acetylated histone H4 (Ac-HH4). Selective induction of hyperacetylated tubulin was observed indicating inhibition of HDAC6. (b) Concentration-dependent hyperacetylation of acetylated tubulin without increases in acetylated histone H3 and H4.

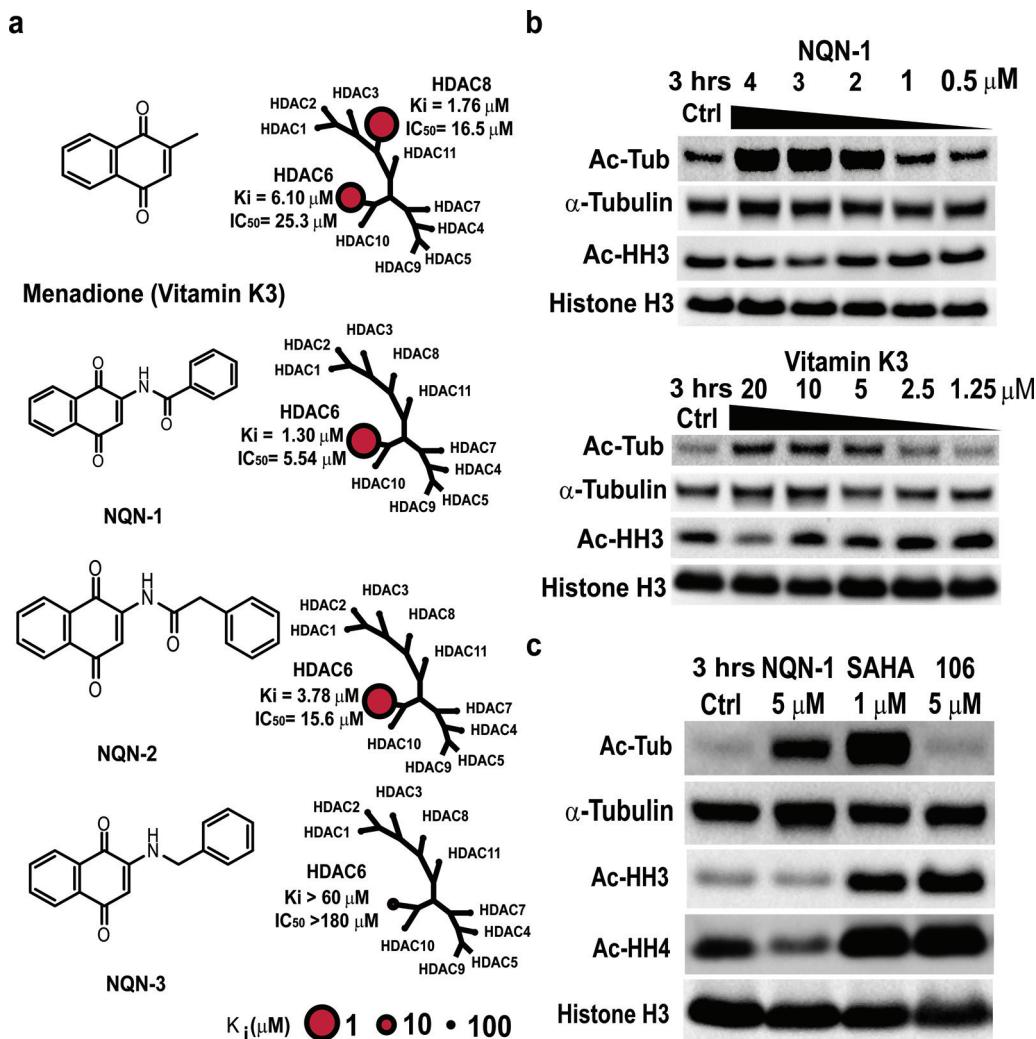


Figure 4. Selective HDAC6 inhibition of naphthoquinone analogues. (a) Inhibition profiles of naphthoquinone analogues. (b) Concentration-dependent induction of acetylated tubulin in AML MV4-11 cells. (c) NQN-1 selectively induces hyperacetylated tubulin without an increase in acetylated histone level. SAHA (class I HDAC and HDAC6 inhibitor) induces both acetylated tubulin and histone; HDAC inhibitor 106 (HDAC3 selective inhibitor) induces only hyperacetylated histone H3/H4.

