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A Selective Phenelzine Analogue Inhibitor of Histone Demethylase LSD1

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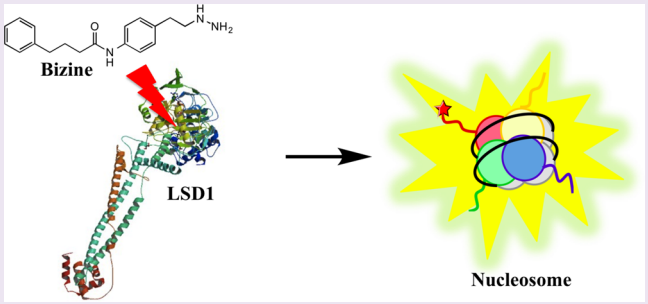
Supporting Information

ABSTRACT: Lysine-specific demethylase 1 (LSD1) is an epigenetic enzyme that oxidatively cleaves methyl groups from monomethyl and dimethyl Lys4 of histone H3 (H3K4Me1, H3K4Me2) and can contribute to gene silencing. This study describes the design and synthesis of analogues of a monoamine oxidase antidepressant, phenelzine, and their LSD1 inhibitory properties. A novel phenelzine analogue (bizine) containing a phenyl-butyrylamide appendage was shown to be a potent LSD1 inhibitor *in vitro* and was selective versus monoamine oxidases A/B and the LSD1 homologue, LSD2. Bizine was found to be effective at modulating bulk histone methylation in cancer cells, and ChIP-seq experiments revealed a statistically significant overlap in the H3K4 methylation pattern of genes affected by bizine and those altered in LSD1−/− cells. Treatment of two cancer cell lines, LNCaP and H460, with bizine conferred a reduction in proliferation rate, and bizine showed additive to synergistic effects on cell growth when used in combination with two out of five HDAC inhibitors tested. Moreover, neurons exposed to oxidative stress were protected by the presence of bizine, suggesting potential applications in neurodegenerative disease.



Reversible histone lysine methylation is a major mechanism for regulating chromatin dynamics and gene expression. Lysine-specific demethylase 1 (LSD1), the first histone demethylase identified, is responsible for oxidatively cleaving one or two methyl groups from Lys4 of histone H3 (H3K4).[1−7] In this way, LSD1 is thought to play a role in gene silencing, since methylation of H3K4 in promoter regions is a well-established chromatin mark linked to transcriptional activation.[8,9] Since its discovery, LSD1 histone demethylase activity has been investigated as a pharmacologic target for cancer and other diseases. It has been found that LSD1 levels are often elevated in various cancers, including prostate, non-small cell lung, and ER-negative breast cancer.[10−12] Moreover, a variety of tumor suppressors that have been shown to be silenced in cancer by epigenetic mechanisms could theoretically be reactivated by LSD1 blockers,[13−16] as has been achieved with histone deacetylase and DNA methyltransferase inhibitors.[17]

LSD1 is a 90 kDa flavin-bound enzyme that belongs to the amine oxidase protein superfamily, which uses molecular oxygen as a cosubstrate and generates hydrogen peroxide and formaldehyde as byproducts (Figure 1A).[1,7,18,19] Based on its enzymatic mechanism, LSD1 cannot demethylate trimethylated H3 Lys4 (H3K4Me3), but members of the iron-dependent Jmj histone demethylases are known to serve this function.[1,20] In addition to the C-terminal amine oxidase catalytic domain, LSD1 also contains an N-terminal SWIRM domain and a 105

