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Design, synthesis, and activity of HDAC inhibitors with a *N*-formyl hydroxylamine head group☆

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Abstract—Histone deacetylases (HDAC) are promising targets for cancer chemotherapy. HDAC inhibitors are thought to act in part by disrupting normal cell cycle regulation, resulting in apoptosis and/or differentiation of transformed cells. Several HDAC inhibitors, which contain hydrophobic tails and the Zn^{2+} chelator hydroxyamic acid as a head group, are potent inhibitors of HDACs both in vitro and in vivo. In this study, a related class of compounds with a *N*-formyl hydroxylamino head group has been synthesized and their ability to inhibit HDACs have been assayed in biochemical and cellular assays. These compounds were found to have comparable activities to suberoylanilide hydroxyamic acid (SAHA) in HDAC enzymatic assays and histone hyperacetylation cellular assays.

Inhibitors of histone deacetylases (HDAC) represent a new class of potential small molecule cancer therapeutics. Among the hybrid polar class of HDAC inhibitors, suberoylanilide hydroxyamic acid (SAHA) has demonstrated both in vitro and in vivo efficacy against several types of cancer and is in clinical trials.[1–3] The molecular basis of HDAC inhibition by SAHA has been revealed by the X-ray co-crystal structure of SAHA and a HDAC related polyaminohydrolase (HDLP).[4] The aliphatic chain of SAHA inserts into the narrow hydrophobic cleft of the enzyme active site, and the polar head group bearing the hydroxyamic acid serves as a bidentate chelator of the catalytic Zn^{2+} ion bound in the active site. Other Zn^{2+} chelators have been used to inhibit Zn^{2+} dependent enzymes, including the isomeric *N*-formyl hydroxylamino moiety which has been used in inhibitors of angiotensin converting enzyme (ACE) and neural endopeptidase (NEP).[5] We therefore hypothesized that replacement of the hydroxyamic acid group of SAHA and other HDAC inhibitors with a *N*-formyl hydroxylamine should not adversely affect their ability to inhibit HDAC, but may lead to a novel HDAC inhibitor with improved biological properties (Fig. 1).

Based on the co-crystal structure of SAHA and HDLP, several compounds were designed which mimic the hydrophobic tail group and aliphatic chain of SAHA, but have a *N*-formyl hydroxylamino group replacing the hydroxyamic acid. These compounds should pack into the hydrophobic cavity in a similar fashion to SAHA, allowing the *N*-formyl hydroxylamine group to form a bidentate chelate to Zn^{2+}. The synthesis of these compounds is illustrated in Scheme 1.[6] Hydroxy-carboxylic acids **1** with varying chain lengths ($n = 5–7$) were first converted to the corresponding mesylate **2** in approximately 75% yield. Coupling of **2** with a number of anilines was carried out using PyBOP/DIEA to yield **3** in 53–65% yield. Nucleophilic displacement of the mesylate by *O*-benzylhydroxylamine was carried out by heating the reaction at 80 °C in DMF in 41–50% yield. The resulting secondary amine **4** was acylated or formylated to give **5** (R_2 = Me, OMe, or H). Final deprotection of the benzyl group under hydrogenation conditions completed the synthesis of **6**. Using this synthetic route, analogues TWZ101 to TWZ108 were made in overall yield of 10–20%.

To synthesize analogues with varying hydrophobic groups, another method was devised in which the hydrophobic moiety was introduced at the last step of the synthesis (Scheme 2).[7] The primary bromide of *N*-Boc-1-amino-5-bromopentane **7** was displaced by *O*-benzylhydroxylamine to afford **8** in 77% yield. Subsequent formylation of the secondary amine by in situ

