A Novel Class of Small Molecule Inhibitors of HDAC6

Elizabeth S. Inks, Benjamin J. Josey, Sean R. Jesinkey and C. James Chou#

From the Department of Pharmaceutical and Biomedical Sciences, Medical University of South Carolina, South Carolina College of Pharmacy, 280 Calhoun St. MSC140 QF307 Charleston, SC 29425

#To whom correspondence may be addressed. E-mail: chouc@musc.edu Tel: 843-792-1289 Fax: 843-792-1617

Supplementary materials includes:
LOPAC HDAC Inhibition titrations, NQN-1 Titration and Timed Western blots, Hsp90 Immuno-precipitation on other HDAC inhibitors, Selective HDAC6 inhibitors' effects on Ac-HH3 and Ac-HH4, Simple naphthoquinones HDAC6 IC_{50}s and MV4-11 toxicities, HDAC6 over-expression and attenuation of NQN-1 toxicity, K_m of HDAC10 and HDAC11, and experimental methods for compound synthesized and characterization data.

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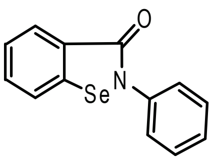
Supplementary Materials
LOPAC HDAC Inhibiton Profiles

Class I and IIb Substrate | Class IIa Substrate



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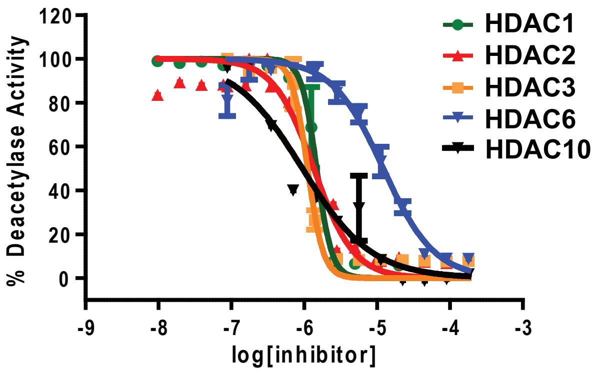

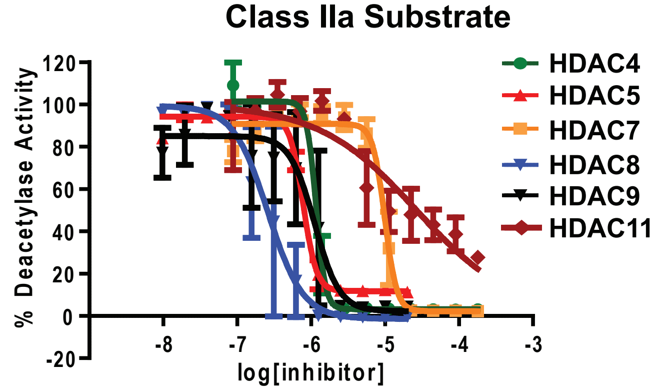

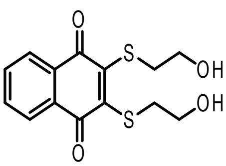

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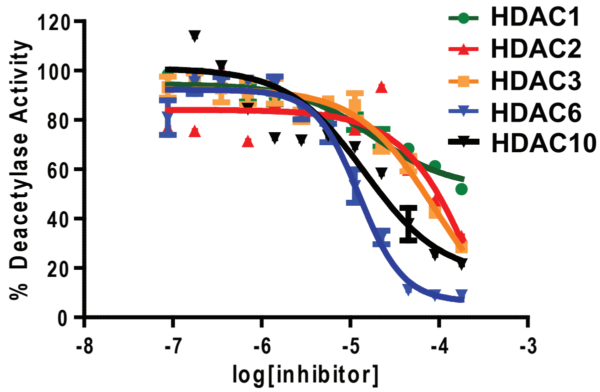

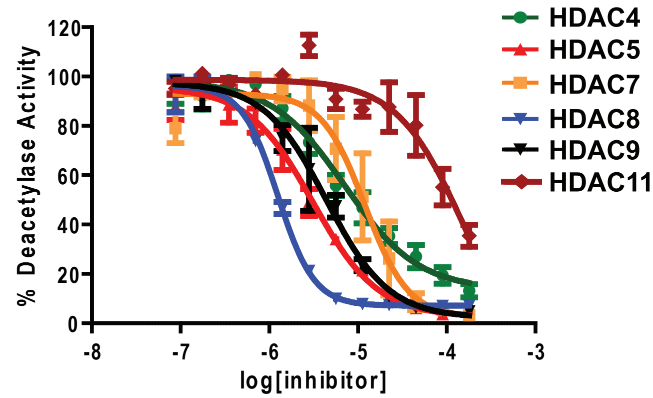

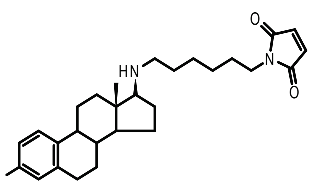

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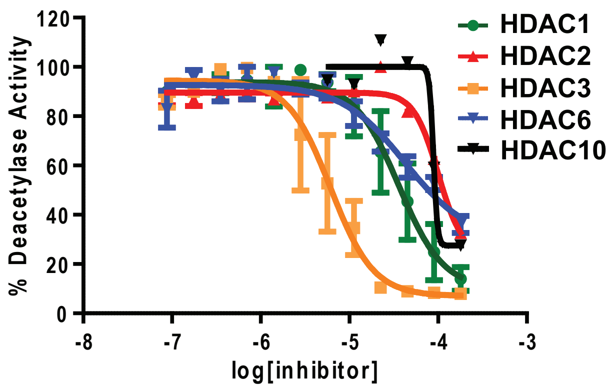