H3, histone H4, and tubulin acetylation in MV4-11 cells. Only one out of the five compounds induced tubulin acetylation at 10 μ M (Figure 3a). NSC-95397 induces hyperacetylated tubulin without an increase in acetylated histone levels. Concentration-dependent induction of acetylated tubulin by NSC-95397 was observed, indicating concentration-dependent inhibition (Figure 3b).

Selective HDAC6 Inhibitors from the LOPAC Library Screen. NSC-95397 contains a central naphthoquinone structure. Naphthoquinone is the central structural motif of many natural products. Most notably it is the central structure for vitamin K. We purchased and synthesized analogues of NSC-95397 to assess their HDAC6 inhibition including vitamin K1/K2/K3, NQN-1 (PPM-18), NQN-2, and NQN-3. Vitamin K1 and K2 only inhibited HDAC6 above 200 μ M

(data not shown), while vitamin K3, 2-methyl-naphthoquinone, selectively inhibited HDAC6 and HDAC8 at low micromolar levels (Figure 4a). NQN-1 (2-benzyl-amino-naphthoquinone), a known anti-inflammatory agent, displayed a highly selective inhibitory profile for HDAC6 and a slight increase in inhibition potency (IC_{50} of $5.54 \pm 0.90 \mu\text{M}$ and K_i of $1.30 \pm 0.22 \mu\text{M}$) and did not inhibit other HDACs in our panel (Figures 4a, 5a,

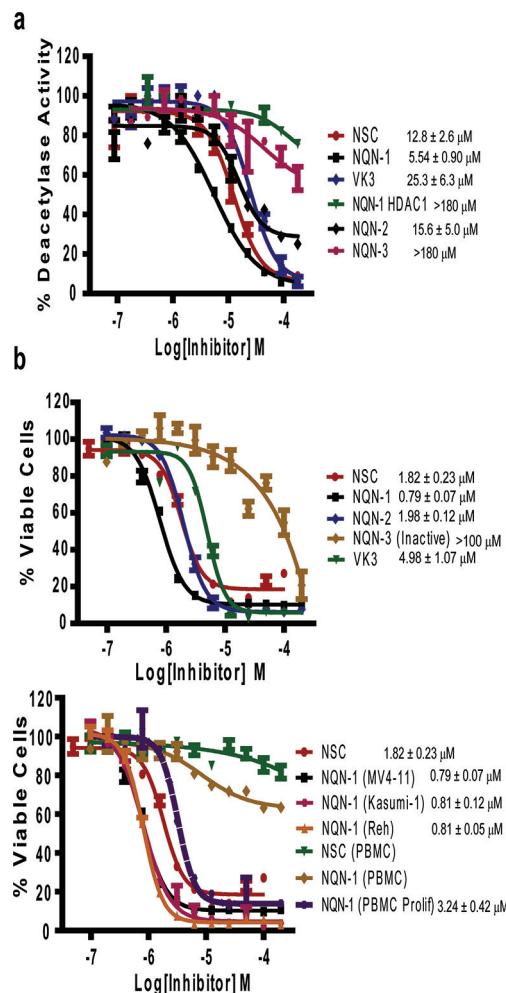


Figure 5. HDAC inhibition titration and selective toxicity against leukemia cells. (a) NQN-1 and analogues display selective inhibition against HDAC6. Minimal inhibitory activity was observed for other HDACs (HDAC1 shown and Supporting Information). (b) NQN analogues cause selective toxicity against leukemia cells consistent with their ability to inhibit HDAC6 *in vitro*. The least potent inhibitor of HDAC6, NQN-3, did not inhibit MV4-11 proliferation (>100 μM). Resting PBMC viability was not affected by either NQN-1 or NSC treatments. PBMC proliferation was inhibited at 3.24 μM, which is 4-fold higher than the toxicity against AML cells *ex vivo*.