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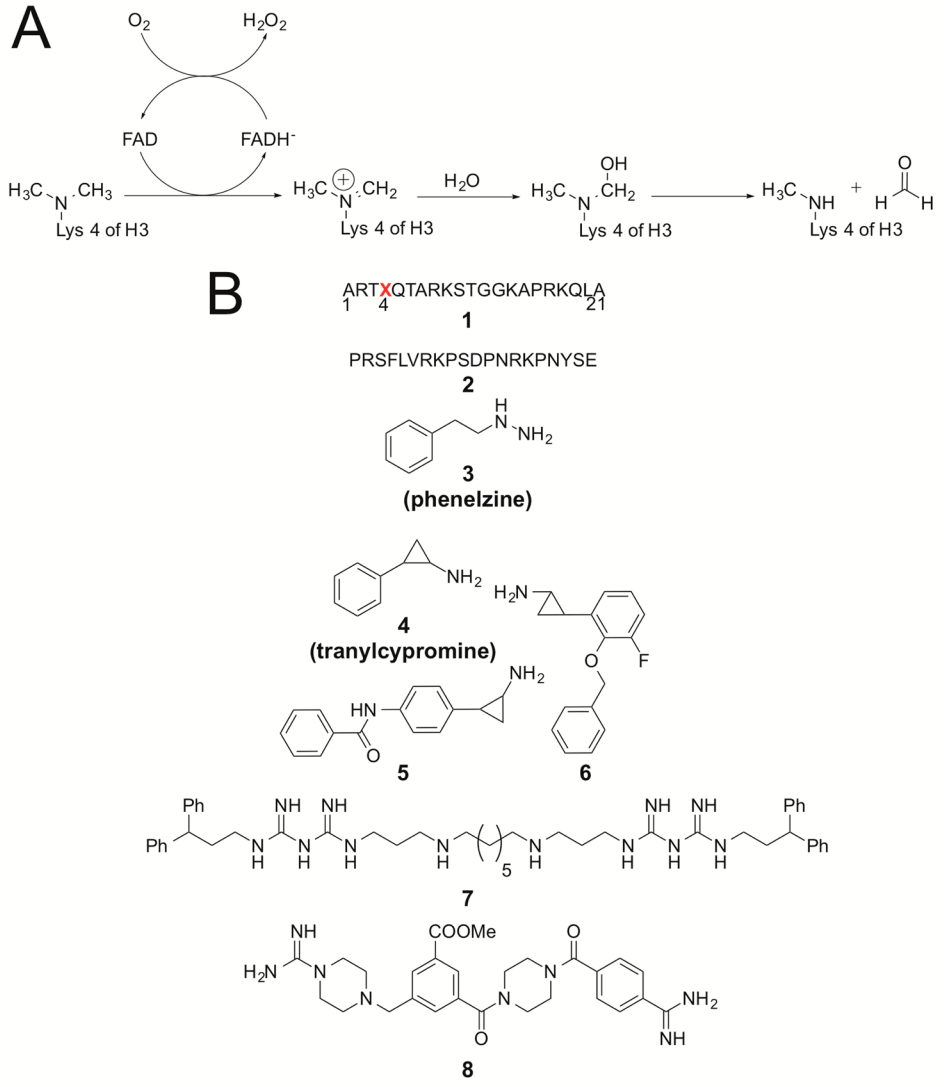

Figure 1. (A) LSD1 demethylation mechanism. (B) LSD1 inhibitor structures published previously: (**1**) Histone H3-21mer peptides with various modified lysine residues, **X**; (**2**) N-terminal SNAIL1 20-mer peptide; (**3**) phenelzine; (**4**) tranylcypromine; (**5**, **6**) tranylcypromine analogues; (**7**) polyamine analogue; (**8**) guanidinium-containing compound.

aa Tower domain insert, which is located in the amine oxidase domain that can bind CoREST. In cells, LSD1 is often found in CoREST complexes that include HDAC1/2.[4,21−25] The LSD1 homologue, LSD2, also catalyzes demethylation of H3K4Me1 and H3K4Me2 but lacks the CoREST binding Tower domain insert and exhibits significant sequence and local structure differences compared to LSD1.[26,27] Mechanistically and structurally, LSD1 is also related to the flavin-dependent monoamine oxidases (MAO A/B), as well as polyamine oxidase enzymes.[15,25,28]

Several prior LSD1 demethylase inhibitors have been reported including peptides (**1**, **2**), MAOIs and derivatives thereof (**3**−**6**), polyamines (**7**), and guanidine containing compounds (**8**) (Figure 1B).[2,29−40] One strategy that has shown promise has been the development of tranylcypromine analogues.[37,38] Tranylcypromine is a classical MAO inhibitor and mechanism-based inactivator involving an oxidative cyclopropylamine ring-opening reaction, used for the treatment of clinical depression, and is weakly potent as an LSD1 mechanism-based inactivator ($K_{i(inact)}$ = 0.5 mM, k_{inact} = 0.67 min^{-1}).[41−43] However, it has been shown that tranylcypromine can be modified by aryl attachment to produce more selective LSD1 inhibitors with enhanced potency.[32,33,44−48] Nevertheless, no LSD1 inhibitor has yet been evaluated in clinical trials.