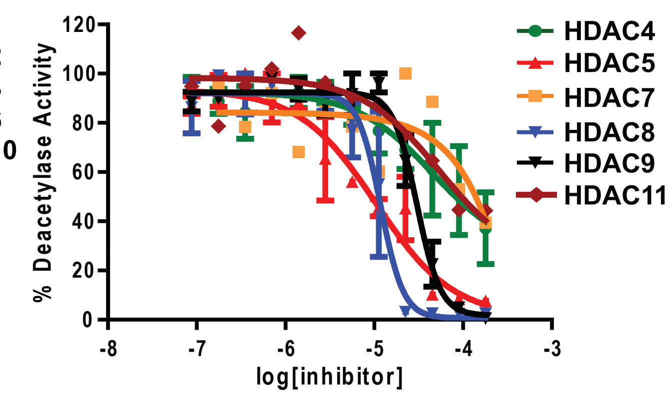

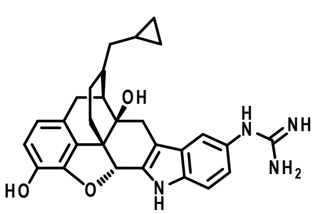

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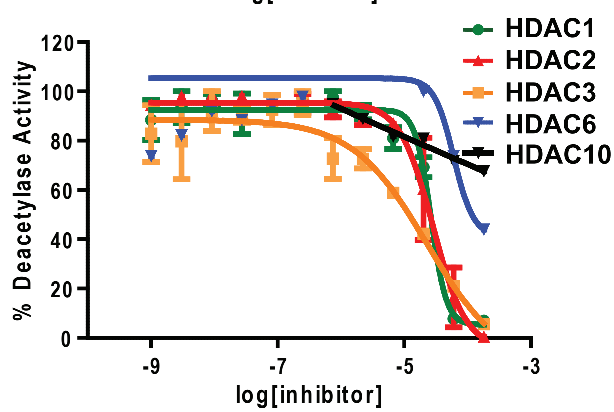
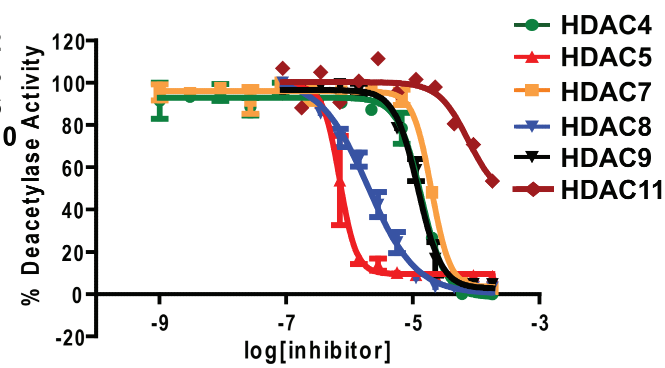
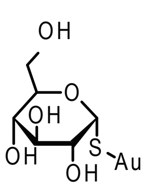
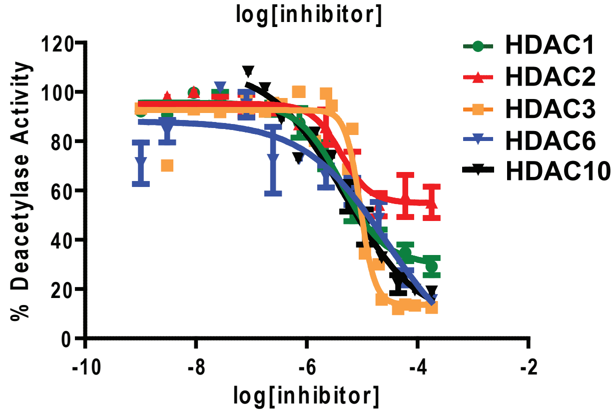
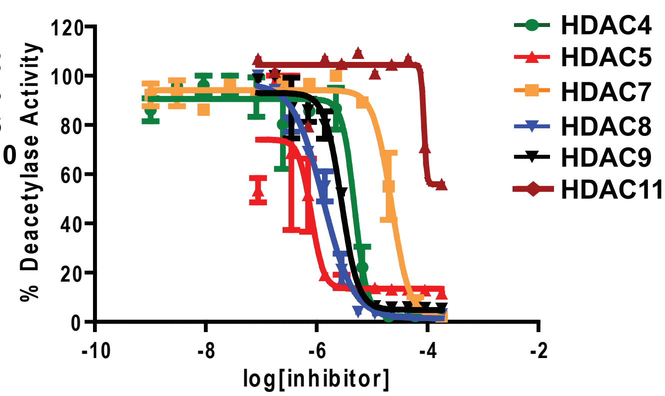