and Supporting Information). NQN-2, an analogue of NQN-1 with one carbon bond extension, also selectively inhibited HDAC6 with a slight loss in its HDAC6 inhibition potency. NQN-3 is the least active analogue of NQN-1 with the carbonyl functional group removed. NQN-3 has little HDAC6 inhibitory activity with $IC_{50} > 180 \mu\text{M}$ and little cellular toxicity (Figure 4a, 5a, and 5b). Simple acetylated 2-acetamido-1,4-naphthoquinone (NQN-4) and 1,4-naphthoquinone were also examined for their HDAC6 inhibition and cellular toxicity (Supporting Information). Figure 5b shows the concentration-

dependent toxicity of MV4-11 cells by the NQN compounds as measured by MTS cell proliferation/toxicity assay. The NQN compounds that show micromolar HDAC6 inhibition displayed cell killing activity consistent with their HDAC6 inhibitory abilities. NQN-3 loses both its HDAC6 inhibition and cell killing abilities with a single key carbonyl functional group removed from NQN-1, suggesting HDAC6's role in selective leukemia toxicity. Although the exact compound concentration and its distribution in cells is unknown, the cell killing concentration over which the compounds are active coincides with the K_i values measured *in vitro*. All active compounds are able to induce concentration-dependent hyperacetylated tubulin consistent with inhibition of HDAC6 (Figures 4b and 5a). The specific acetylation of tubulin and histones are also assessed in comparison SAHA (HDAC1, 2, 3, and 6 inhibitor) and inhibitor 106 (HDAC3 selective inhibitor) (Figure 4c).²⁸ As expected, NQN-1 selectively induced hyperacetylated tubulin without an increase in histone H3 and H4 acetylation. However, NQN-1 unexpectedly caused a decrease in acetylated histone H4, and similar results were also observed for tubastatin A and tubacin with decreases in acetylated histones (Supporting Information). One possible hypothesis for this is that HDAC6 might control the activity of other HDACs or histone acetyltransferases (HATs), which have also been reported to be regulated through acetylation.²⁹ Further studies of how HDAC6 and HDAC6 inhibitors affect histone acetylation are currently underway. Because of the selectivity and potency of NQN-1, we chose NQN-1 as a representative for more detailed follow-up assays.

Selective Toxicity in Acute Myeloid Leukemia. The exact molecular basis for cell line specificity of different anticancer agents is not well understood. SAHA is particularly selective against cutaneous T-cell lymphoma. HDAC6 inhibition and knock-down has been shown to be effective against multiple myeloma and for enhancing the toxicity of the Hsp90 inhibitor, 17-AAG, against acute myeloid leukemia.^{30,31} To determine whether our hit compounds can selectively kill leukemia cells over normal peripheral blood mononuclear cells (PBMC), we compared the cell killing activity of NSC-95397 and NQN-1 in MV4-11 (AML), Kasumi (AML), Reh (ALL), and PBMC from healthy donors. Figure 5b shows that NSC-95397 and NQN-1 are inactive against nonproliferating PBMC (no mitogen stimulation), while NQN-1 inhibits proliferating PBMC at 3.2 μM. NQN-1 showed selective killing of MV4-11 and leukemia cells, but this effect is almost nonexistent against nonproliferating PBMC and 4-fold over mitogen-stimulated proliferating PBMC. This result suggests proliferation is one of the factors that contribute to the selective cellular toxicity of our inhibitors. However, many factors can also contribute to the differences in cell-line toxicity of a given compound such as differences in cellular uptake, metabolism differences, or potential off-target interaction with specific cellular components in cells. Optimization of NQN-1 to improve its leukemia cell specificity will be of great interest for further studies.

Effect of Active NQN-1 on the Levels of Hsp90 Acetylation, Hsp70, FLT-3, STAT5, and Erk phosphorylation. To be more explicit in the mechanism of action, it is critical to explore cellular pathways affected by the inhibitors. HDAC6 is known to control Hsp90 chaperone function through acetylation of its lysine residues and affects its client protein stabilities, *i.e.*, FLT-3, BCR-ABL, Raf-1, Akt, *etc.*^{22,31,32} Inhibition of HDAC6 and selective knock-down of HDAC6 have been shown to reduce mutant FLT-3 and Erk