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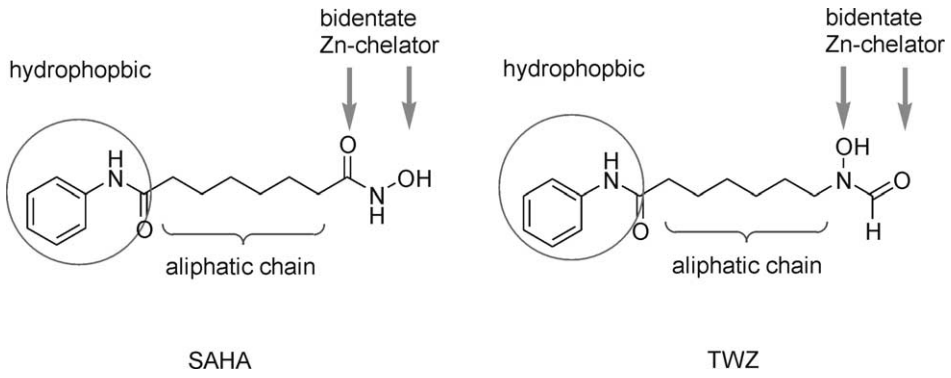
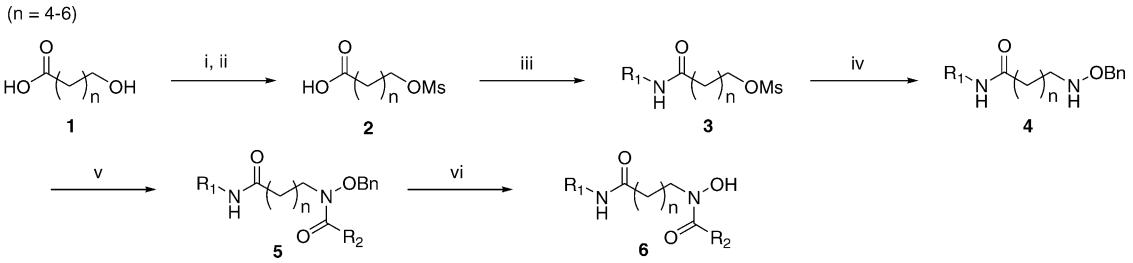
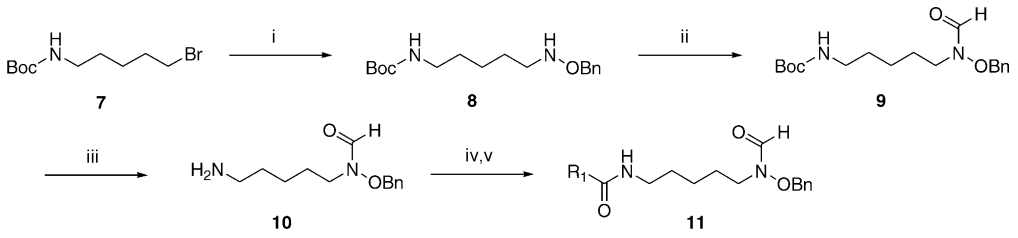


Figure 1. Structures of SAHA and TWZ analogues.



Scheme 1. (i) MsCl, Et_3N; (ii) NaOH, (63–83% over two steps); (iii) R_1NH_2, PyBOP, DIEA, THF, (53–65%); (iv) $BnONH_2$, DMF, 80 °C, (41–50%); (v) for R_2 = Me, OMe: R_2COCl, DIEA, DCM, (>95%); for R_2 = H: HCOOH, CDI, DCM, 0 °C, (>95%); (vi) H_2, Pd/C, MeOH/THF (80–95%).



Scheme 2. (i) $BnONH_2$, K_2CO_3, DMF, 80 °C, (77%); (ii) HCOOH, CDI, DCM, 0 °C, (>95%); (iii) 10% TFA/DCM, (95%); (iv) R_1COCl, DIEA, DCM; (v) H_2, Pd/C, (70% over twp steps).