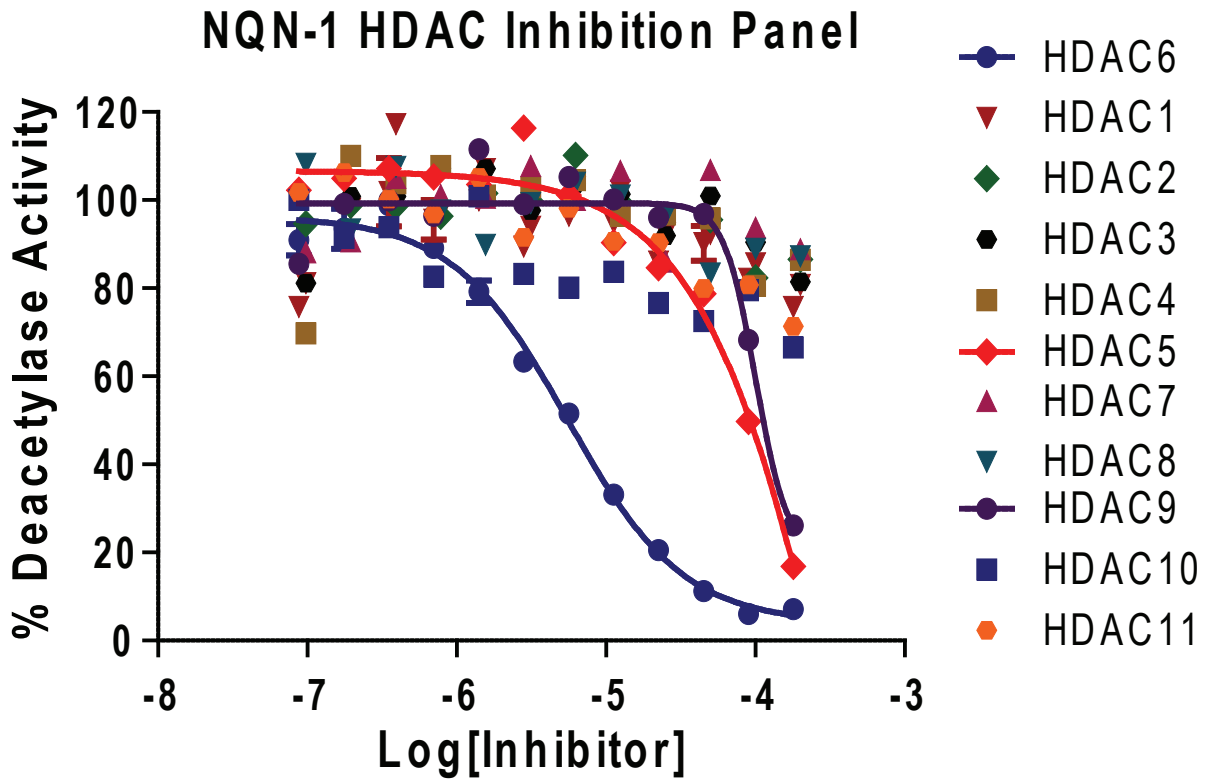


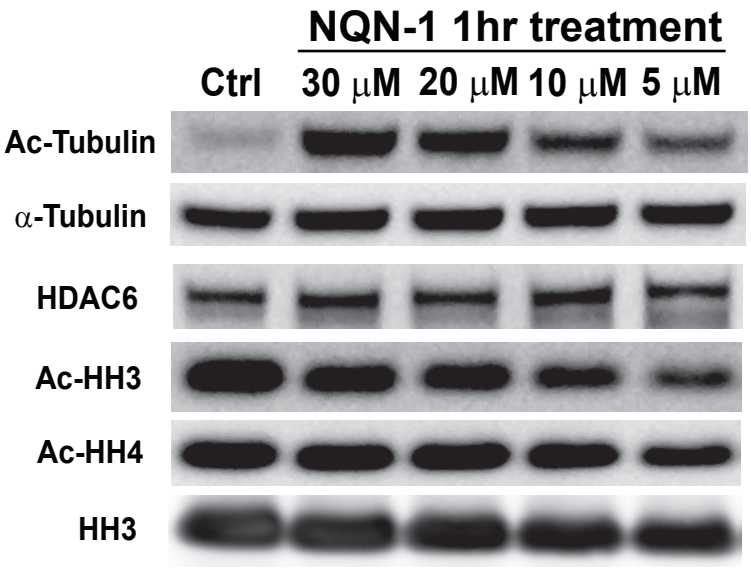
Aurothioglucose

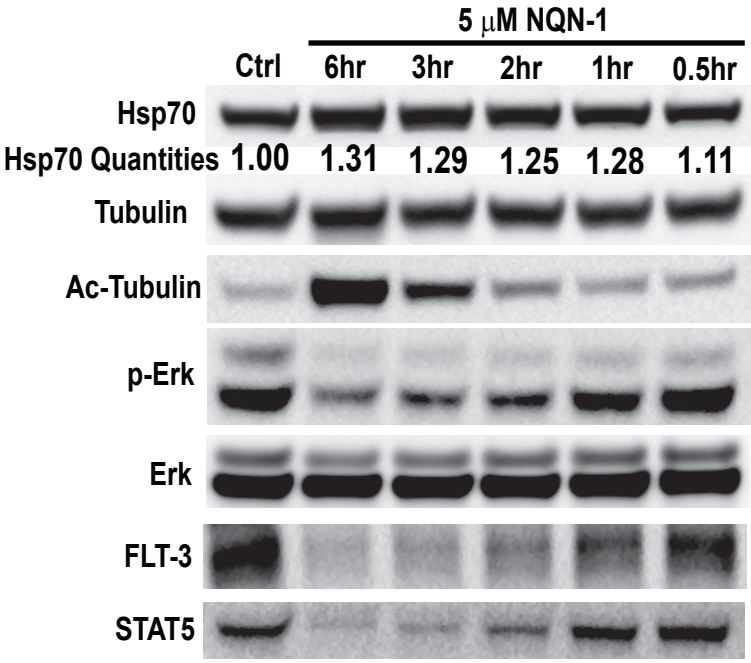




NQN-1 titration and timed treatments

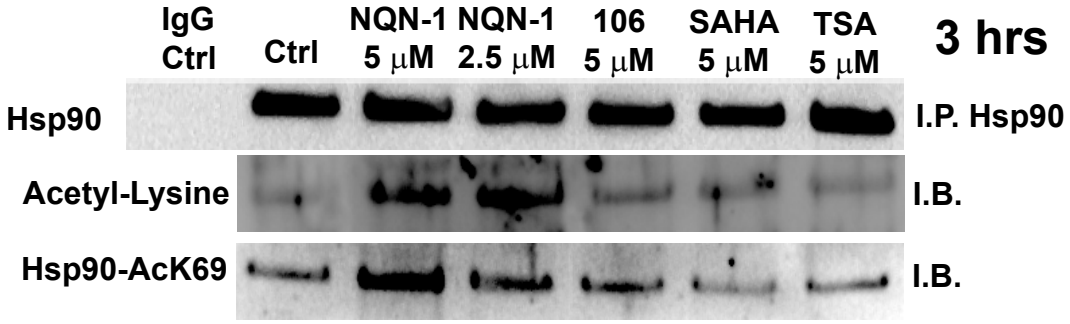




