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Studies of Benzamide- and Thiol-Based Histone Deacetylase Inhibitors in Models of Oxidative-Stress-Induced Neuronal Death: Identification of Some HDAC3-Selective Inhibitors

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We compare three structurally different classes of histone deacetylase (HDAC) inhibitors that contain benzamide, hydroxamate, or thiol groups as the zinc binding group (ZBG) for their ability to protect cortical neurons in culture from cell death induced by oxidative stress. This study reveals that none of the benzamide-based HDAC inhibitors (HDACIs) provides any neuroprotection whatsoever, in distinct contrast to HDACIs that contain other ZBGs. Some of the sulfur-containing HDACIs, namely the thiols, thioesters, and disulfides present modest neuroprotective activity but show toxicity at higher concentrations. Taken together, these data demonstrate that the HDAC6-selective mercaptoacetamides that were reported previously provide the best protection in the homocysteic acid model of oxidative stress, thus further supporting their study in animal models of neurodegenerative diseases.

Introduction

The amino-terminal tails of histones are subject to a variety of post-translational modifications, and in particular through the acetylation of lysine residues. Histone deacetylases (HDACs) and histone acetyl transferases (HATs) determine the pattern of histone acetylation, which together with other dynamic, sequential post-translational modifications such as methylation and phosphorylation represent a "code" that can be recognized by transcriptional factors that are involved in the regulation of gene expression.[1] The ability of HATs and HDACs to modify non-histone substrates and participate in multi-protein complexes also contributes to the regulation of gene transcription, cell cycle progression, differentiation, and stress responses.[2]

Perturbation of acetylation homeostasis is emerging as a central event in the pathogenesis of a host of common diseases. HDAC inhibitors represent an exciting new class of chemotherapeutics that regulate gene expression by enhancing the acetylation status of histones or other non-histone substrates. Although initially derived from the study of differentiation agents, HDAC inhibitors have now been shown to be potent inducers of growth arrest and apoptotic cell death,[3] and thus the application of these agents in cancer therapy has been explored. Several such inhibitory agents, including suberoylanilide hydroxamic acid (SAHA) and depsipeptide (FR901228)[4] have reached clinical trials, and SAHA has been approved by the FDA for use in cutaneous T-cell lymphoma (CTCL).[5]

In contrast to their anticancer effects, HDAC inhibitors have also been shown to exhibit valuable neuroprotective properties in both cellular and animal models of brain injury such as stroke[6,7] and ischemia.[8–10] Mechanisms leading to cell death during cerebral ischemic injury are complex and include excitotoxicity, ionic imbalances, oxidative/nitrosative stress, and inflammation. HDAC inhibitors have been shown to attenuate these disease processes through multiple pathways. Moreover, an increasing number of reports suggest the potential for using HDAC inhibitors to treat chronic neurological disorders such as the polyglutamine-expansion diseases,[4] amyotrophic lateral sclerosis,[11] spinal muscular atrophy,[12] and Alzheimer's disease.[13] The wide range of protective properties of certain HDAC inhibitors in a host of neurological disease models suggests that the loss of HAT activity (or gain of HDAC activity), decreased histone (or other protein) acetylation, and transcriptional dysregulation might be common underlying mechanisms in neurodegeneration.

The utility of HDAC inhibitors in medicine appears to be tremendous, but further translation of these ideas to the clinic will ultimately require the design of potent, isozyme-selective, and drugable molecules that have a minimum of side effects. In mammalian cells, there are now 11 HDACs that operate by zinc-dependent mechanisms (class I includes HDAC1, -2, -3, and -8, class II includes HDAC4, -5, -6, -7, -9, and -10, and clas-

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s IV includes HDAC11).[1,14,15] Class I HDACs are ubiquitously expressed, are predominantly in nucleus, and mainly function as transcriptional co-repressors that are linked to cell proliferation and survival.[16] The distribution of the class II enzymes are more tissue specific; this suggests distinct functions in cellular differentiation and developmental processes.[17] Although the connections between certain HDAC isoforms and pathophysiology are still evolving, accumulating data suggest that targeting specific HDACs might ameliorate certain disease conditions while limiting side effects.[18,19]

In contrast to the now fairly extensive study of structural diverse HDAC inhibitors as cancer therapeutics, only a few prototypical HDAC inhibitors, including the hydroxamates such as trichostatin A (TSA) and SAHA, together with short-chain fatty acids such as phenylbutyrate and valproic acid have been studied in neurological disease models. One major concern in testing relatively nonselective HDAC inhibitors in such disease models relates to the ability to disassociate the neuroprotective actions of HDAC inhibition from any toxicity that might be caused by the inhibition of inappropriate isoforms, or from off-target effects. To pursue our aims to create unique HDAC inhibitors as potential candidates for use in CNS disorders, we have compared the protective effect of some mercaptoacetamide-based inhibitors with hydroxamate-based inhibitors in the HCA-induced model of oxidative stress.[20,21] Two mercaptoacetamides, **1** and **2** (Figure 1) were identified that showed complete neuroprotection in a range from 10 to 50 μM, whereas all the hydroxamates showed bell-shaped dose–response curves and a narrow safety threshold with the highest protection being achieved around 1 μM. Isoform inhibition studies revealed that some of the mercaptoacetamides are relatively selective for HDAC6 over the class I HDACs, which might correlate with their lower toxicity. Also, previously the toxic effect of TSA was found to be linked to its exposure time; a pulsed exposure mode resulted in durable neuroprotection without toxicity in a cortical neuron model (2 h).[6] The evaluation of the druglike properties of one of these mercaptoacetamide-based HDAC inhibitor has shown that it possess favorable solubility, lipophilicity, permeability and plasma-stability features.[22] Moreover, we have tested one of the mercaptoacetamide-based HDAC inhibitors, 4-dimethylamino-*N*-[5-(2-mercaptoacetylamino)pentyl]benzamide (DMA-PB), in a rat model of traumatic brain injury, and found that DMA-PB is able to increase histone H3 acetylation, decrease the microglia inflammatory response, and preserve neurons when administered immediately following TBI.[23]

Herein we compare the neuroprotection profiles of newly synthesized benzamide-based inhibitors with structurally related hydroxamate and sulfur-based inhibitors, with the aim to further elucidate the protective effects of HDAC inhibitors that contain different ZBGs in cultured neurons. The prototypical benzamide-based HDAC inhibitor, MS-275, selectively inhibits class I HDACs and is in phase I/II clinical studies for cancer chemotherapy (Figure 2).[24,25] MS-275 has also been shown to increase the Ac-H3–*RELN* and Ac-H3–GAD_{67} promoter interaction in the frontal cortex, which might be relevant to both schizophrenia and bipolar disorder.[26] Another benzamide, BML-210, is able to induce the accumulation of acetylated histone, but it has no effect on the acetylation status of α-tubulin.[27] BML-210 and its analogues were reported to reverse gene silencing in Friedreich's ataxia.[28] Furthermore, the benzamide K-183 attenuated the decrease of cardiac output and the increase of effective arterial elastance in the hypertrophied heart, although it failed to prevent the formation of isoproterenol-induced cardiac hypertrophy.[29]

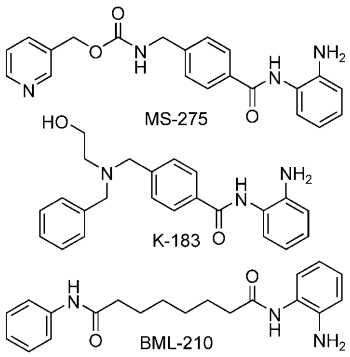


Figure 2. Known benzamide-based HDAC inhibitors.

The synthesis of new benzamides that bear either phenylthiazolyl or triazolylphenyl CAPs (end-group for surface recognition)[21,30,31] are reported herein, and some of these are shown to be HDAC3-selective inhibitors. The ability of these compounds to protect cortical neurons from HCA-induced oxidative stress is examined in turn. As will be apparent, the present results demonstrate that these benzamides, as well as the hydroxamates, display significant toxicity to primary cortical neurons in culture and thus provide little or no protection against oxidative stress-induced neuronal death. These results are contrasted with the neuroprotective activity that has been shown by HDACIs that contain a thiol group as the ZBG.

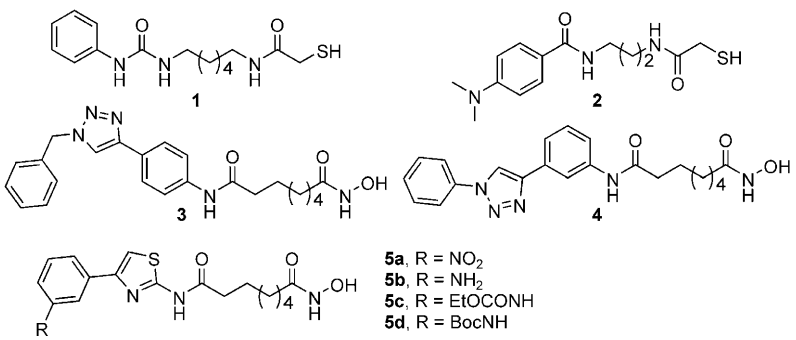


Figure 1. Mercaptoacetamide-based and parent hydroxamate-based HDAC inhibitors.

