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Hydroxamate-based Histone Deacetylase Inhibitors Can Protect Neurons from Oxidative Stress via an HDAC-independent Catalase-like Mechanism

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Summary

Histone deacetylase (HDAC) inhibitors have shown enormous promise for treating various disease states, presumably due to their ability to modulate acetylation of histone and non-histone proteins. Many of these inhibitors contain functional groups capable of strongly chelating metal ions. We demonstrate that several members of one such class of compounds, the hydroxamate-based HDAC inhibitors, can protect neurons from oxidative stress via an HDAC-independent mechanism. This previously unappreciated antioxidant mechanism involves the in situ formation of hydroxamate-iron complexes that catalyze the decomposition of hydrogen peroxide in a manner reminiscent of catalase. We demonstrate that while many hydroxamate-containing HDAC inhibitors display a propensity for binding iron, only a subset form active catalase mimetics capable of protecting neurons from exogenous H_2O_2. In addition to impacting stroke and neurodegenerative disease research, these results highlight the possibility that HDAC-independent factors might play a role in the therapeutic effects of hydroxamate-based HDAC inhibitors.

Graphical Abstract

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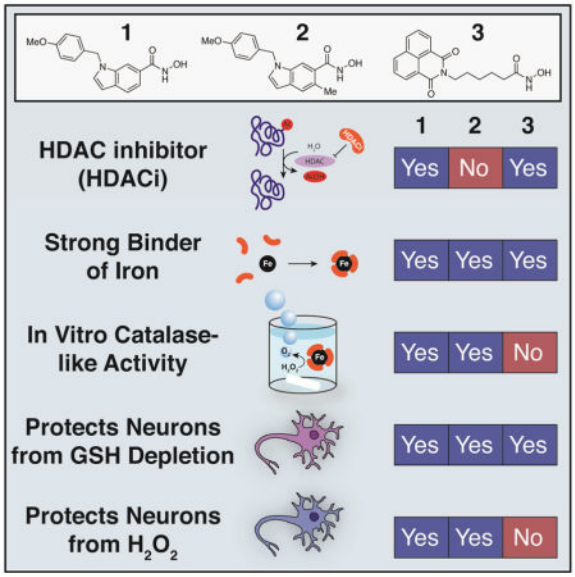

Introduction

The deleterious effects of oxidative stress contribute to the neuronal death associated with a variety of brain disorders including Parkinson's disease, Alzheimer's disease, and stroke (Uttara et al., 2009). It is believed that these effects can be mitigated by promoting oxidative defense mechanisms through the manipulation of epigenetic factors (Schweizer et al., 2013). This conclusion is due in large part to the effectiveness of small molecule inhibitors of histone deacetylases (HDACs) in various models of neuroprotection (Ryu et al., 2003; Kim et al., 2007; Shein et al., 2009; Butler et al., 2010; Fleiss et al., 2012; Lu et al., 2013). HDACs catalyze the removal of acetyl groups from the ε-nitrogens of lysine residues, a posttranslational modification that can profoundly impact cellular processes ranging from cytoskeletal reorganization (Piperno et al., 1987) to gene expression (Spange et al., 2009). With the exception of the NAD-dependent sirtuins (Class III), HDACs are metalloenzymes (Class I: HDACs 1, 2, 3, and 8; Class II: HDACs 4, 5, 6, 7, 9, and 10: Class IV: HDAC11). This means that the vast majority of known HDAC inhibitors (HDACi) contain a metal-binding group of varied chelating ability (e.g., hydroxamic acids, *o*-aminoanilides, ketones, etc.) (Pan et al., 2012); however, the chemical properties associated with such functional groups are often overlooked when studying the biological effects of HDAC inhibitors. Herein, we demonstrate that the iron-binding ability of several known hydroxamate-based HDAC inhibitors is necessary, but not sufficient, to explain their capacity to protect neurons from exogenous H_2O_2. Instead, our data suggests that only a subset of these iron-binding small molecules can form complexes capable of catalyzing the disproportionation of H_2O_2 into water and the less potent cellular oxidant, molecular oxygen.