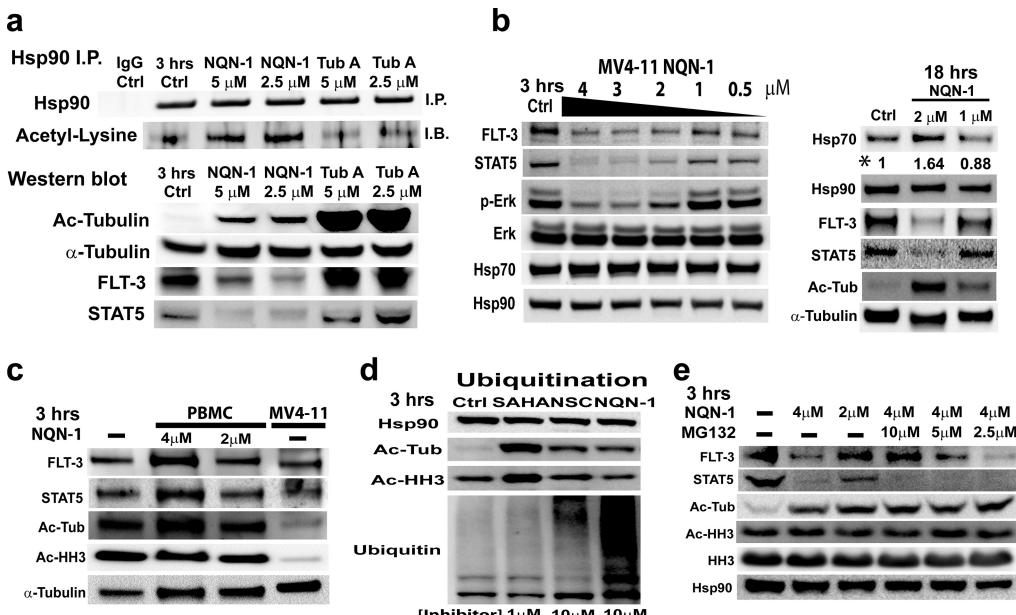


Figure 6. NQN-1 effects on Hsp90 acetylation, mutant FLT-3, STAT5, and Erk phosphorylation. (a) NQN-1 induces Hsp90 acetylation and causes degradation of FLT-3 and STAT5 and a decrease in the level of phospho-Erk 3 h post inhibitor treatment in MV4-11 cells. (b) NQN-1 decreases FLT-3, STAT5, and phospho-Erk in a concentration-dependent manner. Increase in Hsp70 level was observed only after prolonged treatment, 18 h (*quantification with spot density). (c) Tubulin acetylation level did not increase and normal FLT-3 and STAT5 did not decrease when PBMC was treated with NQN-1. Significantly higher acetylated tubulin and histone H3 levels were observed in PBMC ($n = 4$ normal donors) in comparison to malignant MV4-11 cells. (d) NQN-1 and NSC treatment resulted in an increase in total ubiquitinated protein level in MV4-11 cells. (e) The proteasome inhibitor MG132 rescued the depletion of mutant FLT-3 when co-administered at 10 μ M. However, the proteasome inhibitor did not prevent depletion of STAT5, indicating a proteasome-independent degradation pathway that is separate from the Hsp90 function.

phosphorylation and increase total ubiquitinated protein in cells. We compared our selective HDAC6 inhibitor, NQN-1, with recently reported selective HDAC6 inhibitor, tubastatin A, with >1000 selectivity over HDAC1 using immunoprecipitation (I.P.) and immunoblot techniques.³³ NQN-1 inhibits HDAC6 and induces Hsp90 acetylation 3 h following treatment. No induction of Hsp90 acetylation was observed in tubastatin A treated cells, although higher induction of tubulin acetylation was observed for tubastatin A than for NQN-1 (Figure 6a). Western blots on total cell extracts of MV4-11 cell, containing the mutant FLT-3 and constitutively active STAT5 (a downstream effector of tyrosine activation with a key role in AML),^{34,35} Figure 6a and b, shows FLT-3 and STAT5 protein levels decrease within 3 h following NQN-1 treatment but not for tubastatin A. This is consistent with the inhibitor's ability to induce Hsp90 acetylation. Similar results have also been reported by Bali *et al.* for another selective HDAC6 inhibitor, tubacin, which showed tubacin does not significantly increase Hsp90 acetylation even after 16 h post inhibitor treatments.^{22,36} We have also examined the ability of other HDAC inhibitors to induce Hsp90 acetylation 3 h post-treatment. Surprisingly, SAHA, TSA, and inhibitor 106 did not induce Hsp90 acetylation or reduce FLT-3 and STAT5 levels, suggesting a direct link to Hsp90 acetylation and FLT-3 and STAT5 depletions (Supporting Information). Contrary to previous proteomic studies, SAHA was reported to induce acetylation of some residues of Hsp90 after 24 h in MV4-11 cells.³ HDAC inhibitors are known to down-regulate HDAC expression through transcriptional controls;^{37–39} therefore, it is possible that prolonged treatment of SAHA would decrease the level of HDAC6 in MV4-11 resulting in Hsp90 hyperacetylation and inhibition after 24 h. Other thiolated HDAC6 inhibitors, which substitute a thiol for the hydroxamic acid moiety, have only