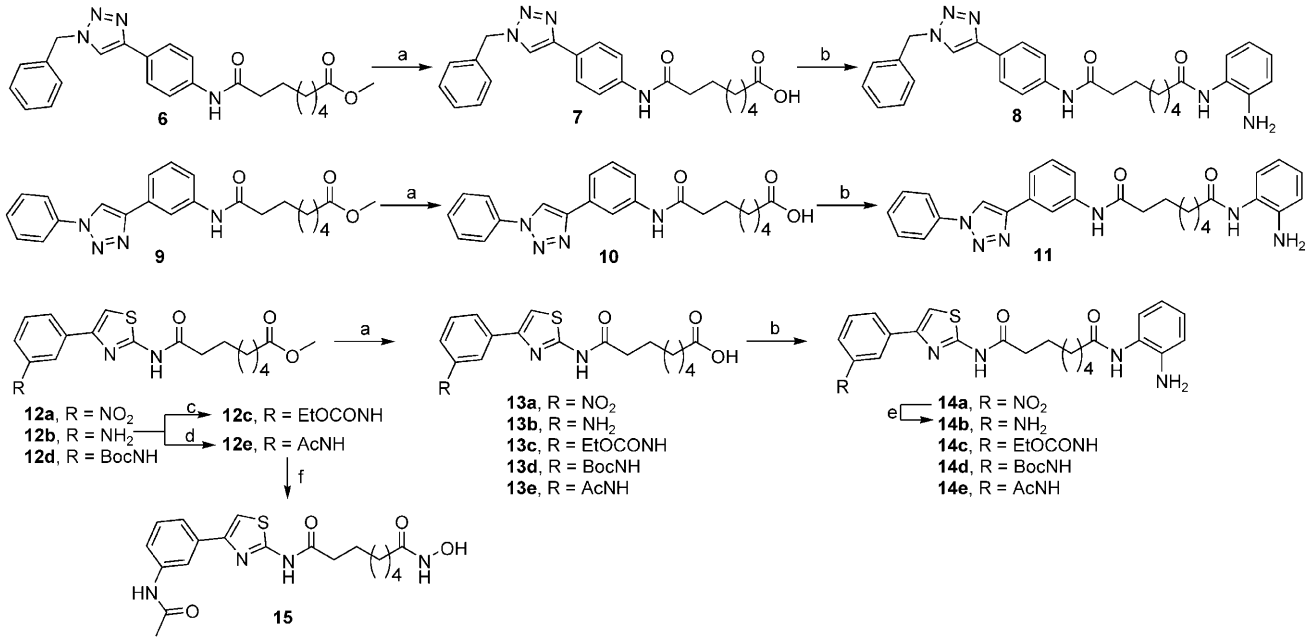
Results and Discussion

Chemical synthesis

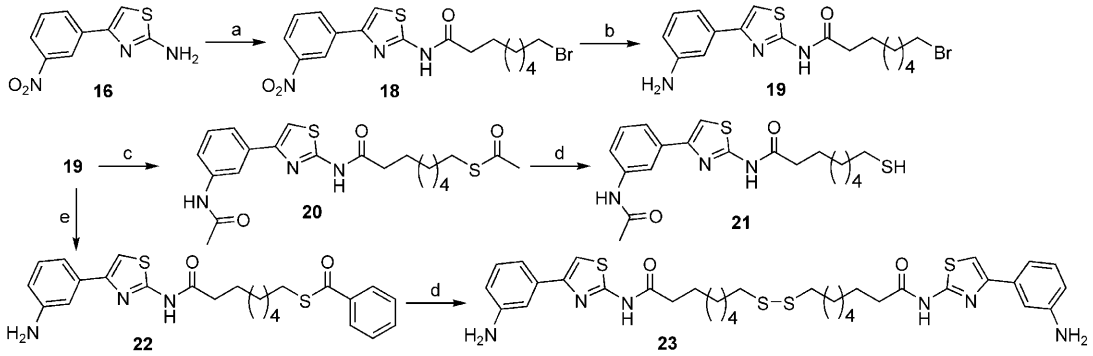
Syntheses of the phenylthiazolyl and triazolylphenyl-based benzamide ligands are outlined in Scheme 1 and require straightforward coupling of the corresponding acids with *o*-phenylenediamine. Thus ester **6**[30] was hydrolyzed by LiOH to acid **7**,[30] which was coupled with *o*-phenylenediamine by 1-hydroxy-7-azabenzotriazole (HOAt) and 4-dimethylaminopyridine (DMAP) to afford benzamide **8**. Other benzamide ligands **11**, **14a**, **14c**, **14d**, and **14e** were obtained by similar procedures. The amine **14b** was obtained from the nitro compound **14a** by catalytic hydrogenation. Treatment of the ester **12e**[21] with NH_2OH and KOH in methanol afforded the hydroxamate **15**, which was used as one of the control ligands in our study.

Scheme 2 outlines the synthesis of the prodrug-based thioester and disulfide ligands by starting from 4-(3-nitrophenyl)thiazol-2-ylamine (**16**) and 8-bromooctanoic acid (**17**) by using $POCl_3$ in pyridine to give compound **19**. Treatment of bromide **19** with excess potassium thioacetate for two days gave the doubly acetylated product **20**. Subsequent hydrolysis of **20** by 10% NaOH gave free thiol **21**, which was characterized by ESIMS and HRMS. Similarly, the thioester **22** was obtained by treating the bromide **19** with thiol benzoic acid and potassium carbonate in DMF. Compound **22** was hydrolyzed and oxidized to the disulfide **23**, which was characterized as the dimer by ESIMS and HRMS.

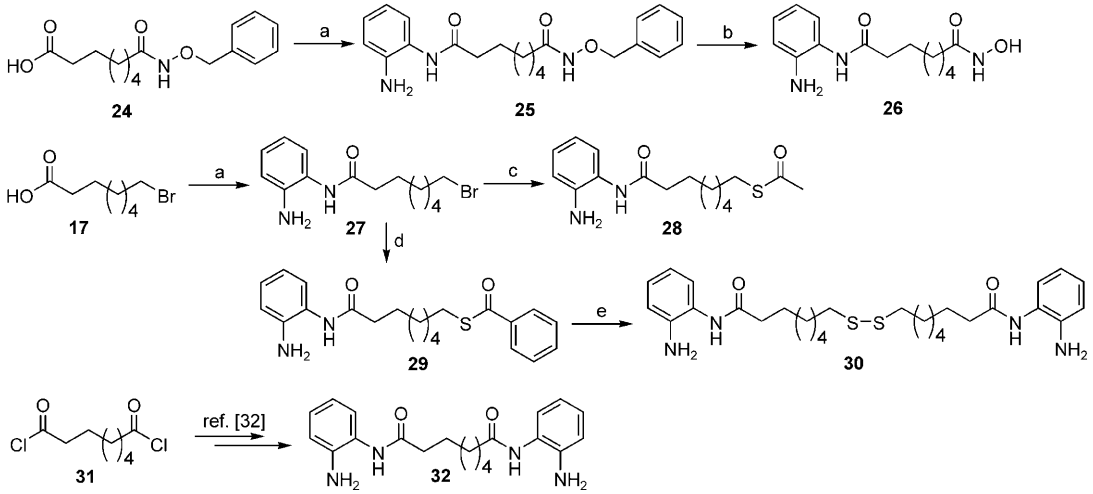
Scheme 3 outlines the synthesis of some ligands that contain two ZBGs. Ligand **26** can be viewed as a SAHA analogue that contains an aminobenzamide CAP and a hydroxamate ZBG. Ligand **30** contains a 2-aminobenzamide as both the CAP group and the ZBG. To synthesize these compounds, 7-benzyloxycarbamoylheptanoic acid (**24**) was coupled with *o*-phenylenediamine by using HOAt and DMAP to give **25**, which was converted into the hydroxamate **26** by catalytic hydrogena-



Scheme 1. Synthesis of ligands **8**, **11**, **14b**–**e**, and **15**: a) LiOH, RT; b) *o*-phenylenediamine, HOAt, Et_3N, DMAP, RT; c) AcCl, Et_3N, 0 °C; d) ethyl chloroformate, THF, reflux, 10 h; e) H_2, 10% Pd/C, RT; f) NH_2OH, KOH, RT.



Scheme 2. Synthesis of ligands **20**, **21**, **22**, and **23**: a) **17**, $POCl_3$, pyridine, 0 °C; b) H_2, 5% Pd/C, RT; c) KSAc (20 equiv), 48 h, RT; d) 10% NaOH, air, 12 h; e) PhCOSH, K_2CO_3, 12 h, RT.



Scheme 3. Synthesis of ligands **25**, **26**, **28**, **29**, **30**, and **32**: a) *o*-phenylenediamine, HOAt, Et_3N, DMAP, RT; b) H_2, 10% Pd/C, RT; c) KSAc, 12 h, RT; d) PhCOSH, K_2CO_3, 12 h, RT; e) 10% NaOH, air, 12 h, RT.

tion. The thioesters **28** and **29** and disulfide **30** were prepared from *o*-phenylenediamine and 8-bromooctanoic acid (**17**) according to the methods that are provided in Scheme 2. The symmetrical octanedioic acid bis-[(2-aminophenyl)amide] (**32**) was prepared according to a published procedure[32] and used as a control in the neuroprotection assays.