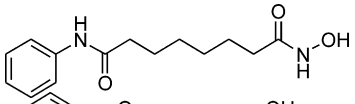
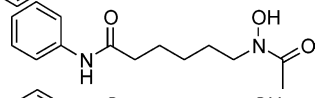
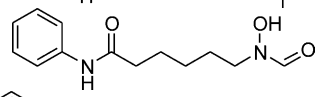
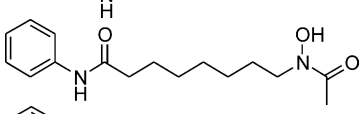
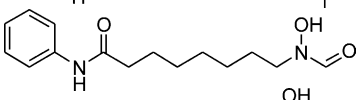
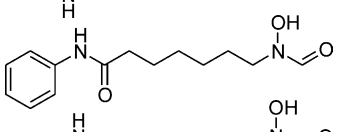
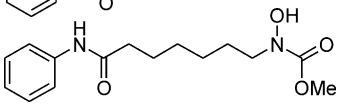
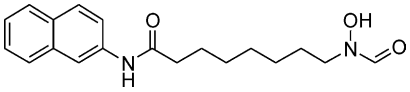
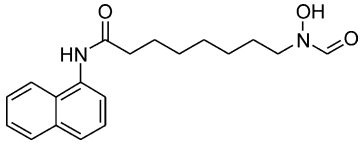
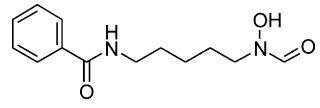
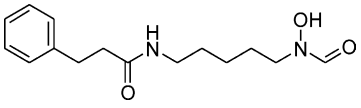
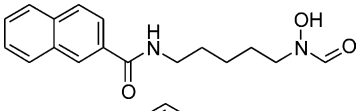
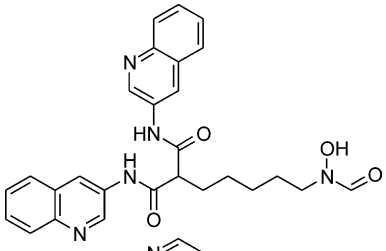
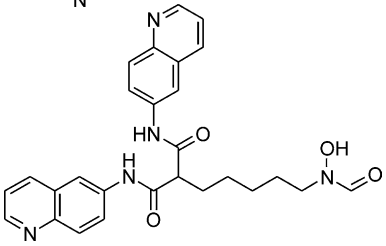
activation of formic acid using carbonyl diimidazole (CDI) yielded compound **9** in nearly quantitative yield. The Boc group was then deprotected by treatment with 10% TFA/DCM to expose the primary amine (**10**). Condensation of **10** with various acid chlorides followed by benzyl deprotection resulted in the synthesis of **11** (TWZ109 to TWZ111) in 49% overall yield. SAHA was also synthesized as a reference compound for biological assays.[8]

Enzymatic activities of HDAC1, HDAC2, HDAC6, HDAC8, as well as total HDAC from nuclear extracts, were tested in fluorescence-based HDAC enzymatic assays (Table 1).[9,10] SAHA was used as a positive control in all of the assays. Changes in the Zn^{2+} chelating group have significant effects on HDAC inhibition. Derivatives with the formyl head group (R_2 = H) inhibited all HDACs with IC_{50}'s in the low micromolar range: a 15- and 150-fold decrease in activity against HDAC2 and HDAC8 relative to the corresponding hydroxyamic acid (SAHA) (Table 1). Compounds with acetyl (R_2 = Me) or methyl carbamate (R_2 = OMe) groups are inactive against all of the HDACs tested (IC_{50} > 100 μM). This latter result is consistent with modeling experiments, which suggest that the active site is too small to accommodate a methyl or methoxy group. However, it is not clear why the *N*-formyl hydroxylamino head group caused a dramatic loss in activity. Further investigations using X-ray crystallography are in progress.

In contrast to the head groups, structural modifications of the hydrophobic tail had less effect on binding affinity. For example, the length of the aliphatic chain (n = 4–6) does not seem to have a significant effect on inhibition activity. However, the structure of the hydrophobic group has a moderate affect on activity. Increasing steric size from phenyl (TWZ104) to naphthyl (TWZ107 and 108) while keeping the alkyl chain constant improved the inhibitory activity 2- to 10-fold. Increasing the flexibility of the hydrophobic group led to a significant decrease in HDAC inhibition (TWZ110). Finally, reversal of the amide bond on the hydrophobic end (TWZ109 and 111) slightly lowered the activity.

Several SAHA analogues with increased potency have been reported which retain the hydroxyamic acid group,

Table 1. Inhibitory activity of TWZ analogues in enzymatic assays

	Structure	HDAC1 (μM)	HDAC2 (μM)	HDAC6 (μM)	HDAC8 (μM)	HDAC (extract) (μM)
SAHA		0.048	0.073	0.10	0.29	0.10
TWZ 101		n.d.[a]	> 100	> 100	> 100	n.d.
TWZ 102		n.d.	19	2.8	6.8	n.d.
TWZ 103		n.d.	> 100	> 100	> 100	n.d.
TWZ 104		n.d.	7.8	5.5	2.8	n.d.
TWZ 105		n.d.	11	n.d.	4.0	n.d.
TWZ 106		n.d.	> 100	> 100	> 100	n.d.
TWZ 107		0.81	0.89	0.91	0.78	4.0
TWZ 108		5.7	5.9	3.1	0.65	50
TWZ 109		30	26	4.3	22	> 100
TWZ 110		49	41	30	9.7	> 100
TWZ 111		1.3	1.4	0.60	7.0	4.4
TWZ 112		0.16	0.20	0.78	0.69	0.97
TWZ 113		0.18	0.27	0.62	0.80	0.75

[a] n.d., not determined.