Results and Discussion

The Development of Chemical Tools for Studying HDACs

As part of our general interest in using chemical tools to study the function of the various Zn-dependent HDAC isoforms (Gregoretti et al., 2004), we amassed a collection of HDAC inhibitors and analogs, including appropriately designed negative control compounds, belonging to different chemical classes with varying potencies and selectivities (Figure 1). Initially, we tested several of these compounds in a glutathione (GSH) depletion assay that models oxidative stress (Murphy et al., 1990; Ratan et al., 1994; Ratan and Baraban, 1995) and found that many were effective in protecting murine cortical neurons from death (Figure S1). The non-selective hydroxamate-based inhibitors were particularly potent as they conferred full protection at nanomolar concentrations.

Several Hydroxamate-based HDAC Inhibitors Exhibit HDAC-independent Antioxidant Properties

Because the GSH depletion assay requires the use of immature neurons lacking functional NMDA receptors to prevent excitotoxicity (Murphy et al., 1990), we next decided to test the neuroprotective properties of these HDAC inhibitors by subjecting fully mature cortical neurons to H_2O_2 (Figure 2A), a direct oxidative insult that mimics the concentration of H_2O_2 released during stroke (Hyslop et al., 1995). Surprisingly, we found that the non-selective inhibitor LBH-589 was able to fully protect neurons from a H_2O_2 insult though other non-selective inhibitors with similar potencies could not (Figure 2A). We ruled out the possibility of differential toxicity accounting for the observed neuroprotection because in the absence of insult, these compounds did not cause any cell death at the concentrations and time frame employed in the assay (Figure 2C). These results provided the first piece of evidence suggesting that there might be multiple mechanisms by which hydroxamate-based HDAC inhibitors can exert neuroprotective effects.

Upon closer examination, we discovered that the non-selective HDAC inhibitor LBH-589, the HDAC8-selective inhibitor PCI-34051 (Balasubramanian et al., 2008), and the HDAC6-selective inhibitor tubastatin A (Butler et al., 2010) protected neurons from H_2O_2 at relatively high concentrations (>10 μM) (Figure 2B), results that seemed inconsistent with their exceptional HDAC potencies (IC_{50}s < 15 nM). Furthermore, we recently reported that PCI-34051 protects neurons from GSH depletion via an opaque, but HDAC-independent mechanism (Sleiman et al., 2014), and we hypothesized that such a mechanism might play a role in the neuroprotective effects of LBH-589 and tubastatin A as well. This hypothesis was supported by the fact that other HDAC6- (i.e., BRD3493 and BRD9757) and HDAC6/8-selective (i.e., BRD3954) inhibitors were unable to protect cortical neurons from the deleterious effects of H_2O_2 (Figure 2A), even at concentrations known to induce tubulin acetylation (Wagner et al., 2013; Olson et al., 2013), the known cellular substrate for HDAC6 (Hubbert et al., 2002).

In order to establish whether or not the inhibition of HDAC6 and/or HDAC8 is involved in neuroprotection, we synthesized BRD3067 and BRD3811 (Olson et al., 2014) as negative control compounds structurally related to tubastatin A and PCI-34051, respectively. These

compounds contain a single methyl group ortho to the hydroxamic acid moiety that precludes entry into the enzyme's active site, but does not interfere with the metal-chelating ability of the hydroxamate. Both negative control compounds protected neurons from H_2O_2 (Figure 2A) despite their inability to inhibit HDACs (Figure 1B), thus demonstrating that structurally related hydroxamate-based HDAC inhibitors exert neuroprotective effects via an HDAC-independent mechanism. Additionally, we noted that BRD3493 and BRD9287, two analogs of tubastatin A and PCI-34051, respectively, did not exhibit any neuroprotective properties (Figure 2A). These compounds retained the phenyl or indole hydroxamic acid portion of their respective parent compound, but lacked the associated aromatic methylene substituent. We were intrigued as to why such small structural changes would have such dramatic effects on the ability of these compounds to protect neurons from oxidative stress, and we set out to link these structural characteristics to the differential functional responses elicited by these compounds.