We further note here that some structurally related free thiols (much like the mercaptoacetamides that were first reported by us) and thioester-based prodrugs have been reported previously by Suzuki and Miyata. Their studies show that these inhibitors can induce the accumulation of both acetylated histone H4 and acetylated α-tubulin, modulate the generation of reactive oxygen species (ROS),[33] and show low-micromolar inhibitory activity against several cancer cell lines.[34–36] For comparison purposes, we have also examined the utility of this class of HDACIs in the neuroprotection models.

HDAC isoform inhibition assay

The inhibitory effects of new benzamides against the various HDAC isoforms were determined by using a fluorescence-based assay in which electrophoretic separation of substrate and product was carried out by using a microfluidic system followed by quantitation of fluorescence intensity in the substrate and product peaks. The assays were performed by using isolated HDAC isoforms that had been expressed as 6×His-tagged fusion proteins in a baculovirus expression system in Sf9 cells. HDAC1, -2, -3, -6, and -8 were expressed as full-length fusion proteins. The HDAC10 fusion protein was expressed as a carboxy-terminal deletion of 38 amino acids (residues 632–669). HDAC3 was co-expressed with a fragment of the SMRT gene (residues 395–489) to generate enzymatically active protein. The data are presented as IC_{50} values. TSA was used as a positive control. The recently published inhibitory data for MS-275 against a panel of recombinant HDACs are also presented for comparison (Table 1).[37]

As apparent from Table 1, the newly synthesized benzamide-based ligands **8**, **11**, **14b**, **14c**, **14d**, **14e**, **25**, and **32** selectively inhibit HDAC3 over the other tested HDAC isoforms. Consistent with the previously reported data for MS-275, the newly synthesized benzamides selectively inhibit the class I HDACs.

Table 1. HDAC inhibitory activity of the new benzamide-bearing ligands, TSA, and MS-275.

	IC_{50} [μM][a]					
Compd	HDAC1	HDAC2	HDAC3	HDAC8	HDAC10	HDAC6
3	0.113	0.678	0.287	3.97	0.188	0.0048
4	0.018	0.064	0.0072	2.78	0.0277	0.0096
5b	0.0038	0.0265	0.0021	1.95	0.0037	0.0033
5c	0.0025	0.0245	0.0013	0.787	0.0021	0.00079
5d	0.0042	0.021	0.0017	2.58	0.0057	<0.00017
15	0.00189	0.0061	0.0021	1.17	0.00161	0.00307
MS-275[b]	0.181	1.155	2.311	>10	NA	>10
TSA	0.004	0.014	0.002	1.380	0.005	0.001
8	>30	>30	1.50	>30	>30	>30
11	>30	>30	0.12	>30	>30	>30
14b	2.49	0.147	0.0603	>30	2.91	>30
14c	24.8	0.88	0.060	>30	>30	>30
14d	4.2	1.1	0.16	>30	5.30	1.50
14e	3.20	0.20	0.030	>30	3.0	11.2
25	13.8	4.49	0.552	>30	13.73	>30
26	0.0672	0.101	0.0211	2.36	0.0493	0.00993
32	14.64	4.8	0.988	>30	16.02	>30
SAHA	0.0874	0.213	0.0295	2.29	0.0918	0.0174

[a] The isoform inhibition was tested at Nanosyn [http://www.nanosyn.com/ (accessed March 10, 2009)]; NA = not assayed. [b] Data from reference [37].

The structural difference between our new benzamides and MS-275, especially as it relates to the difference in the linker region (alkyl versus aromatic linker), might explain the observed HDAC3 selectivity, although more extensive structure–activity relationship (SAR) comparisons will be needed. Because of the instability of the free thiol form of compounds **21**, **23**, and **30**, it is difficult to accurately assay their inhibitory action by using the purified enzymes. Thus, they were synthesized as their prodrug ester forms (compounds **20**, **23**, **28**, and **29**), and the HDAC inhibitory activity of these prodrugs was then assessed by measuring their ability to induce histone H4 acetylation together with their ability to protect immature cortical neurons against oxidative-stress-induced death.

Hyperacetylation of histone protein

To investigate whether the neuroprotective concentrations of the HDAC inhibitors that were used result in histone acetylation in neurons and thus, potentially, changes in gene transcription, the relative acetylation levels of histone H4 were evaluated after treatment with the HDAC inhibitors (Figure 3).

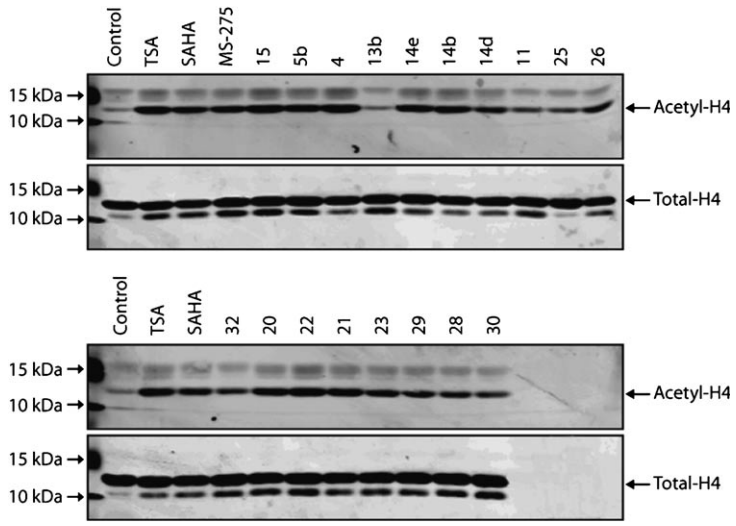


Figure 3. Acetylation levels of histone H4 after treatment with some of the HDAC inhibitors.

Histone proteins were acid-precipitated from cell nuclear extracts that were obtained from rat primary cortical neurons that were treated with controls and the new ligands at 10 μM for 8 h. Western blot analysis by using acetyl-histone H4-specific antibodies (Upstate Cell Signaling Solutions, Charlottesville, VA) demonstrates that acetylation of H4 histone proteins is promoted by some but not all of the HDAC inhibitors. The hydroxamates **4**, **5b**, and **15** induce significant increases in histone acetylation, as do the thiol-based ligands **20**–**23**. Compounds **11** and **25** were found to induce only a marginal increase in H4 acetylation, which suggests that the inhibition of HDAC3 might not play an important role in H4 acetylation, at least in the cases that were studied here. The acid **13b** is an intermediate in the synthesis of compound **14b**. Because it lacks an effective ZBG, it fails to induce an increase in H4 acetylation, and thus serves as a negative control in this study.

Neuroprotective activity

A comparison of the neuroprotective ability of the benzamide-based ligands to the corresponding hydroxamate and sulfur-based ligands was assessed by using an in vitro model of oxidative-stress-induced neurodegeneration in primary cortical neurons.[38] In this model, neurodegeneration is induced by the presence of a 5 mM concentration of the glutamate analogue, homocysteate (HCA), which depletes the cellular antioxidant glutathione by the competitive inhibition of cyst(e)ine uptake at the level of the plasma membrane cystine–glutamate antiporter system xc−. Because cysteine is required for the synthesis of glutathione, the inhibition of its uptake results in glutathione depletion. Cellular redox homeostasis therefore becomes disrupted, and the accumulation of endogenously produced and unopposed oxidants results in neuronal degeneration between 12 and 24 h. Importantly, primary neurons at this early developmental stage lack ionotropic and metabotropic receptors and are not susceptible to excitotoxicity, rather death is induced by accumulation of unopposed free radicals and the neurons exhibit a number of apoptotic features.

The dose-dependent neuroprotection graphs for the new HDACIs reported herein by using the HCA–cortical neuron model of oxidative stress over 48 h are summarized in Figure 4. These graphs are divided into five groups according to the nature of their ZBGs. For comparison purposes, data for two mercaptoacetamide-based HDACIs **1** and **2** are provided. As is apparent, these mercaptoacetamides show a gradated increase in neuroprotection from HCA toxicity as the dosage of the HDACI is increased, with no apparent toxicity caused by the HDACIs when tested in the absence of HCA. The majority of the hydroxamates (compounds **3**, **4**, **5b**, **5c**, and **5d**) show significant toxicity resulting in approximately bell-shaped curves with the greatest protection being reached at approximately 1 μM, which is consistent with the data that we reported previously for other hydroxamates. In contrast, the benzamide-based HDACIs, including MS-275, display considerable toxicity on their own and show no ability to block the toxicity of HCA. The differential protective effects of these diverse HDACIs appear to be related to the chemical nature of their ZBG as well as to their HDAC isozyme selectivity. Because the benzamides **8** and **11** show HDAC3-selectivity, we tentatively conclude that the inhibition of HDAC3 has little effect against oxidative stress in this model, however, we cannot rule out the possibility that our results stem from off-target effects or even from cellular metabolism of the benzamides to toxic (semi)quinone imines.[39] Compound **14c**, like MS-275, is a class-I-selective inhibitor, and it also fails to provide neuroprotection. These data further support our previous contention that neuroprotection in the cortical neuron model can best be achieved by certain class-II-se-