Table 2. TWZ inhibitory activities (EC_{50}) in cellular assays

	Histone Hyperacetylation	Proliferation			
		U-937	Jurkat	Hepatocyte	BMC
SAHA	0.7 μM	33 μM	2.3 μM	> 500 μM	150 μM
TWZ112	5.1 μM	40 μM	11 μM	41 μM	78 μM
TWZ113	10 μM	100 μM	31 μM	> 500 μM	130 μM

but which have a larger hydrophobic tail.[11] Thus, an inccrease in the size of the hydrophobic tail might compensate for the loss in activity of the TWZ analogues. The bis-aminoquinoline hydrophobic group was selected to test this notion. TWZ112 and TWZ113 were synthesized as described in Scheme 1. The IC_{50}'s of TWZ112 and TWZ113 were between 150–800 nM against different HDACs (Table 1). These IC_{50} values are approximately 10- to 50-fold lower than previous hydroxylamine analogues, but 2- to 5-fold higher than SAHA in the enzymatic assays. These two compounds, as well as SAHA, were tested in a histone hyperacetylation assay in cells (Table 2).[12] Consistent with the in vitro inhibition activity, the EC_{50}'s were 0.7, 5, and 10 μM for SAHA, TWZ112, and TWZ113, respectively. Cell proliferation assays were also performed on two human leukemia cell lines (U-937 and Jurkat)[13] and two types of human primary cells (hepatocytes and bone marrow cells)[14] (Table 2). TWZ112 and TWZ113 did exhibit cytotoxicity effects in the cancer cells tested, but with a two- to ten-told lower activity than SAHA (Table 2). This result is consistent with the enzymatic and histone hyperacetylation assay results. The toxicity of TWZ113 against hepatocytes and bone marrow cells is comparable to that of SAHA, while TWZ112 has increased cytotoxicity against hepatocytes ($EC_{50} = 41$ μM).

In conclusion, replacing the hydroxyamic acid group on SAHA with an *N*-formylhydroxylamino head group led to inhibitors of HDAC, but with a fifty-fold drop in potency. This loss in activity could be offset by increasing the size of the hydrophobic region to afford HDAC inhibitors with low micromolar activity in cellular histone hyperacetylation assays. The anti-proliferation effects of TWZ112 and TWZ113 in leukemia cells and additional toxicity tests in hepatocytes and bone marrow cells indicated these compounds have a comparable therapeutic window to SAHA. Further in vitro and in vivo assays will be required to determine whether these compounds offer enhanced pharmacological properties relative to SAHA.