Next, we found that LBH-589, tubastatin A, and PCI-34051 were able to protect rat cortical neurons from a H_2O_2 insult even in the presence of actinomycin D or cycloheximide (Figure 2D), confirming that the mechanism by which these small molecules protect cells from H_2O_2 is independent of transcription and translation, and thus, not dependent on gene expression changes resulting from HDAC inhibition. Furthermore, we noticed that the neuroprotective effects of PCI-34051 and BRD3811 could be attenuated by the addition of $ZnCl_2$ (unpublished results), suggesting an important role for metal chelation. This hypothesis was further strengthened by the observation that only compounds containing hydroxamic acids, and not other metal-binding groups such as *o*-aminoanilides or ketones, were protective against H_2O_2 (Figure 2A). Additionally, it should be noted that the neuroprotective properties of these compounds seem to be limited to oxidative stress as PCI-34051 was unable to protect neurons from other forms of stress including treatment with NMDA, glutamate, 6-OHDA, 3-NP, camptothecin, or staurosporine (unpublished results).

Not All Hydroxamate-based HDAC Inhibitors Are Strong Binders of Iron

As it is well established that Fenton chemistry can play a critical role in deleterious oxidative stress (Kohen and Nyska, 2002; Prousek, 2007; Thomas et al., 2009), and these compounds are neuroprotective at the approximate concentration of free chelatable cellular iron (Fiedler et al., 2007), we reasoned that the metal chelating property of these hydroxamic acids is crucially important for protecting neurons from a H_2O_2 insult. To test this hypothesis, we measured the ability of each of these compounds to bind iron using an *in vitro* calcein assay (Figure 3) and found that several of them could bind metals in cells (Figure S2A) (Cabantchik et al., 1996). Briefly, upon binding to iron, the fluorescence of calcein is partially quenched. Compounds that are strong chelators of iron can effectively compete with calcein for the available iron, ultimately decreasing the amount of iron-bound calcein and increasing calcein fluorescence. Somewhat surprisingly, we found that not all hydroxamic acid-containing compounds were able to effectively bind iron (Figure 3). For instance, BRD3493 and BRD9287, the analogs of tubastatin A and PCI-34051, respectively, that did not exhibit any neuroprotective properties, also did not effectively compete with calcein for free chelatable iron. Not surprisingly, the compounds that were the most effective

iron binders were also the best at protecting cortical neurons from an Fe/8-hydroxyquinoline complex, a membrane permeable iron insult (Jonas and Riley, 1991) (Figure S2B). This effect could be due to either the sequestration of redox active iron within the cell, or the rapid stripping of iron from 8-hydroxyquinoline to form cell-impermeable complexes. Regardless, these studies confirm the results of our *in vitro* iron-binding assays.

The Ability to Bind Iron is Not Sufficient to Protect Neurons From H_2O_2

While all compounds protective in the H_2O_2 neuroprotection assay were effective chelators of iron, the non-selective inhibitor scriptaid and its structurally related negative control nullscript were able to bind iron and protect cells from an iron insult (Figure 3 and Figure S2B), but were unable to protect neurons from H_2O_2 (Figure 2). Therefore, we deemed iron chelation necessary, but not sufficient to protect neurons from exogenous H_2O_2. It is possible that a reduction in Fenton chemistry plays a small role in the neuroprotective effects of these compounds; however, based on the scriptaid and nullscript results, we hypothesized that the complexes formed upon iron chelation might be exerting a more profound effect than simply the act of sequestering iron itself.

HDAC Inhibitors Display Catalase-like Activity in the Presence of Iron

Inspired by previous reports (Baker et al., 1998; Rauen et al., 2004; Sustmann et al., 2007), we hypothesized that some of these hydoxamic acid-containing compounds might form catalase mimetic complexes upon binding intracellular iron, thus facilitating the degradation of H_2O_2 and making them particularly well-suited to protect neurons from a H_2O_2 insult. To test this idea, we performed an *in vitro* assay (Figure 4A) that uses chemiluminescent detection of H_2O_2 (Figure S3A) to measure changes in H_2O_2 concentration and compared these hydroxamate-iron complexes to the known catalase mimetic EUK 134 (Baker et al., 1998). Iron sulfate ($FeSO_4$) itself causes a slight reduction in H_2O_2 levels after 2h. However, when $FeSO_4$ is combined with certain hydroxamic acid-containing HDAC inhibitors or analogs, these compounds serve as ligands for the iron and form complexes leading to the rapid decomposition of H_2O_2 (Figure 4C). Although the most potent complexes caused the final concentration of H_2O_2 to fall below the linear range of the assay (Figure S3B), we were able to estimate that 0.00005 equivalents of one of these iron complexes (e.g., iron complexes utilizing LBH-589 or PCI-34051 as ligands) relative to H_2O_2 is able to reduce the levels of oxidant by six orders of magnitude (from >1 M to ~1 μM) in only 2h. Importantly, in the absence of additional $FeSO_4$, these compounds do not cause the decomposition of H_2O_2 (Figure S3C), thus indicating that it is the complexes, and not the ligands themselves that are responsible for performing the catalysis.