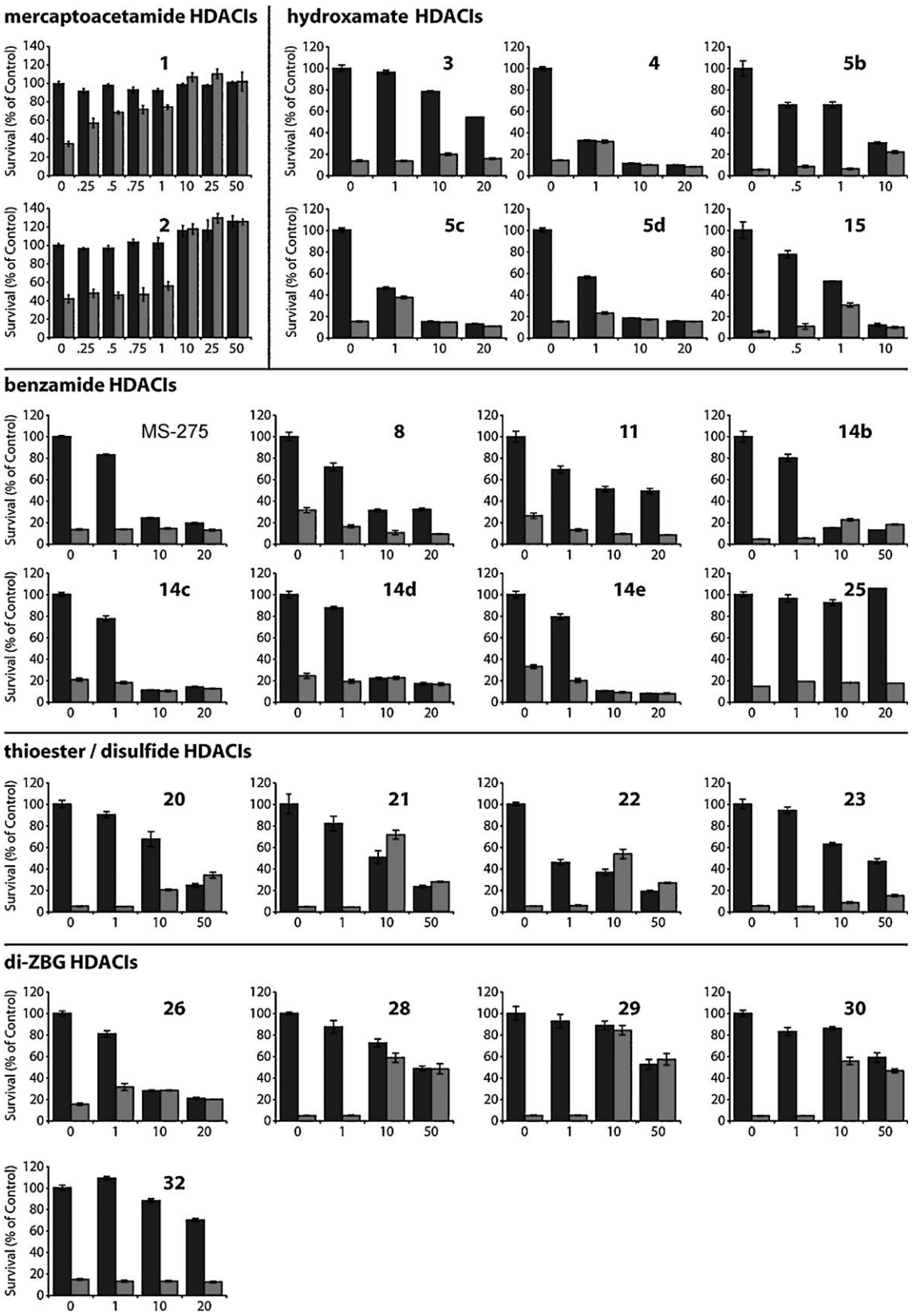


Figure 4. Dose-dependent neuroprotection for structurally diverse HDAC inhibitors and controls in the HCA–cortical neuron model in the absence (blue bars) or presence (red bars) of HCA.

lective mercaptoacetamides, and in particular, those acting through the inhibition of HDAC6, which may, in turn, be coupled to the hyperacetylation of HSP90. The thioesters **20** and **22**, free thiol **21**, and disulfide-based (**23**) HDACIs show toxicity

at the higher concentrations, but **20**–**22** show modest neuroprotective activity at concentrations of about 10 μM. Clearly, however, these particular sulfur-based compounds do not appear to be very useful as neuroprotective agents. The neuroprotective activity that was found for compounds **26**, **28**, **29**, **30**, and **32**, which contain two ZBG groups depends on the nature of the ZBG. Compound **26**, which contains both a hydroxamate and a benzamide group as well as **32**, which contains two benzamide groups are unable to protect neurons from HCA toxicity. Compounds **28**, **29**, and **30**, which contain both benzamide and thioester or thiol ZBGs (assuming the disulfide **30** is reductively cleaved to thiol, as is known to be the case for the drug FK-228)[40] show modest neuroprotection that is similar to that shown by the thioester-based HDACIs. The present results thus complement other published work in the HDAC field, and thus add a cautionary note to the selection of HDACIs that are used in studies in animal models of neurodegenerative diseases. Of all the compounds that we have tested, the mercaptoacetamides like **1** and **2** offer the best levels of neuroprotection. Whereas HDAC3-selective compounds do not protect neurons in culture against oxidative stress, they might be efficacious against other types of stress or underlying factors of neurodegeneration in vivo. For example, targeting HDAC3 in Friedreich's ataxia can correct the frataxin gene expression deficiency.[41]

Conclusions

In summary, we have synthesized a series of structurally unique benzamide-containing HDAC inhibitors that represent analogues of our previously reported highly potent phenylthiazolyl- and triazolylphenyl-bearing HDAC inhibitors. Some members of this class of benzamides were found to be reasonably selective for HDAC3 in isolated enzyme inhibition assays. We have compared the neuroprotection profiles of these HDAC3-selective benzamides as well as other less-selective benzamides with structurally related HDAC inhibitors that bear hydroxamates or thiol groups as the ZBGs. We found that none of these benzamide-based HDACIs were able to provide any degree of neuroprotection, whereas the thioester-containing ligands were modestly neuroprotective. The benzamides thus behave like the hydroxamate-containing HDACIs in their toxicity to cortical neurons making it difficult to assess their ability to protect from HCA-induced oxidative stress. These data strongly support the contention that neuroprotection in the cortical neuron model of oxidative stress can best be achieved by class-II-selective agents, such as observed with certain mercaptoacetamides, through the inhibition of HDAC6.

Experimental Section

Chemistry

General. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker spectrometer at 300/400 and 75/100 MHz, respectively, by using TMS as an internal standard. Standard abbreviation indicating multiplicity was used as follows: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintuplet, m = multiplet and br = broad. HRMS data were obtained with a Q-TOF-2TM instrument (Micromass). TLC was performed with Merck 250 mm 60 F_{254} silica gel plates. Preparative TLC was performed with Analtech 1000 mm silica gel GF plates. Column chromatography was performed by using Merck silica gel (40–60 mesh). HPLC was carried out on ACE AQ columns (100×4.6 mm and 250×10 mm), with detection at 254 nm on a Shimadzu SPD-10A VP detector; flow rate: 2.0–3.5 mL min^{-1}; from 10% CH_3CN in H_2O to 100% CH_3CN with 0.05% TFA.

7-[4-(1-Benzyl-1*H*-[1,2,3]triazol-4-yl)phenylcarbamoyl]heptanoic acid (7): LiOH·H_2O (0.199 g, 4.75 mmol) was added to a solution of compound **6**[30] (0.100 g, 0.23 mmol) in a 1:1 mixture of MeOH (5 mL) and H_2O (5 mL), and the mixture was stirred at room temperature for 1 h. The reaction was acidified by dropwise addition of 1 N HCl to pH 5, and the product was extracted with EtOAc. The organic layer was washed with H_2O and brine, dried over Na_2SO_4, and then filtered. The solvent was evaporated to give acid **7** (0.078 g, 89%). ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 1.29 (br s, 4H), 1.49 (t, J = 6.8 Hz, 2H), 1.58 (t, J = 6.8 Hz, 2H), 2.19 (t, J = 7.6 Hz, 2H), 2.30 (t, J = 7.2 Hz, 2H), 5.62 (s, 2H), 7.38–7.35 (m, 5H), 7.65 (d, J = 8.8 Hz, 2H), 7.75 (d, J = 8.4 Hz, 2H), 8.52 (s, 1H), 9.94 (s, 1H), 11.98 ppm (br s, 1H); ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 24.8, 25.3, 28.7, 28.8, 34.0, 36.8, 53.4, 119.6, 121.3, 125.7, 126.0, 128.3, 128.5, 129.2, 136.4, 139.4, 147.0, 171.7, 174.9 ppm.