modest preference for HDAC6 and thus were not compared in this study.^{33,40,41} In PBMC, little hyperacetylated tubulin was observed 3 h post-treatment, and normal FLT-3 and STAT5 protein levels were not affected by the treatment (Figure 6c), which is consistent with a previous study using tubacin.³⁰ The authors attribute the lack of tubacin-induced tubulin hyperacetylation to the low expression of HDAC6 in PBMC. This is consistent with our observation that acetylated tubulin and acetylated histone H3 in normal PBMC are several folds higher than the malignant AML MV4-11 cells, indicating either PBMC has a low expression of HDAC6 or HDAC6 is inactive in PBMC population from healthy donors (Figure 6c). This result suggests that HDAC6 activity and inhibition affects normal cells differently than leukemia cells and offers an explanation to the selective nature of the inhibitor. Moreover, the inhibition of Hsp90 chaperone activity due to HDAC6 inhibition increases Hsp70 protein level after 6 h (Figure 6b and Supporting Information), which is consistent with previous reports of increased Hsp70 by HDAC6 siRNA knock-down.

Previous studies have also shown Hsp90 client protein degradation is mainly mediated through proteasomal pathways with increased cellular ubiquitinated protein levels.^{22,30,31} NQN inhibitors also increase the level of ubiquitinated proteins within 3 h following treatment (Figure 6d). To further elucidate the role HDAC6 inhibition and proteasomal pathways play in FLT-3 (a known client protein of Hsp90) and STAT5 degradation, proteasome inhibitor MG132 was used to rescue FLT-3 degradation caused by HDAC6 inhibition and loss of Hsp90 chaperone function. As expected, FLT-3 degradation is rescued by 10 μ M MG132 co-treatment. This is consistent with our hypothesis of HDAC6 inhibition affecting Hsp90 client protein through proteasome degradation (Figure 6e). However, MG132 did not prevent STAT5 degradation, indicating that

HDAC6 inhibition might also play a role in controlling the function of other proteases, which will be investigated further.

Conclusion. In this study, we have shown that it is possible to identify novel HDAC inhibitors with potential for improved isozyme selectivity that do not conform to the canonical structural motifs of known HDAC inhibitors.¹⁹ We demonstrated that HDAC class-specific substrates and highly active recombinant HDAC enzymes can be used to screen a compound library in high-throughput-compatible format. Using this assay to screen 1280 compounds, we have identified, for the first time, a class of small molecule inhibitors of HDAC6 with IC₅₀'s in the micromolar range that do not contain either hydroxamic acid or benzamide metal chelating groups.

In human AML cells, the prototype compound NQN-1 induced hyperacetylation of tubulin and Hsp90 within 3 h following treatment, which the known hydroxamic acid based inhibitor, tubastatin A, does not. It also caused degradation of the Hsp90 client protein, mutant FLT-3, as well as constitutively active STAT5, which are known to be critical for leukemia cell proliferation.^{32,34} The degradation of mutant FLT-3 is proteasome-dependent and can be rescued with the proteasome inhibitor MG132, while STAT5 degradation is proteasome-independent and requires further investigation. Additionally, the compound shows moderately selective toxicity for leukemia cells *versus* normal proliferative PBMC and is inactive against nonproliferative PBMC. Similarly, the HDAC6-selective inhibitor tubacin has been shown to selectively induce apoptotic cell death in multiple myeloma and acute lymphoblastic leukemia cells with a lesser effect on normal PBMC.^{30,42} Namdar *et al.* recently reported that tubacin only slows the growth but does not induce cell death of normal human foreskin fibroblast and transformed human prostate and breast cancer cells.⁴³ These studies suggest HDAC6 inhibition offers potential selective toxicity against cancers of leukemic or plasma origins.