It is important to note that not all hydroxamic acids are capable of serving as a precursor to a catalase-mimetic complex. Some do not bind iron strongly (e.g., BRD9287, BRD3493, and BRD9757) while others are strong iron binders yet the complexes they form are unable to perform the catalysis (e.g., scriptaid and nullscript). While the exact structures of these hydroxamate-based catalase mimetic complexes have not yet been elucidated, all competent ligands share similar structural features. These ligands are invariably either an aryl or cinnamyl hydroxamic acid with a pendant substituent. For reasons that are presently unclear,

alkyl and alkenyl hydroxamic acids in the presence of iron do not display catalase-like activity.

The reaction of several of these catalase-mimetic complexes, such as the one formed from PCI-34051 and iron, with H_2O_2 is so vigorous that the product of that reaction (i.e., molecular oxygen) is immediately visualized as obvious effervescence (Figure 4B) (Fung and Petrishko, 1973). This is in stark contrast to complexes formed by compounds like scriptaid, which do not catalyze the decomposition of H_2O_2 (Figure 4C), and thus do not cause effervescence (Figure 4B). Importantly, the results of our *in vitro* H_2O_2 decomposition assay are in accord with the results of our H_2O_2 neuroprotection assay, that is to say that the compounds that form complexes capable of catalyzing the decomposition of H_2O_2 *in vitro* are also those that protect cortical neurons from a H_2O_2 insult. Although the iron complex of BRD9287 did show some catalase-like activity *in vitro* (Figure 4C), it did not *in cells* (Figure 2A). This is likely due to *in vitro* conditions driving the formation of the catalase mimetic complex. *In cells*, the low concentration of free chelatable iron coupled with the low affinity of BRD9287 for iron (Figure 3) is likely to preclude the formation of the active catalyst, thus explaining the inability of BRD9287 to protect neurons from a H_2O_2 insult (Figure 2A). Therefore, we propose that hydroxamate-containing molecules must 1) display a propensity for binding cellular iron, and 2) form iron-complexes capable of catalyzing the disproportionation of H_2O_2 in order for these compounds to exhibit sufficient antioxidant properties to protect cells from exogenous H_2O_2.

When compared to the state of the art commercially available catalase mimetic EUK 134 (Baker et al., 1998), a Mn-complex whose antioxidant properties have been shown to drastically extend the life of *C. elegans* (Melov et al., 2000), the hydroxamate-iron complexes described here display similar catalase-like activity. In fact, they are approximately 10 times more potent than EUK 134, as 1 mM EUK 134 was necessary to produce a decrease in H_2O_2 concentration comparable to the decrease produced by 0.1 mM of the hydroxamate-iron complexes (Figure 4C). Furthermore the *in situ* formation of catalase mimetic complexes from hydroxamates confers two obvious advantages over simply utilizing a Mn complex like EUK 134, 1) it obviates the need to introduce toxic metals into the cells, and 2) it has the added benefit of sequestering any excess redox-active iron that may be engaging in deleterious Fenton chemistry.

Next, we assayed the effects of hydroxamate-based HDAC inhibitors and associated analogs on cellular levels of reactive oxygen species (ROS) using CM-H_2DCFDA, a fluorescein derivative whose fluorescence intensity is increased in the presence of ROS (Figure S4). Initially, when rat cortical neurons are treated with H_2O_2, levels of cellular ROS appear to decrease (Figure S4). This somewhat counterintuitive result can be rationalized by a rapid H_2O_2-induced increase in antioxidant defense mechanisms. Compounds that form catalase mimetic complexes *in vitro* and protect neurons from a H_2O_2 insult either delay or completely prevent this initial decrease in fluorescence, presumably by sufficiently lowering intracellular H_2O_2 levels (Figure S4). In time, the antioxidant defense mechanisms of cells not treated with catalase mimetics are overwhelmed and the cells ultimately succumb to the swelling and rupture characteristic of H_2O_2-induced necrosis. Finally, we treated cells for 24h (in the absence of H_2O_2) with several non-selective HDAC inhibitors to assess changes

in endogenous levels of ROS. While LBH-589 was able to lower endogenous ROS levels, the two compounds of this series that did not form catalase mimetic complexes *in vitro* (i.e., scriptaid and SAHA) could not (Figures 4D and 4E), thus confirming that iron sequestration alone is not sufficient to reduce endogenous ROS levels.