Octanedioic acid (2-aminophenyl)amide-[4-(1-benzyl-1*H*-[1,2,3]-triazol-4-yl)phenyl]amide (8): HOAt (0.066 g, 0.45 mmol), Et_3N (0.21 mL, 1.50 mmol) and DMAP (0.018 g, 0.15 mmol) were added sequentially to a stirred solution of acid **7** (0.061 g, 0.15 mmol) and *o*-phenylenediamine (0.162 g, 1.50 mmol) in dry DMF (10 mL) at room temperature, and the stirring was continued overnight. The mixture was diluted with EtOAc, washed with H_2O, satd $NaHCO_3$ solution, satd NH_4Cl solution, and brine, dried over Na_2SO_4, filtered, and concentrated. The crude material was purified by preparative HPLC to give compound **8** (0.030 g, 40%). HPLC purity: 95%; R_f = 0.25 (EtOAc); ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 1.34 (br s, 4H), 1.60 (br s, 4H), 2.32–2.28 (m, 4H), 4.80 (s, 2H), 5.62 (s, 2H), 6.52 (t, J = 8.0 Hz, 1H), 6.70 (d, J = 8.0 Hz, 1H), 6.87 (t, J = 8.0 Hz, 1H), 7.14 (d, J = 7.2 Hz, 1H), 7.40–7.33 (m, 5H), 7.65 (d, J = 8.8 Hz, 2H), 7.75 (d, J = 8.8 Hz, 2H), 8.53 (s, 1H), 9.08 (s, 1H), 9.96 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO, 90 MHz): δ = 25.8, 26.0, 29.3, 36.5, 37.2, 53.7, 116.6, 116.9, 120.0, 121.6, 124.3, 126.1, 126.3, 126.5, 128.7, 128.9, 129.6, 136.8, 139.8, 142.7, 147.3, 171.9, 172.1 ppm; ESI-HRMS: *m/z* calcd for $[C_{29}H_{32}N_6O_2+H]^+$: 497.2659; found: 497.2659 $[M+H]^+$.

Octanedioic acid (2-aminophenyl)amide-[3-(1-phenyl-1*H*-[1,2,3] triazol-4-yl)phenyl]amide (11): Compound **11** (yield 30%, two steps) was prepared from ester **9**[30] according to the procedure for the preparation of compound **8** from compound **6.** HPLC purity: 95%; R_f = 0.30 (EtOAc); ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 1.36 (br s, 4H), 1.63–1.61 (m, 4H), 2.36–2.29 (m, 4H), 4.81 (br s, 1H), 6.53 (t, J = 8.0 Hz, 1H), 6.71 (d, J = 8.0 Hz, 1H), 6.88 (t, J = 8.0 Hz, 1H), 7.14 (d, J = 4.0 Hz, 1H), 7.41 (t, J = 8.0 Hz, 1H), 7.65–7.50 (m, 5H), 7.98 (d, J = 8.0 Hz, 2H), 8.27 (s, 1H), 9.09 (s, 1H), 9.26 (s, 1H), 10.03 ppm (S, 1H); ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 25.5, 25.6, 28.9, 36.1, 36.8, 116.3, 116.6, 119.3, 120.4, 120.7, 124.0, 125.7, 125.8, 126.1, 129.1, 129.7, 130.3, 131.0, 137.0, 140.3, 142.3, 147.7, 171.5, 171.8 ppm. ESI-HRMS: *m/z* calcd for $[C_{28}H_{30}N_6O_2+H]^+$: 483.2503; found: 483.2502 $[M+H]^+$.

7-[4-(3-Ethoxycarbonylaminophenyl)thiazol-2-ylcarbamoyl]heptanoic acid methyl ester (12c): A mixture of **12b**[21] (0.200 g, 0.55 mmol) and ethyl chloroformate (0.600 g, 5.53 mmol) in THF (20 mL) was reflux for 5 h. The solvent was then evaporated in vacuo, and the residue was dissolved in EtOAc, washed with satd

$NaHCO_3$ solution and brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:2) to give compound **12c** (0.178 g, 74%). R_f = 0.80 (hexane/EtOAc, 1:1); ^{1}H NMR (CD_3OD, 400 MHz): δ = 1.33 (t, J = 7.0 Hz, 3H), 1.41 (brs, 4H), 1.64 (t, J = 7.0 Hz, 2H), 1.73 (t, J = 7.0 Hz, 2H), 2.34 (t, J = 7.3 Hz, 2H), 2.49 (t, J = 7.4 Hz, 2H), 3.66 (s, 3H), 4.21 (q, J = 7.0 Hz, 2H), 7.37–7.29 (m, 3H), 7.57 (d, J = 7.3 Hz, 1H), 8.03 ppm (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz): δ = 13.5, 24.3, 24.7, 28.3, 28.4, 33.2, 35.0, 50.5, 60.4, 107.3, 116.0, 117.8, 120.3, 125.8, 128.6, 135.2, 139.2, 149.5, 154.7, 157.9, 172.3 ppm.

7-[4-(3-Acetylaminophenyl)thiazol-2-ylcarbamoyl]heptanoic acid methyl ester (12e): Acetyl chloride (0.156 g, 1.99 mmol) was added dropwise to a solution of **12b** (0.180 g, 0.49 mmol) and Et_3N (0.70 mL, 4.97 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C. This solution was stirred at this temperature for 2 h, then diluted with EtOAc and washed thoroughly with satd aq NH_4Cl solution and brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 3:2) to give compound **12e** (0.120 g, 59%). R_f = 0.31 (hexane/EtOAc, 1:1); ^{1}H NMR ([D$_6$]DMSO, 300 MHz): δ = 1.28 (brs, 4H), 1.59–1.49 (m 4H), 2.05 (s, 3H), 2.28 (t, J = 7.2 Hz, 2H), 2.44 (t, J = 7.2 Hz, 2H), 3.57 (s, 3H), 7.32 (t, J = 7.8 Hz, 1H), 7.41 (d, J = 8.1 Hz, 1H), 7.47 (s, 1H), 7.54 (d, J = 7.5 Hz, 1H), 8.22 (s, 1H), 9.98 (s, 1H), 12.23 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO, 75 MHz): δ = 24.9, 25.1, 25.3, 28.9, 29.0, 34.0, 35.6, 52.0, 108.7, 117.4, 119.3, 121.3, 129.8, 135.6, 140.5, 149.6, 158.7, 169.1, 172.4, 174.1 ppm.

7-[4-(3-Ethoxycarbonylaminophenyl)thiazol-2-ylcarbamoyl]heptanoic acid (13c): Compound **13c** (yield 75%) was prepared from ester **12c**[21] according to the procedure for the preparation of compound **7**. ^{1}H NMR (CD_3OD, 400 MHz): δ = 1.33 (t, J = 7.0 Hz, 3H), 1.43 (brs, 4H), 1.65 (t, J = 7.0 Hz, 2H), 1.75 (t, J = 7.0 Hz, 2H), 2.31 (t, J = 7.3 Hz, 2H), 2.51 (t, J = 7.4 Hz, 2H), 4.21 (q, J = 7.0 Hz, 2H), 7.35–7.29 (m, 3H), 7.58 (d, J = 7.4 Hz, 1H), 8.02 ppm (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz): δ = 13.5, 24.4, 24.8, 28.4, 33.4, 35.1, 60.4, 107.2, 116.0, 117.8, 120.3, 128.6, 135.2, 139.2, 149.5, 154.7, 157.9, 172.4, 176.2 ppm.

Octanedioic acid (2-aminophenyl)amide-[4-(3-nitrophenyl)thiazol-2-yl]amide (14a): Compound **14a** (yield 24%, two steps) was prepared from ester **12a**[21] according to the procedure for the preparation of compound **11**. R_f = 0.40 (MeOH/EtOAc, 5:95); ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 1.33 (brs, 4H), 1.61 (brs, 4H), 2.30 (t, J = 6.9 Hz, 2H), 2.46 (t, J = 6.9 Hz, 2H), 4.81 (s, 2H), 6.51 (t, J = 7.5 Hz, 1H), 6.70 (d, J = 7.8 Hz, 1H), 6.87 (t, J = 7.5 Hz, 1H), 7.14 (d, J = 7.8 Hz, 1H), 7.72 (t, J = 8.1 Hz, 1H), 7.90 (s, 1H), 8.16 (d, J = 8.1 Hz, 1H), 8.33 (d, J = 7.5 Hz, 1H), 8.72 (s, 1H), 9.08 (s, 1H), 12.33 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 25.4, 26.0, 29.2, 29.3, 35.7, 36.5, 111.2, 116.7, 117.0, 120.9, 123.1, 124.4, 126.1, 126.5, 131.2, 132.5, 136.7, 142.7, 147.1, 149.1, 159.2, 171.9, 172.6 ppm.