All of the active compounds derived from the screen targeting HDAC6 contain the central 1,4-naphthoquinone ring system. In the recombinant enzyme and cellular assays performed to date, NQN-1 displayed the strongest potency and selectivity at inducing AML cell death, HDAC6 inhibition, and Hsp90 acetylation. Interestingly, the carbonyl linking the naphthoquinone and the phenyl groups is required for its HDAC6 inhibitory activity and toxicity against leukemia cells, while vitamin K3, with a simple 2-methyl-1,4-naphthoquinone structure, inhibits HDAC6 with IC₅₀ of 25 μ M. We theorize that although the phenyl group from NQN-1 improves the specificity toward HDAC6, the larger phenyl group might not be ideal. This is supported by the potency decrease imparted by a single methylene linker extension in NQN-2. The lack of toxicity of NQN-3 and the attenuation of the NQN-1 toxic effects by overexpression of HDAC6 (Supporting Information) suggest that the toxicity is target-specific and is unlikely due to nonspecific toxicity from the 1,4-naphthoquinone core. Further structural investigation will be done to improve both the affinity and specificity of this unique class of HDAC6 inhibitors.

Using compounds identified and derived from the novel high-throughput screen developed in this study, we demonstrated that inhibition of HDAC6 and interference with Hsp90 chaperone function results in the degradation of critical proteins and attenuation of signaling cascades that are known to promote leukemia cell growth. This data, combined with demonstrated selective toxicity of the compounds toward AML cells, provides a strong rationale for the development of novel

small molecule inhibitors of HDAC6. This unique class of HDAC6 inhibitor already shows micromolar activity in various *in vitro* and *ex vivo* assays. Future generations of inhibitors with increased potency and selectivity for HDAC6 not only will be useful chemical probes for investigations seeking to elucidate the isozyme's involvement in various pathologies but also have the potential to be developed into novel leukemia and cancer therapeutics.

METHODS

Materials. Recombinant human HDAC1, HDAC2, HDAC3/N-CoR2, HDAC5, HDAC6, HDAC8, HDAC9, and HDAC10 expressed in baculovirus were purchased from BPS Bioscience. Recombinant human HDAC4 and HDAC7 were purchased from Millipore, and HDAC11 was purchased from Enzo Life Sciences. Acetylated-Lys(acetyl)-7-amino-methylcoumarin (acetyl-Lys(Ac)-AMC) was purchased from MP Biomedicals. Acetyl-Lys(trifluoroacetyl)-7-amino-methylcoumarin (acetyl-Lys(TFA)-AMC) was purchased from Bachem. Leucine, glycine, lysine acetyl, and trifluoroacetyl substrates (Ac-KGL and TFA-KGL substrates) were synthesized as previously described (Supporting Information). Suberoylanilide hydroxamic acid (SAHA) and Tubastatin A were purchased from Cayman Chemical, and Tubacin was purchased from Enzo Life Science. Diphenyl acetic hydroxamic acid was synthesized as previously described.²⁴ All LOPAC chemicals were purchased from Sigma-Aldrich and dissolved with DMSO to a minimum of 10 mM stock solution. All other chemicals were purchased from Sigma-Aldrich. NQN-1 was purchased from Sigma-Aldrich and synthesized in our laboratory (Supporting Information). NQN-2, NQN-3, and NQN-4 were synthesized as described and characterized with ¹H NMR and LC-MS/MS (Supporting Information).

Cells and Antibodies. MV4-11, Kasumi-1, and Reh leukemia cells were purchased from American Type Culture Collection (ATCC). Normal PBMCs were purchased from Cellular Technology Inc. MV4-11 is a human AML cell line expressing mutant FLT-3. MV4-11 was cultured in recommended media supplemented with 10% heat-inactivated fetal bovine serum. PMBC was thawed with serum-free wash buffer and cultured in standard culture media. All cells were grown in a humidified atmosphere with 5% CO₂ at 37 °C. Acetylated tubulin, tubulin, acetylated-HH4, acetylated HH3, FLT-3, STAT5, Erk, phos-Erk, ubiquitin, Hsp90, and horseradish peroxide (HRP) secondary antibodies used for immunoprecipitation and Western blot were purchased from Santa Cruz Biotechnology. Hsp70 and acetylated-lysine antibodies were purchased from Cell Signaling Technology. Histone H3 antibody was purchased from Abcam, and acetylated-Hsp90 lysineK69 antibody was purchased from Alpha Diagnostic International.