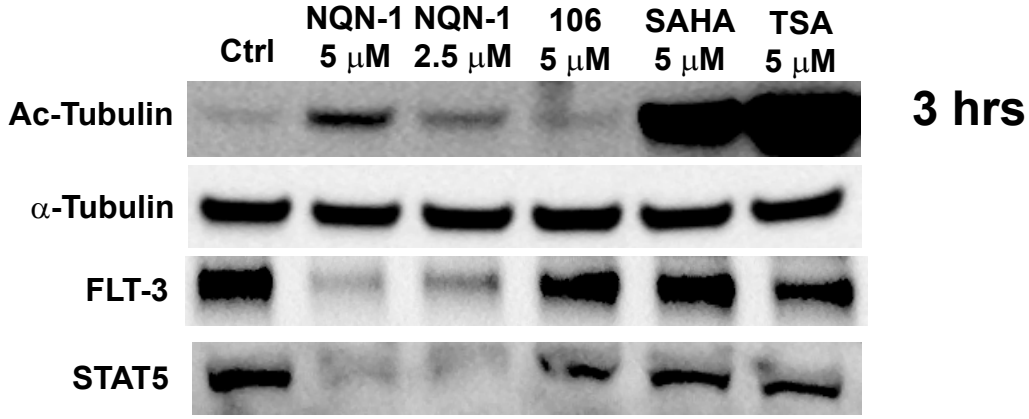


Hsp90 I.P. and Acetyled Hsp90 Level of different HDAC inhibitors

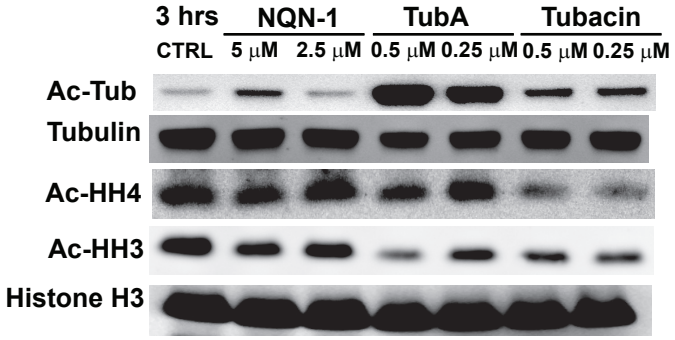
Hsp90 I.P. followed with Ac-Lysine Immunoblot