Octanedioic acid (2-aminophenyl)amide [4-(3-aminophenyl) thiazol-2-yl]amide (14b): A suspension of compound **14a** (0.045 g, 0.096 mmol) and Pd/C (10 wt%, 10 mg) in EtOH (10 mL) was stirred under a H_2 atmosphere at room temperature for 5 h. The catalyst was removed by filtration through a pad of Celite and washed thoroughly with MeOH. The solvent was evaporated, and the residue was purified by column chromatography on silica gel (EtOAc/hexane, 3:1) to give compound **14b** (0.024 g, 56%). HPLC purity: 95%; R_f = 0.43 (EtOAc); ^{1}H NMR (CD_3OD, 400 MHz): δ = 1.41 (brs, 4H), 1.71 (brs, 4H), 2.48–2.37 (m, 4H), 6.68–6.66(m, 2H), 6.83 (d, J = 8.1 Hz, 1H), 7.11–6.98 (m, 3H), 7.25–7.21 ppm (m, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ = 23.6, 24.3, 27.3, 27.4, 33.9, 34.6, 105.6, 111.6, 113.7, 114.8, 116.0, 117.0, 122.6, 124.6, 125.7, 127.8, 134.1, 140.7, 146.3, 149.0, 156.6, 171.2, 172.5 ppm; ESI-HRMS: m/z calcd for $[C_{23}H_{27}N_5O_2S_1+H]^+$: 438.1958; found: 438.1954 $[M+H]^+$.

(3-{2-[7-(2-Aminophenylcarbamoyl)heptanoylamino]thiazol-4-yl}-phenyl)carbamic acid ethyl ester (14c): Compound **14c** (yield 42%) was prepared from acid **13c**[21] according to the procedure for the preparation of compound **8**. HPLC purity: 95%; R_f = 0.52 (EtOAc); ^{1}H NMR (CD_3OD, 400 MHz): δ = 1.32 (t, J = 7.2 Hz, 3H), 1.49–1.47 (m, 4H), 1.77–1.75 (m, 4H), 2.44 (t, J = 7.2 Hz, 2H), 2.52 (t, J = 7.6 Hz, 2H), 4.20 (q, J = 7.2 Hz, 2H), 6.74–6.72 (m, 1H), 6.86–6.84 (m, 1H), 7.08–7.01 (m, 2H), 7.36–7.30 (m, 3H), 7.57 (d, J = 7.2 Hz, 1H), 8.01 ppm (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz): δ = 15.0, 25.1, 25.6, 28.8, 35.3, 36.1, 60.6, 108.4, 116.1, 116.3, 116.6, 118.2, 120.3, 124.0, 125.7, 126.1, 129.4, 135.3, 140.0, 142.3, 149.2, 154.0, 158.3, 171.5, 172.0 ppm; ESI-HRMS: m/z calcd for $[C_{26}H_{31}N_5O_4S_1+H]^+$: 510.2169; found: 510.2063 $[M+H]^+$.

(3-{2-[7-(2-Aminophenylcarbamoyl)heptanoylamino]thiazol-4-yl}-phenyl)carbamic acid *tert*-butyl ester (14d): Compound **14d** (yield 17%, two steps) was prepared from acid **13d**[21] according to the procedure for the preparation of compound **8**. HPLC purity: 95%; R_f = 0.52 (EtOAc); ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 1.36 (brs, 4H), 1.53 (s, 9H), 1.70 (brs, 4H), 2.38 (brs, 2H), 2.54 (brs, 2H), 6.83 (s, 1H), 7.08–6.94 (m, 3H), 7.30–7.23 (m, 3H), 7.40–7.35 (m, 2H), 7.87 (s, 1H), 8.43 ppm (brs, 1H); ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 24.8, 25.3, 27.2, 28.4, 28.5, 35.0, 35.6, 107.2, 116.0, 117.8, 120.0, 125.6, 126.8, 128.5, 135.1, 139.4, 149.6, 153.9, 157.8, 172.4 ppm; ESI-HRMS: m/z calcd for $[C_{28}H_{35}N_5O_4S_1+H]^+$: 538.2482; found: 538.2475 $[M+H]^+$.

Octanedioic acid [4-(3-acetylaminophenyl)thiazol-2-yl]amide-(2-aminophenyl)amide (14e): Compound **14e** (yield 29%, two steps) was prepared from acid **13e**[21] according to the procedure for the preparation of compound **8**. HPLC purity: 95%; R_f = 0.28 (MeOH/EtOAc, 5:95); ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 1.34 (brs, 4H), 1.63–1.61 (m, 4H), 2.34 (t, J = 7.6 Hz, 2H), 2.46 (t, J = 7.2 Hz, 2H), 6.87 (t, J = 8.0 Hz, 1H), 6.98 (d, J = 8.0 Hz, 1H), 7.06 (t, J = 8.0 Hz, 1H), 7.21 (d, J = 8.0 Hz, 1H), 7.32 (t, J = 8.0 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.48 (s, 1H), 7.54 (d, J = 8.0 Hz, 1H), 8.23 (s, 1H), 9.52 (s, 1H), 10.00 (s, 1H), 12.26 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 24.4, 25.0, 25.6, 28.8, 35.3, 36.1, 108.3, 116.4, 116.8, 117.0, 118.9, 120.9, 124.1, 125.7, 126.1, 129.4, 135.2, 140.1, 142.0, 149.2, 158.3, 168.7, 171.5, 172.0 ppm. ESI-HRMS: m/z calcd for $[C_{25}H_{29}N_5O_3S+H]^+$: 480.2063; found: 480.2061 $[M+H]^+$.

Octanedioic acid [4-(3-acetylaminophenyl)thiazol-2-yl]amide hydroxyamide (15): KOH (2.55 g, 45.6 mmol) was added at 40 °C over 10 min to a solution of hydroxylamine hydrochloride (3.16 g, 45.6 mmol) in MeOH (20 mL). The mixture was cooled to 0 °C and filtered. Compound **12e** (0.092 g, 0.22 mmol) was added to the filtrate followed by KOH (0.255 g, 4.56 mmol), and the solution was stirred at room temperature for 30 min. The mixture was extracted with EtOAc, the organic layer was washed with satd aq NH_4Cl solution and brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by preparative HPLC to give compound **15** (0.034 g, 36%). HPLC purity: 95%; ^{1}H NMR (CD_3OD, 300 MHz): δ = 1.39 (brs, 4H), 1.62 (t, J = 6.6 Hz, 2H), 1.71 (t, J = 6.9 Hz, 2H), 2.08 (t, J = 7.5 Hz, 2H), 2.13 (s, 3H), 2.47 (t, J = 7.2 Hz, 2H), 7.34–7.28 (m, 2H), 7.43 (d, J = 8.1 Hz, 1H), 7.61 (d, J = 7.5 Hz, 1H), 8.12 ppm (s, 1H); ^{13}C NMR (CD_3OD, 75 MHz): δ = 21.2, 23.6, 23.9, 27.1, 27.2, 31.1, 33.9, 68.9, 106.3, 116.2, 118.0, 120.3, 127.4, 134.0, 137.6, 148.2, 156.8, 169.1, 170.4, 171.3 ppm; ESI-HRMS: m/z calcd for $[C_{19}H_{24}N_4O_4S_1+H]^+$: 405.1591; found: 405.1590 $[M+H]^+$.

8-Bromooctanoic acid [4-(3-nitrophenyl)thiazol-2-yl]amide (18): $POCl_3$ (0.758 g, 4.94 mmol) was added to a solution of **16** (0.911 g, 4.11 mmol) and **17** (0.919 g, 4.11 mmol) in dry pyridine (30 mL) at 0 °C dropwise. This solution was stirred at this temperature for 1 h, then diluted with EtOAc and washed thoroughly with satd aq $KHSO_4$ and brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:2) to give compound **18** (0.715 g, 40%). R_f = 0.26 (hexane/EtOAc, 1:1); ^{1}H NMR ([D$_6$]DMSO, 300 MHz): δ = 1.37–1.30 (m, 6H), 1.61 (br s, 2H), 1.78 (t, J = 6.3 Hz, 2H), 2.46 (t, J = 7.2 Hz, 2H), 3.52 (dt, J = 6.6 and 2.4 Hz, 2H), 7.72 (dt, J = 8.1 and 2.7 Hz, 1H), 7.92 (d, J = 2.7 Hz, 1H), 8.16 (d, J = 8.1 Hz, 1H), 8.33 (d, J = 7.8 Hz, 1H), 8.72 (d, J = 1.2 Hz, 1H), 12.32 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO, 75 MHz): δ = 25.3, 28.2, 28.6, 29.1, 33.0, 35.6, 36.0, 111.2, 120.9, 123.1, 131.2, 132.5, 136.6, 147.1, 149.2, 159.2, 172.6 ppm.

8-Bromooctanoic acid [4-(3-aminophenyl)thiazol-2-yl]amide (19): Compound **19** (yield 83%) was prepared from **18** according to the procedure for the preparation of compound **14b**. ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 1.37–1.29 (m, 6H), 1.59 (t, J = 6.8 Hz, 2H), 1.78 (t, J = 7.2 Hz, 2H), 2.43 (t, J = 7.2 Hz, 2H), 3.51 (t, J = 6.8 Hz, 2H), 5.11 (br s, 2H), 6.51 (d, J = 6.8 Hz, 1H), 7.07–7.00 (m, 3H), 7.35 (s, 1H), 12.16 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 25.0, 27.7, 28.2, 28.7, 32.6, 35.2, 35.6, 107.3, 111.7, 114.0, 125.8, 129.5, 135.3, 149.2, 150.0, 157.9, 171.9 ppm.