Significance

Our data suggest that hydroxamate-based HDAC inhibitors can exert neuroprotective effects via an HDAC-dependent mechanism, an HDAC-independent mechanism, or a combination of both (Figure 4G). At low nM concentrations near their HDAC IC_{50}s, non-selective inhibitors, such as scriptaid, can protect neurons from GSH depletion via what appears to be an HDAC-dependent mechanism, a conclusion that is supported by the fact that no such protection is observed when the structurally related negative control compound nullscript is employed (Figure S1). Other HDAC inhibitors, such as PCI-34051, protect neurons from oxidative stress at concentrations much greater than their HDAC IC_{50}s, and these compounds engage in an HDAC-independent mechanism outlined in Figure 4F. Upon entering the cell, these hydroxamic acids bind to free cellular iron, potentially mitigating deleterious Fenton chemistry, but more importantly, forming complexes capable of catalyzing the disproportionation of H_2O_2 into water and O_2. Hydroxamate-containing compounds that exhibit catalase-like activity but not HDAC inhibitor activity, such as BRD3811, may prove to be useful in the treatment of diseases such as stroke as these compounds have powerful antioxidant properties, and presumably, will not suffer from the HDAC-related toxicities that have hampered the development of HDAC inhibitors as therapeutics. Of course, exploiting such catalase-like antioxidant effects for therapeutic purposes would necessitate achieving a sufficiently high concentration of hydroxamate *in vivo*. The discovery that hydroxamate-based HDAC inhibitors can exert neuroprotective effects by forming catalase mimetic complexes in cells lays the foundation for future studies regarding the HDAC-independent effects of these important compounds on a variety of disease states involving oxidative stress including immunological disorders and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Several, but not all, HDAC inhibitors display HDAC-independent antioxidant properties
- Not all hydroxamates bind iron equally well
- Several HDAC inhibitor-iron complexes catalyze the disproportionation of H_2O_2 *in vitro*
- *In vitro* catalase-like activity correlates with capacity to protect neurons from H_2O_2