References and notes

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4. Finnin, M. S.; Donigian, J. R.; Cohen, A.; Richon, V. M.; Rifkind, R. A.; Marks, P. A.; Breslow, R.; Pavletich, N. P. *Nature* **1999**, *401*, 188.
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6. A representative synthetic protocol for Scheme 1. To a stirred solution of **1** ($n = 6$) (250 mg, 1.56 mmol) in THF (7 mL) at 0 °C was added dropwise MsCl (0.3 mL, 3.91 mmol) followed by Et_3N (0.65 mL, 4.68 mmol). The reaction was warmed to rt over 5 h. A solution of NaOH (312 mg, 7.8 mmol) in water (7 mL) was added and stirred for an additional 15min. Ether was added to the reaction mixture, and the organic layer was extracted and discarded. The aqueous layer was acidified with 3N HCl (to pH < 1) and extracted three times with ethyl acetate. The combined organic layers were dried with anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude product **2** (320 mg, ca. 86%) was used in the next step without further purification. To a solution of **2** ($n = 6$) (160 mg, ca. 0.73 mmol) in DCM (7 mL) was added 1-aminonaphthalene (104 mg, 0.73 mmol), PyBOP (380 mg, 0.73 mmol), and DIEA (0.25 mL, 1.46 mmol). The reaction was stirred at rt for 8 h and poured into water. The aqueous layer was removed, and the organic layer was washed with saturated aqueous NH_4Cl, saturated aqueous $NaHCO_3$, brine, dried with $MgSO_4$, and concentrated under reduced pressure. The resulting crude product was purified by flash column chromatography on silica gel using 25% ethyl acetate in hexane to give 172 mg (65%) of compound **3** (R_1 = 1-naphthyl, $n = 6$). A stirred solution of **3** (R_1 = 1-naphthyl, $n = 6$) (97 mg, 0.267 mmol) and *O*-benzylhydroxyamine (165 mg, 1.34 mmol) in DMF (5 mL) was heated to 80 °C overnight. The reaction was cooled to ambient temperature, poured into water, and the aqueous layer was extracted three times with ethyl acetate. The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$, and concentrated under reduced pressure. The resulting crude was purified by flash column chromatography on silica gel using 20% ethyl acetate in hexane to give 52 mg (50%) of **4** (R_1 = 1-naphthyl, $n = 6$). For the synthesis of **5** where R_2 = methyl or methoxy, a solution of **4** (R_1 = Ph, $n = 5$) (50 mg, 0.144 mmol) in THF (1.4 mL) was added methyl chloroformate (17 μL, 0.22 mmol) and DIEA (38 μL, 0.22 mmol). The reaction was stirred at rt for 2 h and the solvent was removed under reduced pressure. The resulting crude product **5** (R_1 = Ph, $n = 5$, R_2 = OMe) (ca. > 95% yield by LCMS) was used in the next step without further purification. For the synthesis of **5** where R_2 = H, a solution of carbonyl diimidazole (CDI) (117 mg, 0.72 mmol) in THF (5 mL) at 0 °C was added 95% formic acid (35 μL, 0.72 mmol) and stirred at 0 °C for 30min. Then, a solution of **4** (R_1 = Ph, $n = 5$) (50 mg, 0.144 mmol) in THF (2 mL) was added, and the reaction was stirred at 4 °C overnight. The solvent was removed under reduced pressure, and the resulting crude product **5** (R_1 = Ph, $n = 5$, R_2 = H) (ca. > 95% yield by LCMS) was used in the next step without further purification. Compound **5** was dissolved in MeOH/THF (1:1) and a catalytic amount of 10% Pd/C

was added. The reaction vessel was quickly evacuated and refilled with hydrogen gas three times. The reaction was allowed to stir under hydrogen atmosphere for 5–8 h. The Pd catalyst was filtered through a plug of Celite and washed with MeOH. The filtrate was concentrated and the resulting crude product (ca. 80–95% yield) was purified by HPLC using a C18 reverse phase column with a 15 min gradient of 5–95% acetonitrile/water. The fractions containing the desired product were dried down and prepared as 10 mM DMSO stock solutions. TWZ102. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.49 (d, J = 7.7 Hz, 2H), 7.29 (t, J = 7.7 Hz, 2H), 7.08 (t, J = 7.3 Hz, 1H), 3.49 (t, J = 6.6 Hz, 2H), 2.36 (t, J = 7.0 Hz, 2H), 1.69–1.77 (m, 4H), 1.34–1.42 (m, 2H). Observed $[M+H]^+$ 251.13; calcd for 250.13.