Thioacetic acid *S*-{7-[4-(3-acetylaminophenyl)thiazol-2-ylcarbamoyl]heptyl}ester (20): A solution of **19** (0.060 g, 0.15 mmol) and KSAc (0.345 g, 3.02 mmol) in DMF (5 mL) was stirred at room temperature for 48 h. The mixture was diluted with EtOAc, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:2) to give compound **20** (0.035 g, 53%). HPLC purity: 95%; ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 1.27 (br s, 6H), 1.48 (t, J = 6.4 Hz, 2H), 1.59 (t, J = 6.4 Hz, 2H), 2.05 (s, 3H), 2.30 (s, 3H), 2.43 (t, J = 7.2 Hz, 2H), 2.81 (t, J = 7.2 Hz, 2H), 7.31 (t, J = 8.0 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.47 (s, 1H), 7.53 (d, J = 7.6 Hz, 1H), 8.21 (s, 1H), 9.98 (s, 1H), 12.22 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 24.4, 25.0, 28.3, 28.5, 28.7, 28.8, 29.5, 31.0, 35.2, 108.3, 117.0, 118.9, 120.9, 129.4, 135.2, 140.1, 149.2, 158.3, 168.7, 172.0, 195.7 ppm; ESI-HRMS: m/z calcd for $[C_{21}H_{27}N_3O_3S_2-H]^-$: 432.1421; found: 432.1419 $[M-H]^-$.

8-Mercaptooctanic acid [4-(3-acetylaminophenyl)thiazol-2-yl]amide (21): A solution of 2 N aq NaOH (1 mL, 2.00 mmol) was added to a solution of **20** (0.030 g, 0.07 mmol) in EtOH (2 mL), and the mixture was stirred at room temperature for 12 h. The mixture was poured into H_2O and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 2:1) to give compound **21** (0.017 g, 66%). HPLC purity: 98%; R_f = 0.72 (MeOH/EtOAc, 5:95); ^{1}H NMR (CD$_3$OD, 300 MHz): δ = 1.38 (m, 6H), 1.60 (t, J = 6.3 Hz, 4H), 2.05 (s, 3H), 2.45 (t, J = 8.0 Hz, 2H), 2.68 (t, J = 8.0 Hz, 2H), 7.31 (t, J = 8.0 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.47 (s, 1H), 7.53 (d, J = 7.6 Hz, 1H), 8.23 (s, 1H), 9.98 (s, 1H),12.22 ppm (s, 1H); ^{13}C NMR (CD$_3$OD, 75 MHz): δ = 22.8, 23.9, 25.3, 28.2, 28.8, 29.1, 34.1, 35.5, 107.8, 117.8, 119.5, 121.8, 129.0, 135.6, 139.2, 149.8, 158.3, 170.7, 172.9 ppm; ESI-HRMS: m/z calcd for $[C_{19}H_{25}N_3O_2S_2+Na]^+$: 414.1286; found: 414.1261 $[M+Na]^+$.

Thiobenzoic acid *S*-{7-[4-(3-aminophenyl)thiazol-2-ylcarbamoyl]heptyl}ester (22): Thiobenzoic acid (0.264 g, 1.91 mmol) and K_2CO_3 powder (0.264 g, 1.91 mmol) were added to a solution of **19** (0.120 g, 0.30 mmol) in DMF (10 mL), and the mixture was stirred at room temperature for 12 h. The reaction was diluted with EtOAc and washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:1) to give compound **22** (0.105 g, 78%). HPLC purity: 95%; ^{1}H NMR (CD$_3$OD, 400 MHz): δ = 1.44–1.39 (m, 6H), 1.72–1.62 (m, 4H), 2.47 (t, J = 7.2 Hz, 2H), 3.06 (t, J = 7.6 Hz, 2H), 6.70–6.67 (m, 1H), 7.14 (t, J = 7.6 Hz, 1H), 7.27–7.22 (m, 3H), 7.47 (t, J = 7.6 Hz, 2H), 7.59 (t, J = 7.6 Hz, 1H), 7.93 ppm (d, J = 7.2 Hz, 2H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ = 24.8, 28.2, 28.3, 28.6, 29.2, 35.1, 106.7, 112.7, 114.8, 115.9, 126.6, 128.3, 128.8, 133.1, 135.3, 137.1, 147.4, 150.1, 157.7, 172.4, 192.0 ppm; ESI-HRMS: m/z calcd for $[C_{24}H_{27}N_3O_2S_2-H]^-$: 452.1471; found: 452.1469 $[M-H]^-$.

8-{7-[4-(3-Aminophenyl)thiazol-2-ylcarbamoyl]heptyldisulfanyl}-octanoic acid [4-(3-aminophenyl)thiazol-2-yl]amide (23): Compound **23** (yield 58%) was prepared according to the procedure for the preparation of compound **30**. R_f = 0.76 (MeOH/EtOAc, 5:95); HPLC purity: 95%; ^{1}H NMR (CD$_3$OD, 400 MHz): δ = 1.20–1.12 (m, 8H), 1.32–1.25 (m, 8H), 1.52–1.48 (m, 4H), 1.65–1.62 (m, 4H), 2.08 (t, J = 7.2 Hz, 4H), 2.65 (t, J = 6.8 Hz, 4H), 6.68–6.65 (m, 2H), 7.10 (s, 2H), 7.17 (d, J = 1.2 Hz, 2H), 7.21–7.19 (m, 4H), 11.24 ppm (s, 2H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ = 24.6, 28.0, 28.6, 28.7, 28.9, 35.8, 39.1, 107.7, 112.6, 115.0, 116.5, 129.8, 135.2, 146.8, 149.7, 159.2, 171.6 ppm; ESI-HRMS: m/z calcd for $[C_{34}H_{44}N_6O_2S_4-H]^-$: 695.2335; found: 695.2335 $[M-H]\ ]^-$.

Octanedioic acid (2-aminophenyl)amide benzyloxyamide (25): Compound **25** (yield 28%) was prepared from acid **24** and phenyldiamine according to the procedure for the preparation of compound **8**. HPLC purity: 95%; R_f = 0.72 (acetone); ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 1.27 (br s, 4H), 1.49 (t, J = 6.4 Hz, 2H), 1.58 (t, J = 6.4 Hz, 2H), 1.94 (t, J = 7.2 Hz, 2H), 2.32 (t, J = 7.6 Hz, 2H), 4.77 (s, 2H), 6.88 (t, J = 7.2 Hz, 1H), 6.99 (d, J = 7.6 Hz, 1H), 7.06 (t, J = 8.0 Hz, 1H), 7.21 (d, J = 7.6 Hz, 1H), 7.38–7.33 (m, 5H), 9.53 (s, 1H), 10.94 ppm (br s, 1H); ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 25.2, 25.4, 28.7, 28.8, 32.6, 36.0, 77.1, 125.8, 126.4, 128.6, 128.7, 129.1, 172.1 ppm; ESI-HRMS: m/z calcd for $[C_{21}H_{27}N_3O_3+H]^+$: 370.2125; found: 370.2124 $[M+H]^+$.

Octanedioic acid (2-aminophenyl)amide hydroxyamide (26): A suspension of compound **25** (0.060 g, 0.16 mmol) and Pd/C (10 wt%, 10 mg) in EtOAc (10 mL) was stirred under H_2 atmosphere at room temperature for 2 h. The catalyst was removed by filtration through a pad of Celite and washed thoroughly with MeOH. The solvent was evaporated, and the residue was purified by preparative HPLC to give compound **26** (0.016 g, 35%). HPLC purity: 95%; ^{1}H NMR (CD$_3$OD, 400 MHz): δ = 1.41–1.39 (m, 4H), 1.68–1.63 (m, 2H), 1.76–1.72 (m, 2H), 2.12 (t, J = 7.2 Hz, 2H), 2.50 (t, J = 7.2 Hz, 2H), 7.35–7.31 (m, 1H), 7.39–7.37 ppm (m, 1H), 7.42–7.41 (m, 2H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ = 24.8, 25.0, 28.3, 28.4, 32.2, 35.5, 123.4, 125.4, 126.3, 127.0, 128.4, 131.0, 171.6, 174.4 ppm; ESI-HRMS: m/z calcd for $[C_{14}H_{21}N_3O_3+H]^+$: 280.1655; found: 280.1655 $[M+H]^+$.

8-Bromooctanoic acid (2-aminophenyl)amide (27): Compound **27** (yield 30%) was prepared from 8-bromooctanoic acid **17** and phenyldiamine according to the procedure for the preparation of compound **8**. ^{1}H NMR (CDCl$_3$, 400 Hz): δ = 1.37 (br s, 4H), 1.49–1.43 (m, 2H), 1.75–1.67 (m, 2H), 1.90–1.83 (m, 2H), 2.35 (t, J = 7.6 Hz, 2H), 3.41 (t, J = 6.8 Hz, 2H), 6.80–6.77 (m, 2H), 7.05 (t, J = 8.0 Hz, 1H), 7.15 (d, J = 7.2 Hz, 1H), 7.46 ppm (br s, 1H); ^{13}C NMR (CDCl$_3$, 100 Hz): δ = 25.6, 27.9, 28.4, 29.0, 32.6, 33.9, 36.8, 118.1, 119.5, 124.3, 125.3, 127.1, 140.7, 171.9 ppm.