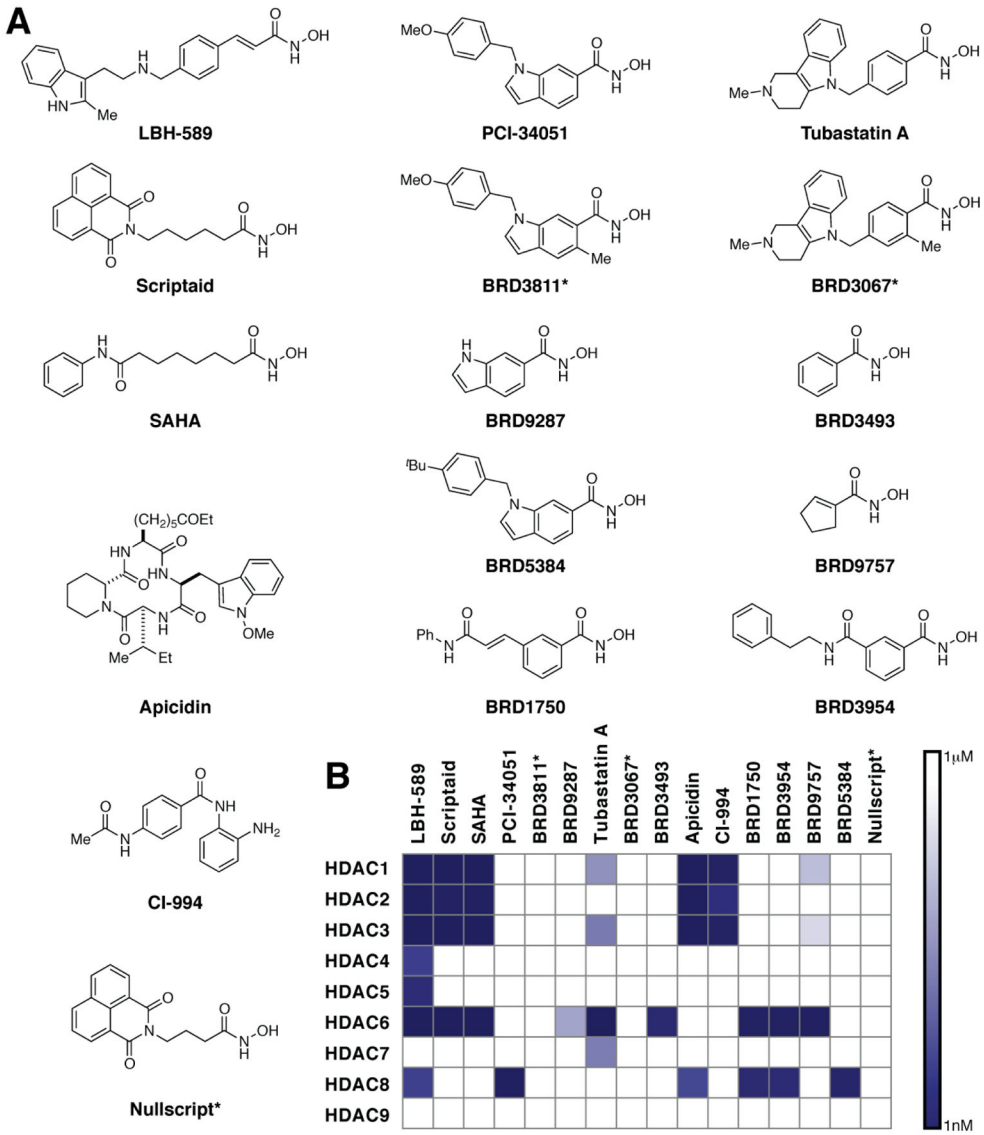

Figure 1. Chemical tools for studying the role of HDACs in neuroprotection

Chemical structures (A) and HDAC potencies (IC_{50}s) (B) of chemical tools used in this study. Absolute potency values can be found in Table S1. *Denotes a negative control compound based on the parent structure. See also Table S1.

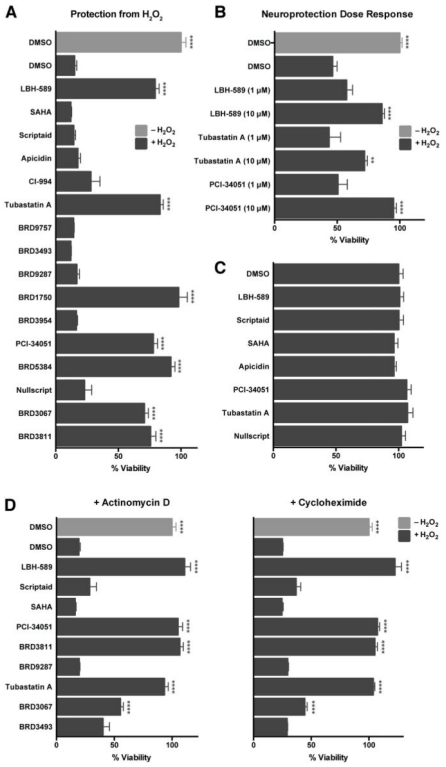

Figure 2. Hydroxamate-based HDAC inhibitors protect murine cortical neurons from oxidative stress through an HDAC-independent mechanism

(A) Rat E18 cortical neurons were simultaneously treated with 150 μM H_2O_2 and compounds (30 μM). Cell death was assayed 24h later via an MTT assay. Several negative control compounds based on the structures of known HDAC inhibitors exhibited robust protection of cortical neurons from H_2O_2-induced death, despite lacking HDAC inhibitory activity. (B) Protection from H_2O_2 occurs in a dose-dependent manner. (C) Compounds do not induce cell death at the highest concentration (30 μM) and longest time frame (24h) employed in these assays. (D) Protection from H_2O_2 occurs independent of transcription or translation. Assays were performed in the presence of either 2 μg/mL actinomycin D or 10 μg/mL cycloheximide. Data are shown as mean ± SEM. Significance was established relative to DMSO + H_2O_2. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$ from Tukey post hoc test following a one-way analysis of variance (ANOVA). See also Figure S1.