Primary Hit Identification Using Substrate-Specific HDAC Assays and Hit Identification. The LOPAC library was obtained from Sigma-Aldrich. HDAC3 and HDAC7 were preincubated with screening compounds for 2 h and deacetylate reactions were initiated with addition of HDAC substrates (acetyl-Lys(Ac)-AMC for HDAC3 and acetyl-Lys(TFA)-AMC for HDAC7). The reactions were allowed to proceed for 2 h and developed with 25 μ L of trypsin peptidase at 10 mg mL⁻¹. Z'-factor, a quality parameter used to judge high-throughput performance, is determined using known HDAC inhibitors as positive control for both HDAC3 and HDAC7.

Biochemical HDAC Deacetylase Inhibition Assay. Individual IC₅₀ values for each HDAC isozyme were measured with the homogeneous fluorescence release HDAC deacetylase assay. Purified recombinant enzymes were incubated with serial diluted inhibitors at the indicated concentration. The deacetylase activities of class I HDACs 1, 2, 3, 6, and 10 were measured by assaying enzyme activity using AMC-K(Ac)GL substrate and AMC-K(TFA)GL substrate for class IIa HDACs 4, 5, 7, 8, 9, and 11 as previously described.^{15,17} Deacetylated AMC-KGL was sensitive toward lysine peptidase, and free fluorogenic 4-methylcoumarin-7-amide (MCA) was generated, which can be excited at 355 nm and observed at 460 nm. The data was

analyzed on a plate to plate basis in relationship to the control and imported into analytical software (Graphpad Prism).

Compound Libraries and Analogues. LOPAC library was obtained from Sigma-Aldrich. NQN analogues were synthesized as described, characterized with ^1H and ^{13}C NMR, mass spectrometry, and analytical HPLC (Supporting Information).

MTS Colorimetric Cell Proliferation/Toxicity Assay. The inhibitor's effect on cell proliferation and the ability to initiate apoptosis were determined using Promega CellTiter 96 Aqueous One Cell Proliferation assay. The effective dose 50 (EC_{50}) was determined from the inhibitor against cell proliferation, and the data were analyzed with Graphpad Prism.

Immunoprecipitation and Western Blotting. Treated cells were washed with cold Hanks' Balanced Salt Solution (Invitrogen) followed by lysis with a low salt lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.5 Triton X-100, 1X protease and phosphatase inhibitor cocktail, and 1 μM TSA) for 30 min, followed by a 15-s sonication pulse at 30 W. For immunoprecipitation, the lysates were incubated with Hsp90 conjugated Dynabeads (Invitrogen) for 1 h according to the manufacturer instruction. The immunoprecipitates were immunoblotted and tested against Hsp90 (loading control), antiacetylated lysine (acetylation level), and acetylated-Hsp90K69. For Western blot, the cell lysates were denatured with LDS loading buffer (Invitrogen) and run on 4–12% Tris-glycine gradient gels after centrifugation. Total histone H3, acetylated histone H3, acetylated H4, FLT-3, STAT5, Hsp70, Hsp90, and acetylated α -tubulin were assessed with primary antibody, followed by rabbit or mouse IgG-HRP secondary antibody. The blot was developed using Pierce Thermo Dura-ECL reagent (Thermo-Fisher) and visualized using Alpha Innotech gel imager (Cell Biosciences).

Data Analysis. The inhibition of HDAC activity by small-molecule inhibitor candidates was measured as described above. Data were analyzed by nonlinear regression with determination of IC_{50} and standard deviation using GraphPad Prism. K_i values were calculated on the basis of the Cheng-Prusoff equation for standard fast-on/off inhibitor, $K_i = \text{IC}_{50}/(1 + ([S]/K_m))$, with IC_{50} values derived from the nonlinear curve fit of the dose response curves, where K_i is the inhibition constant, [S] is the substrate concentration, and K_m is the Michaelis constant.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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