Western Immunoblot

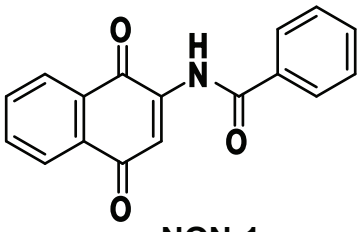


Selective HDAC6 inhibition affects Ac-HH3 and Ac-HH4



Seletive inhibiton of HDAC6 decreases acetylated Histone H4 (Ac-HH4) and acetyalted histone H3 (Ac-HH4). Similar results were observed for HDAC6 knockout and tubacin and tubastatin A treatments in Zhang et al., Mol Cell Biol. 2008 Mar;28(5):1688-701, Namdar et al. Proc Natl Acad Sci U S A. 2010 Nov 16;107 (46):20003-8, Dompierre et al., J Neurosci. 2007 Mar 28;27(13):3571-83, and Butler et al., and J Am Chem Soc. 2010 Aug 11; 132(31):10842-6, although the observations were not explicitly stated

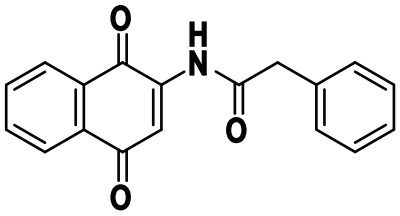
Toxicity of simple naphthoquinones and a non-specific Michael's acceptor in AML MV4-11 cells



NQN-1

HDAC6 K_i = 1.3 μM

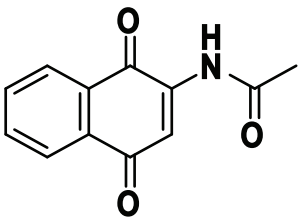
EC_{50} = 0.79 μM



NQN-2

HDAC6 K_i = 3.78 μM

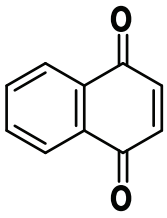
EC_{50} = 1.98 μM



NQN-4

HDAC6 K_i = 4 μM

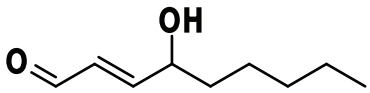
EC_{50} = 4.9 μM



Naphthoquinone (NQ)

HDAC6 K_i > 20 μM IC_{50} > 180 μM

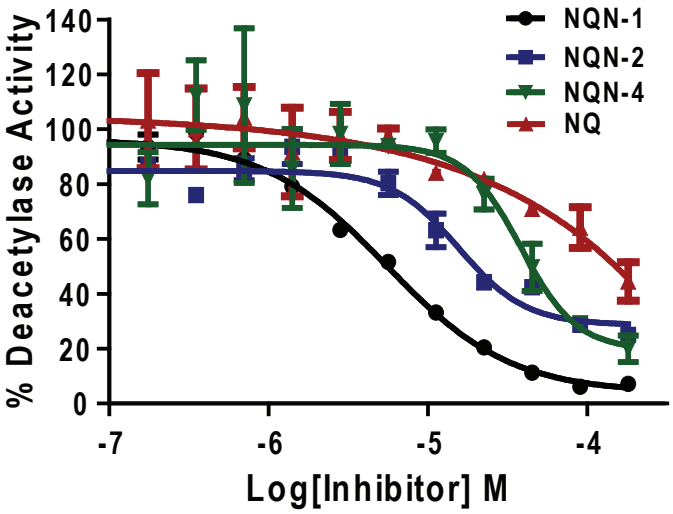
EC_{50} = 15.2 μM

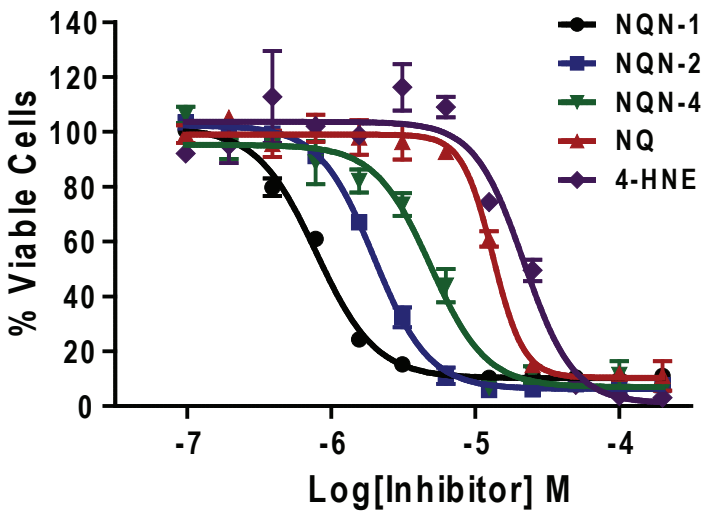


4-Hydroxynonenal (4-HNE)