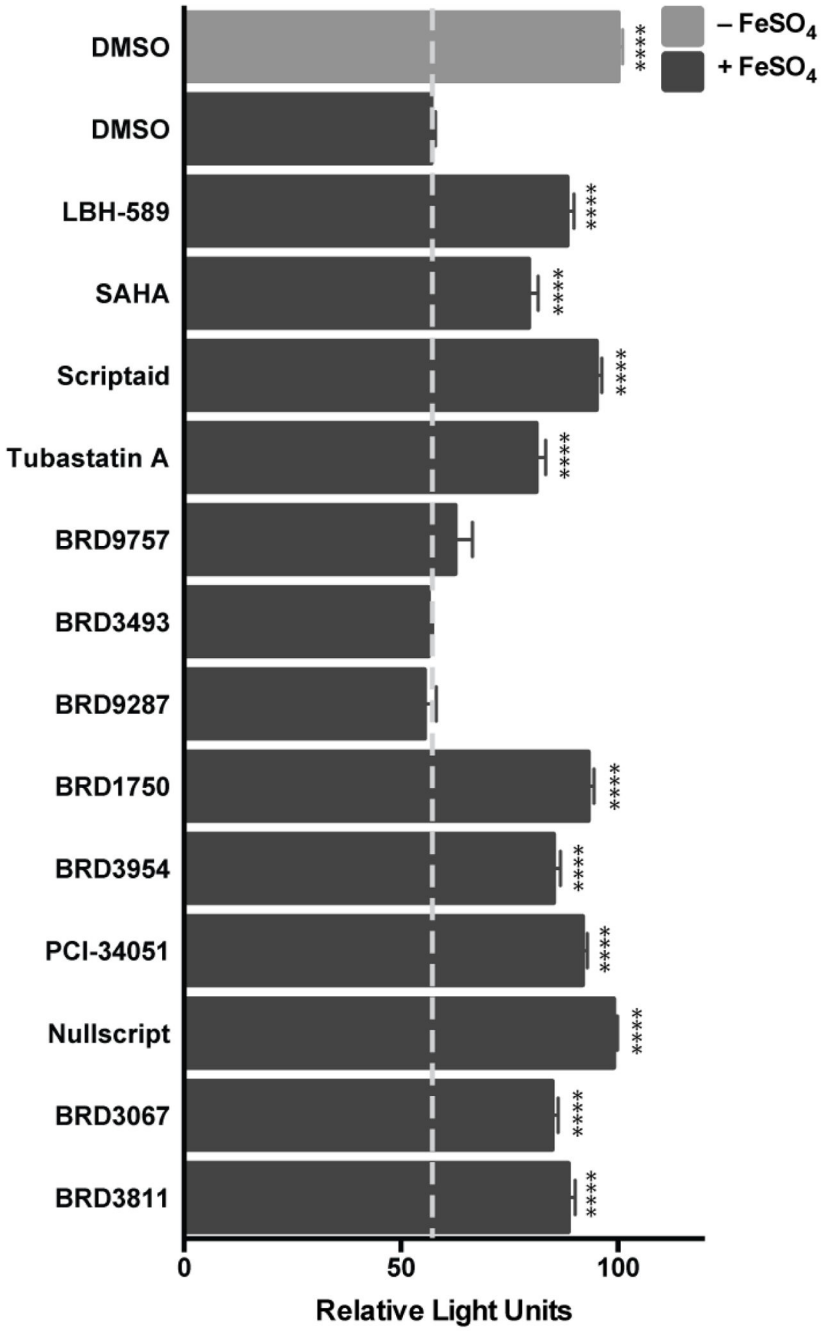

Figure 3. HDAC inhibitors display differential abilities to bind iron

In vitro calcein fluorescence was partially quenched upon the addition of $FeSO_4$. Several, but not all, compounds were able to restore calcein fluorescence through competitive iron binding. Data are shown as mean ± SEM. Significance was established relative to DMSO + $FeSO_4$. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$ from Tukey post hoc test following a one-way analysis of variance (ANOVA). See also Figure S2.