Prior work from our group showed that the antidepressant MAO inhibitor phenelzine is considerably more potent than tranylcypromine as an LSD1 inhibitor.[29] Like tranylcypromine, phenelzine is an LSD1 mechanism-based inactivator, but in this case the key initiating step involves hydrazine rather than cyclopropylamine oxidation. In this manuscript, we generated a series of phenelzine analogues informed by the general concept derived from tranylcypromine studies that linkage of phenelzine to an aromatic functionality might improve its LSD1 potency.[32,33] Below we disclose structure−activity relationships with various phenelzine analogues, ultimately developing a compound with considerable potency and selectivity for LSD1 versus MAO and LSD2 enzymes. This phenelzine analogue bizine was further examined in cell-based assays assessing bulk as well as gene-specific histone methylation changes, antiproliferative activity in several cancer cell lines, and neuroprotection in response to oxidative stress. Below we describe

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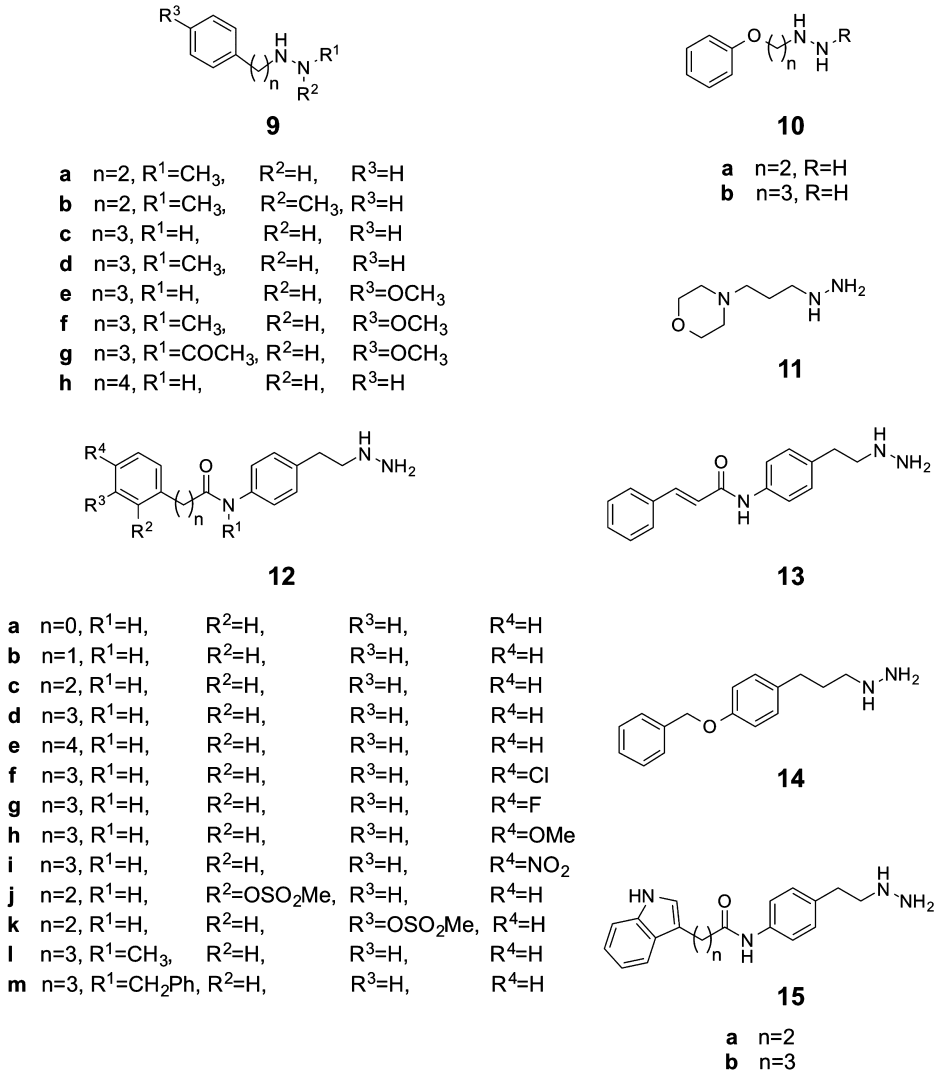

Figure 2. Phenelzine analogues tested as LSD1 inhibitors.