HDAC6 K_i = Not Determined

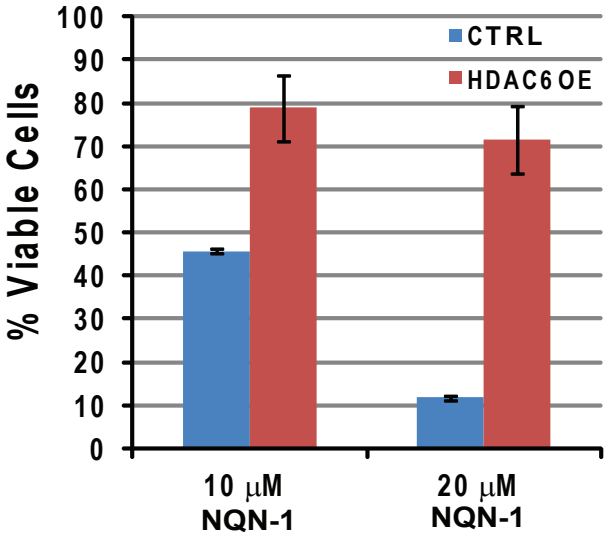
EC50 = 21 μM

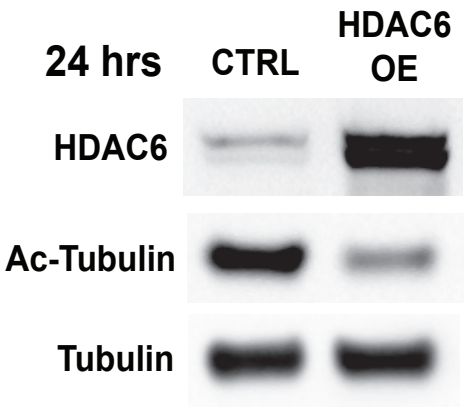


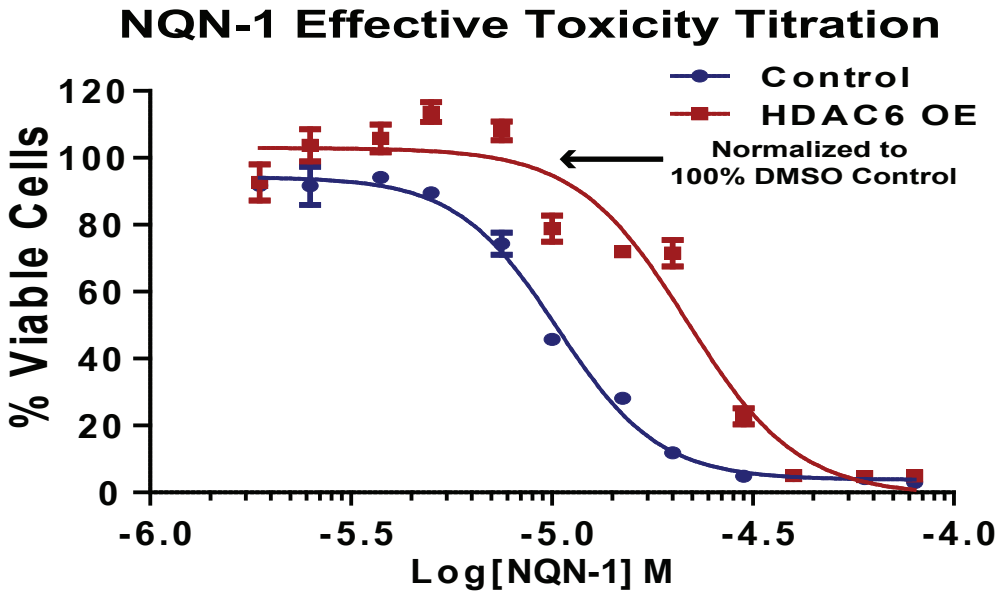


1,4-Naphthoquinone (NQ), 2-Acetamido-1,4-naphthoquinone (NQN-4), and a known Michael's acceptor, 4-hydroxynonenal, was examined for non-specific toxicity against MV4-11 cells. Inhibitors that do not inhibit HDAC6 only affect MV4-11 viability with concentration (EC_{50}) greater than 15 μM (approximately 20 times of NQN-1 EC_{50}). The efficacy against AML MV4-11 cells also correlates with the inhibitor's ability to inhibitor HDAC6.