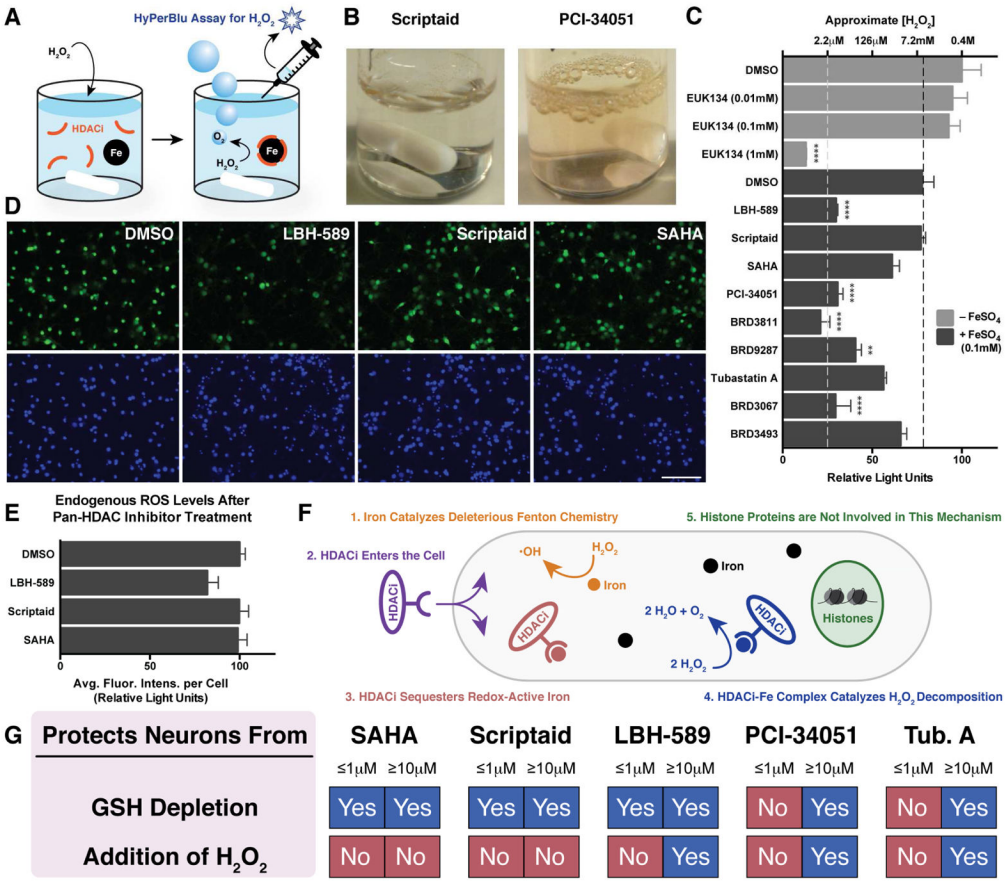

Figure 4. Hydroxamate-based HDAC inhibitor-iron complexes catalyze the decomposition of H_2O_2 and mitigate intracellular ROS levels

(A) Schematic depicting an *in vitro* H_2O_2 decomposition assay. (B) Reactions employing hydroxamate-based HDACi-iron complexes capable of catalyzing the disproportionation of H_2O_2 into water and gaseous O_2 produce effervescence. (C) Some hydroxamate-based HDAC inhibitors (HDACi), in combination with $FeSO_4$, are capable of drastically reducing *in vitro* levels of H_2O_2 while others cannot. The black dotted line indicates the decomposition of H_2O_2 resulting from DMSO + $FeSO_4$ only. Concentrations below the white dotted line fall outside the linear range of this assay. Data are shown as mean ± SEM. Significance was established relative to DMSO + $FeSO_4$. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$ from Tukey post hoc test following a one-way analysis of variance (ANOVA). (D) Rat cortical neurons were treated with HDAC inhibitors at 30 μM for 24h before endogenous levels of intracellular ROS were determined using the ROS-sensitive fluorescein derivative CM-H_2DCFDA. The scale bar denotes 100 μm. (E) Quantification of endogenous ROS levels shows that the catalase mimetic properties of LBH-589 can reduce endogenous ROS levels. Data are shown as mean ± SEM. (F) Schematic depiction of a model for an HDAC-independent neuroprotection mechanism (G) Summary of neuroprotective effects at either low (≤1μM) or high (≥10μM) concentrations. See also Figures S3 and S4.