Thiobenzoic acid *S*-[7-(2-aminophenylcarbamoyl)heptyl]ester (28): A solution of **27** (0.100 g, 0.31 mmol) and KSAc (0.072 g,

0.63 mmol) in DMF (5 mL) was stirred at room temperature for 12 h. The reaction was diluted with EtOAc, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:1) to give compound **28** (0.074 g, 52%). HPLC purity: 95%; $R_f = 0.76$ (EtOAc); ^{1}H NMR ($CDCl_3$, 300 Hz): $\delta = 1.44–1.39$ (m, 4H), 1.71–1.63 (m, 4H), 2.35 (t, $J = 7.2$ Hz, 2H), 3.07 (t, $J = 7.2$ Hz, 2H), 6.79–6.77 (m, 2H), 7.05 (t, $J = 7.8$ Hz, 1H), 7.15 (d, $J = 7.8$ Hz, 1H), 7.57–7.42 (m, 2H), 7.97 ppm (d, $J = 7.5$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 Hz): $\delta =$ 26.1, 29.0, 29.1, 29.3, 29.4, 29.8, 37.2, 118.5, 119.8, 124.7, 125.7, 127.5, 128.9, 133.6, 137.6, 141.2, 172.4, 192.6 ppm; ESI-HRMS: *m/z* calcd for $[C_{21}H_{26}N_2O_2S+H]^+$: 371.1787; found: 371.1785 $[M+H]^+$.

Thiobenzoic acid *S*-[7-(2-aminophenylcarbamoyl)heptyl]ester (29): Thiobenzoic acid (0.264 g, 1.91 mmol) and K_2CO_3 powder (0.264 g, 1.91 mmol) were added to a solution of **27** (0.120 g, 0.38 mmol) in DMF (10 mL), and the mixture was stirred at room temperature for 12 h. The reaction was diluted with EtOAc, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:1) to give compound **29** (0.074 g, 52%). HPLC purity: 95%; $R_f = 0.76$ (EtOAc); ^{1}H NMR ($CDCl_3$, 300 Hz): $\delta = 1.44–1.39$ (m, 4H), 1.71–1.63 (m, 4H), 2.35 (t, $J = 7.2$ Hz, 2H), 3.07 (t, $J = 7.2$ Hz, 2H), 6.79–6.77 (m, 2H), 7.05 (t, $J = 7.8$ Hz, 1H), 7.15 (d, $J = 7.8$ Hz, 1H), 7.57–7.42 (m, 2H), 7.97 ppm (d, $J = 7.5$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 Hz): $\delta = 26.1$, 29.0, 29.1, 29.3, 29.4, 29.8, 37.2, 118.5, 119.8, 124.7, 125.7, 127.5, 128.9, 133.6, 137.6, 141.2, 172.4, 192.6 ppm; ESI-HRMS: *m/z* calcd for $[C_{21}H_{26}N_2O_2S+H]^+$: 371.1787; found: 371.1785 $[M+H]^+$.

8-[7-(2-Aminophenylcarbamoyl)heptyldisulfanyl]octanoic acid (2-aminophenyl)amide (30): A solution of 2 N aq NaOH (2 mL, 4.00 mmol) was added to a solution of **29** (0.050 g, 0.13 mmol) in EtOH (5 mL), and the mixture was stirred at room temperature for 12 h. The mixture was poured into H_2O and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 2:1) to give compound **30** (0.014 g, 39%). HPLC purity: 95%; $R_f = 0.40$ (EtOAc); ^{1}H NMR ($CDCl_3$, 300 Hz): $\delta = 1.37$ (br s, 12H), 1.69 (br s, 8H), 2.34 (t, $J = 7.5$ Hz, 4H), 2.69 (t, $J = 7.2$ Hz, 4H), 6.76–6.74 (m, 4H), 7.04 (t, $J = 7.5$ Hz, 2H), 7.12 (d, $J = 8.1$ Hz, 2H), 7.58 ppm (br s, 2H); ^{13}C NMR ($CDCl_3$, 75 Hz): $\delta = 26.1$, 28.6, 29.3, 29.4, 29.5, 37.2, 39.5, 118.4, 119.7, 124.7, 125.8, 127.5, 141.2, 172.5 ppm; ESI-HRMS: *m/z* calcd for $[C_{28}H_{42}N_4O_2S_2+H]^+$: 531.2821; found: 531.2816 $[M+H]^+$.

Biological assays

HDAC inhibition assays. Purified HDACs were incubated with 1 μM carboxyfluorescein (FAM)-labeled acetylated peptide substrate and test compound for 17 h at 25 °C in HDAC assay buffer that contained 100 mM HEPES (pH 7.5), 25 mM KCl, 0.1% BSA and 0.01% Triton X-100. Reactions were terminated by the addition of buffer that contained 0.078% SDS for a final SDS concentration of 0.05%. Substrate and product were separated electrophoretically by using a Caliper LabChip 3000 system with blue laser excitation and green fluorescence detection (CCD2). The fluorescence intensity in the substrate and product peaks was determined by using the Well Analyzer software on the Caliper system. The reactions were performed in duplicate for each sample. IC_{50} values were automatically calculated by using the IDBS XLFit version 4.2.1 plug-in for Microsoft Excel and the XLFit 4 Parameter Logistic Model (sigmoidal dose–response model): $A+\{(B-A)/[1+(C/x)^D]\}$, in which x is compound concentration, A is the estimated minimum and B is the estimated maximum of percent inhibition, C is the inflection point, and D is the Hill slope of the sigmoidal curve. The standard errors of the IC_{50} values were automatically calculated by using the IDBS XLFit version 4.2.1 plug-in for Microsoft Excel and the formula xf4_FitResultStdError(). (Enzyme was prepared in-house. Peptides were synthesized by Biopeptide Co. Inc. (HDAC1, -2, -6, -8, -10 peptides), and by Synpep (HDAC3 peptide)).

Primary neurons and cell culture. Cell cultures were obtained from the cerebral cortex of fetal Sprague–Dawley rats (embryonic day 17) as described previously.[42] All experiments were initiated 24 h after plating. Under these conditions, the cells are not susceptible to glutamate-mediated excitotoxicity.

Neuron viability assays. For cytotoxicity studies, cells were rinsed with warm PBS and then placed in minimum essential medium (Invitrogen) that contained 5.5 g L^{-1} glucose, 10% fetal calf serum, 2 mM L glutamine, and 100 μM cystine. Oxidative stress was induced by the addition of the glutamate analogue homocysteate (HCA; 5 mM) to the media. HCA was diluted from 100-fold-concentrated solutions that were adjusted to pH 7.5. In combination with HCA, commercially available HDAC inhibitors, or the novel HDAC inhibitors were used at the concentrations specified. Viability was assessed after 48 h by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method.

Histone precipitation and Western blotting. Approximately 1×10^7 treated neurons were incubated in hypotonic lysis buffer (1 mL) that contained 10 mM Tris-HCl pH 8, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM aprotinin, 1 mM pepstatin, and 0.4 mM PMSF for 30 min by rotating at 4 °C. Nuclei were pelleted by centrifugation for 10 min at 10000 rpm (9000 *g*), resuspended in 0.4 N H_2SO_4 (200 μL), and rotated for 12 h at 4 °C. Following centrifugation at 13000 rpm (16000 *g*) for 10 min, the supernatant was transferred to a new tube, and histone proteins were precipitated by adding 100% TCA (66 μL) dropwise followed by a 30 min incubation on ice. Histone proteins were pelleted by centrifugation at 13000 rpm (16000 *g*) for 10 min, washed twice with ice-cold acetone, dried at room temperature for 20–40 min and resuspended in 50 μL H_2O. Total histone proteins (15 μg) were boiled in Laemmli buffer and electrophoresed under reducing conditions on 15% polyacrylamide gel. Histone proteins were transferred to a nitrocellulose membrane (Bio-Rad). Nonspecific binding was inhibited by incubation in Tris-buffered saline with Tween 20 (TBST: 50 mM Tris-HCl, pH 8.0, 0.9% NaCl, and 0.1% Tween 20) that contained 5% nonfat dried milk for at least 1.5 h. Primary antibodies against acetylated histone H3 (Upstate) were diluted 1:1000 in TBST that contained 5% milk and incubated with the membrane overnight at 4 °C followed by incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Acetyl histone H3 immunoreactivity was detected according to the enhanced chemiluminescent protocol (Amersham Biosciences).

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Keywords: benzamides · HDAC inhibitors · neurological agents · oxidative stress · prodrugs

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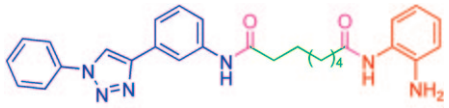
Studies of Benzamide- and Thiol-Based Histone Deacetylase Inhibitors in Models of Oxidative-Stress-Induced Neuronal Death: Identification of Some HDAC3-Selective Inhibitors



11: selectively inhibits HDAC3

Less stress: We compare three structurally different classes of histone deacetylase (HDAC) inhibitors that contain benzamide, hydroxamate, or thiol groups as the zinc binding group (ZBG) for their ability to protect cortical neurons in culture from cell death induced by oxidative stress. Novel benzamide-based ligands selectively inhibit HDAC3 but provide no neuroprotection in the HCA–cortical neuron model of oxidative stress.