Attenuation of NQN-1 toxicity with HDAC6 over-expression

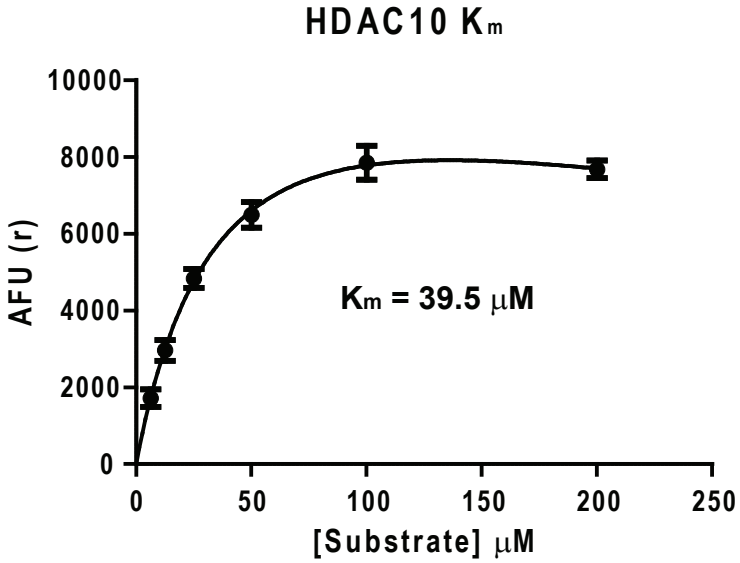


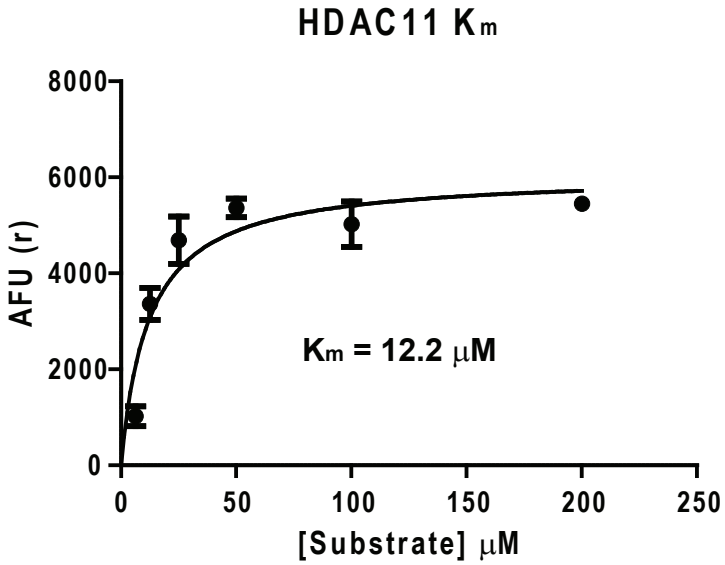




Over-expression (OE) of HDAC6 resulting in attenuation of NQN-1 toxicity in cells. Hek293 cells were transfected with Flag-HDAC6 plasmid and the OE is verified by Western blot. HDAC6 OE did not inhibit cell growth. The cells were then challenged with NQN-1 24 hr after transfection, and significant resistant to the inhibitor was observed at 10 and 20 μM NQN-1 after 24 hrs.

K_m of HDAC10 and HDAC11





***HDAC10 and HDAC11 Kms were not reported previously, Kms determined for HDAC10 and HDAC11 were used for Ki determination**

Experimental Section

Unless otherwise noted, chemicals were commercially available and used as received without further purification. Moisture sensitive reactions were carried out under a dry argon atmosphere. Thin-layer chromatography was performed on precoated silica gel PE SIL G/UV plates (Whatman). Silica gel chromatography was performed using silica gel 60A (Fischer, 230-400 Mesh). High pressure liquid chromatography was performed using a Waters RCM 25 x 10 C-18 column. High resolution ion trap mass spectra were obtained using a Finnigan LCQ Advantage Max mass spectrometer. All the NMR spectra were recorded on a Bruker 400 model spectrometer in either DMSO-d_6 or $CDCL_3$. Chemical shifts (δ) for ^1H NMR spectra are reported in parts per million to residual solvent protons.

2-amino-1,4-naphthoquinone (1). 6.25 g NaN_3 was dissolved in 15 mL H_2O and acidified with 5 mL glacial acetic acid. The NaN_3 solution was added to a solution of 1,4-naphthoquinone (5 g, 29 mmol) dissolved in 100 mL of THF/H_2O (4:1) and stirred at room temperature. After 6 hrs, the reaction was concentrated *in vacuo* and redissolved in ethyl acetate. The resulting solution was washed with 1 M NaOH and saturated NaCl. Multiple extractions were required. The extracts were combined, dried with $MgSO_4$, and concentrated *in vacuo*. The reddish brown residue was purified by column chromatography (silica gel, 50% v/v ethyl acetate/hexane) to yield 4.8 g **1** (96% yield). MS m/z calcd (M+) 173.05, found 173.04. 1H NMR (400 MHz, DMSO-d6) Shift 7.94 (dd, J = 7.15, 19.70 Hz, 2H), 7.82 (dt, J = 1.00, 7.53 Hz, 1H), 7.69 - 7.76 (m, 1H), 5.82 (s, 1H).

N-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)benzamide (NQN-1). 0.3 g (1.73 mmol) **1** and 3 equiv NaH (60% dispersion, 0.2 g) were dissolved in 20 mL dry THF. To this was added 1.5 equiv. benzoyl chloride (301 µL). The reaction was quenched with water and extracted twice with DCM. The organic extracts were combined and washed sequentially with 1 M NaOH, 1 M HCl, and a saturated solution of NaCl. The extract was then dried over Mg_2SO_4 and concentrated *in vacuo*. The resulting powder was further purified by column chromatography (silica gel, 30/70% v/v ethyl acetate/hexane with 1% Et_3N) and crystallized from ethyl acetate/hexane to yield 183 mg small, fine, bright yellow crystals (39% yield). MS m/z calcd (M+) 277.07, found 277.1. 1H NMR (400 MHz, DMSO-d6) Shift 9.74 (s, 1H), 8.10 - 8.15 (m, 1H), 7.87 - 8.07 (m, 5H), 7.79 (s, 1H), 7.67 - 7.74 (m, 1H), 7.57 - 7.65 (m, 2H).). C13-HSQC (400 MHz, DMSO-d6) Shift 126.1, 129.5, 117.0, 128.4, 40.1, 126.9, 126.1, 129.5, 117.1, 128.4, 40.1, 133.3, 129.4, 134.3, 135.3.