7. A representative synthetic protocol for Scheme 2. N-Boc-1-amino-5-bromopentane **7** (1 g, 3.77 mmol) was stirred with *O*-benzylhydroxyamine (1.4 g, 11.3 mmol) and K_2CO_3 (1.6 g, 11.3 mmol) in DMF (15 mL). The reaction was heated to 90C for 3 h and poured into water. The aqueous layer was extracted three times with ethyl acetate and the combined organic layers were washed with brine, dried with anhydrous $MgSO_4$, and concentrated under reduced pressure. The resulting crude was purified by flash column chromatography on silica gel using 1% MeOH in DCM to give 892 mg of **8** (77%). To a solution of carbonyl diimidazole (CDI) (527 mg, 3.25 mmol) in THF (30 mL) at 0 °C was added 95% formic acid (158 μL, 3.25 mmol) and stirred at 0 °C for 30 min. Then, a solution of **8** (200 mg, 0.65 mmol) in THF (10 mL) was added, and the reaction was stirred at 4 °C overnight. The solvent was removed under reduced pressure, and the resulting crude product **9** was treated with 10% TFA/DCM for 1.5 h at rt. The reaction was poured into saturated aqueous K_2CO_3 and the aqueous layer was extracted three times with DCM. The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$, and concentrated under reduced pressure. The resulting product **10** (>95% yield by LCMS) was used in the next step without further purification. To a solution of **10** (50 mg, 0.22 mmol) in DCM (1 mL) was added benzoyl chloride (27 μL, 0.233 mmol) and DIEA (58 μL, 0.33 mmol). The reaction was stirred at rt for 8 h, poured into water, and the aqueous layer was extracted twice with DCM. The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$, and concentrated en vacuo. The resulting crude product was then dissolved in MeOH/THF (1:1; 1mL) and a catalytic amount of 10% Pd/C was added. The reaction vessel was quickly evacuated and refilled with hydrogen gas three times. The reaction was allowed to stir under hydrogen atmosphere for 5 h. The Pd catalyst was filtered through a plug of Celite and washed with MeOH. The filtrate was concentrated and the resulting crude product (ca. 70% yield over two steps) was purified by HPLC using a C18 reverse phase column with a 15 min gradient of 5–95% acetonitrile/water. The fractions containing the desired product were dried down and prepared as 10 mM DMSO stock solutions. TWZ109. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.25 (s, 1H), 7.74–7.76 (m, 2H), 7.72 (br, 1H), 7.40–7.50 (m, 3H), 3.42–3.50 (m, 4H), 1.55–1.78 (m, 4H), 1.27–1.39 (m, 2H). Observed $[M+H]^+$ 251.13; calcd for 250.13.
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9. Protocols for enzymatic assays using purified HDAC1, HDAC2, HDAC6, and HDAC8 were developed and carried out by Syrrx. Detailed methods will be published elsewhere.
10. For the preparation of partially purified HeLa nuclear extract as the total HDAC enzyme source, refer to: Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. *Nucleic Acids Res.* **1983**, *11*, 1475. In vitro HDAC-inhibition assays were performed as follows: HeLa nuclear extract was incubated with Fluor de Lys acetyllysine substrate (Biomol) for 30 min at room temperature in the presence of test compound or DMSO. The reaction was stopped with excess Trichostatin A and developer solution to generate a fluorescent product. The fluorophore is detected on a fluorometric plate reader (Ex 360 nm, Em 460 nm).
11. Breslow, R.; Belvedere, S.; Gershell, L.; Miller, T. A.; Marks, P. A.; Richon, V. M.; Rifkind, R. A. U.S. Patent, US 6511990 B1, **2003**.
12. For detailed procedures used in the histone hyperacetylation assays in cells, refer to: Stockwell, B. R.; Haggarty, S. J.; Schreiber, S. L. *Chem. Biol.* **1999**, *6*, 71. Adherent cells (approx. 5000/well) in a 384-well Greiner polystyrene assay plate were exposed to compound for 0 to 24 h. Cells are washed once with PBS (80 μL) and then fixed (95% ethanol, 5% acetic acid) for 1 min at rt (40 μL). Cells are blocked with 4% BSA for 1 h and washed and stained with anti-acetylated histone antibody followed by washing and incubation with an appropriate secondary antibody conjugated to HRP or fluorophore.
13. U-937 and Jurkat cell lines were obtained from ATCC. They were cultured with RPMI1640, 1 mM Na Pyruvate, MEM essential vitamins, L-glutamine, 10% Fetal Bovine Serum, and 1% penicillin/streptomycin. Cells were plated into a white, clear bottom 96-well plate at 8×10^4 cell per well. Compounds were added at 100, 33, 11, 3.7, 1.2, 0.41, 0.14, 0.05 μM (final concentration). The final volume is 100 μL per well. After incubating at 37 °C for 24 h, 10 μL of Alamar Blue (Trek) was added. After incubation for an additional 2 h, the fluorescence intensities were read using an Acquest (Molecular Device) plate reader.
14. We thank Dr. Mingli Chen (GNF, San Diego, CA) for performing the toxicity assays on human hepatocytes and bone marrow cells.