N-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)-2-phenylacetamide (NQN-2). 0.5 g (2.89 mmol) **1** and 0.1 g NaH (60% dispersion) were dissolved in 20 mL dry THF and stirred at rt for 30 min. To this was added 1.5 equiv phenylacetyl chloride (573 µL) and the

reaction was stirred at rt for 1 hr. The reaction was quenched with water and extracted twice with DCM. The organic extracts were combined and washed sequentially with 1 M NaOH, 1 M HCl, and a saturated solution of NaCl. The extract was then dried over Mg_2SO_4 and concentrated *in vacuo*. The resulting powder was further purified by column chromatography (silica gel, 30/70% v/v ethyl acetate/hexane with 1% Et_3N) and crystallized from ethyl acetate/hexane to yield 33 mg small, light copper colored crystals (39% yield). MS m/z calcd (M+) 291.09, found 291.1. 1H NMR (400 MHz, CHLOROFORM-d) Shift 8.43 (br. s., 1H), 8.01 - 8.15 (m, 2H), 7.88 (s, 1H), 7.79 (dt, J = 1.25, 7.53 Hz, 1H), 7.67 - 7.74 (m, 1H), 7.34 - 7.52 (m, 5H), 3.85 (s, 2H). C13-HSQC (400 MHz, CHLOROFORM-d) Shift 128.1, 134.8, 133.1, 126.4, 126.6, 129.3, 117.1, 129.5, 77.1, 45.1.

2-(benzylamino)naphthalene-1,4-dione (NQN-3). 0.2 g (1.15 mmol) **1** and 0.1 g NaH (60% dispersion) were dissolved in 10 mL dry THF. To this was added 1.5 equiv benzyl bromide (206 µL) and the reaction was refluxed for 16 hrs . The reaction was quenched with water and extracted twice with DCM. The organic extracts were combined and washed sequentially with 1 M NaOH, 1 M HCl, and a saturated solution of NaCl. The extract was then dried over Mg_2SO_4 and concentrated *in vacuo*. The resulting powder was further purified by column chromatography (silica gel, 50/50% v/v ethyl acetate/hexane with 1% Et_3N) and crystallized from ethyl acetate/hexane to yield 25 mg bright orange, shiny, needle-like crystals (8% yield). MS m/z calcd (M+) 263.09, found 263.1. 1H NMR (400 MHz, CHLOROFORM-d) Shift 8.11 (dd, J = 7.78, 14.05 Hz, 2H), 7.76 (dt, J = 1.00, 7.53 Hz, 1H), 7.61 - 7.70 (m, 1H), 7.32 - 7.47 (m, 5H), 6.22 (br. s., 1H), 5.82 (s, 1H), 4.41 (d, J = 5.77 Hz, 2H). C13-HSQC (400 MHz, CHLOROFORM-d) Shift 126.3, 134.8, 131.8, 129.0, 128.1, 77.1, 101.8, 46.7.

N-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)acetamide (NQN-4). 0.15 g (0.88 mmol) was dissolved in 2 mL acetic anhydride along with 0.2 mL glacial acetic acid, and the reaction was refluxed overnight. The reaction was allowed to cool to room temperature and the precipitated product was filtered and crystallized from ethyl acetate and hexane to yield 156 mg of fine yellow crystals (82% yield). MS m/z calcd (M+) 216.06, found 216.1. 1H NMR (400 MHz, DMSO-d6) Shift 9.95 (s, 1H), 8.02 - 8.11 (m, 1H), 7.94 - 8.01 (m, 1H), 7.80 - 7.93 (m, 2H), 7.70 (s, 1H), 2.25 (s, 3H). C13-HSQC (400 MHz, DMSO-d6) Shift 25.27, 39.91,115.96, 134.11, 125.83, 134.10, 125.82.

Modified Tripeptide Substrates. Both substrates were synthesized from Fmoc-Lys(carbamate wang resin)-AMC (Novabiochem San Digo, CA) using standard Fmoc solid phase peptide synthesis protocol. Prior to cleavage from the resin, the free terminal amine was acetylated using 10% acetic anhydride, 10% pyridine in DMF. Peptides were cleaved from the resin using 95% trifluoroacetic acid, 2.5% DCM, and 2.5% DI H_2O. To generate the **acetylated substrate, Ac-Leu-Gly-(AMC)Lys-Ac,** the resulting peptide was stirred for 20 min in 10% acetic anhydride, 10% pyridine in NMP.

To generate the **trifluoroacetylated substrate, Ac-Leu-Gly-(AMC)Lys-TFA,** the resulting peptide was stirred for 20 min in 10% trifluoracetic anhydride, 10% pyridine in NMP. Peptides were purified using high-pressure liquid chromatography and characterized using mass spectrometry. **Ac-Leu-Gly-(AMC)Lys-Ac** MS m/z calcd (M+) 558.29, found 558.1. **Ac-Leu-Gly-(AMC)Lys-TFA** MS m/z calcd (M+) 612.26, found 612.1.