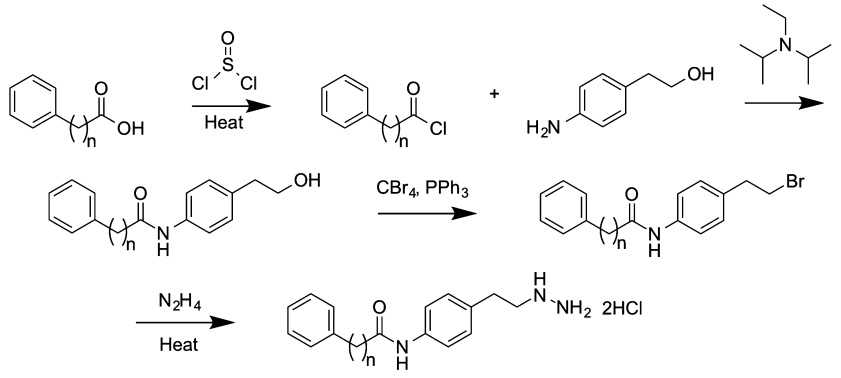

Figure 3. General synthesis of novel phenelzine analogues.

these findings and place their significance in the context of prior LSD1 studies.

RESULTS AND DISCUSSION

Phenelzine Analogue LSD1 Inhibitors. On the basis of prior findings that phenelzine was a moderately potent, mechanism-based inactivator of LSD1, we synthesized a series of phenelzine analogues exploring hydrazine modifications, variations in alkyl chain length and rigidity, phenyl replacement, and phenyl ring substitution (compounds **9**–**15**, Figure 2).[29] Synthetic routes generally involved late stage hydrazine introduction by converting a terminal alkyl hydroxy group to either the corresponding bromide or mesylate followed by hydrazine displacement reactions as exemplified in Figure 3 (additional detailed routes are shown in Supplementary Figures 1–4). Compounds were assayed for their ability to inhibit

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Table 1. Kinetics of Phenelzine Analogue LSD1 Inhibitors

inhibitor	$K_{i(inact)}$ (μM)	k_{inact} (min^{-1})	$k_{inact}/K_{i(inact)}$ (μM^{-1} min^{-1})	IC_{50} (μM)
phenelzine	5.6 ± 1.3	0.35 ± 0.056	0.063 ± 0.018	N/A
9a	N/A	N/A	N/A	85.00
9b	N/A	N/A	N/A	>100.0
9c	5.0 ± 1.1	0.32 ± 0.010	0.064 ± 0.014	N/A
9d	N/A	N/A	N/A	46.74
9e	8.0 ± 3.5	0.15 ± 0.023	0.019 ± 0.0087	N/A
9f	N/A	N/A	N/A	>100.0
9g	N/A	N/A	N/A	N/A
9h	22 ± 3.0	0.12 ± 0.01	0.0055 ± 0.00087	N/A
10a	44 ± 9.7	0.15 ± 0.010	0.0034 ± 0.00079	N/A
10b	12 ± 2.1	0.22 ± 0.020	0.018 ± 0.0036	N/A
11	N/A	N/A	N/A	>100.0
12a	0.28 ± 0.11	0.19 ± 0.036	0.70 ± 0.31	N/A
12b	0.37 ± 0.033	0.20 ± 0.0087	0.54 ± 0.054	N/A
12c	0.26 ± 0.058	0.24 ± 0.022	0.92 ± 0.22	N/A
12d	0.059 ± 0.021	0.15 ± 0.017	2.5 ± 0.96	N/A
12e	0.26 ± 0.11	0.22 ± 0.038	0.86 ± 0.39	N/A
12f	0.156 ± 0.047	0.17 ± 0.018	1.1 ± 0.35	N/A
12g	0.138 ± 0.0.48	0.17 ± 0.020	1.2 ± 0.44	N/A
12h	0.207 ± 0.089	0.26 ± 0.042	1.2 ± 0.57	N/A
12i	0.282 ± 0.076	0.21 ± 0.024	0.74 ± 0.22	N/A
12j	0.204 ± 0.098	0.18 ± 0.034	0.88 ± 0.46	N/A
12k	0.223 ± 0.064	0.17 ± 0.020	0.76 ± 0.24	N/A
12l	2.0 ± 0.73	0.24 ± 0.033	0.12 ± 0.045	N/A
12m	1.6 ± 0.49	0.22 ± 0.025	0.14 ± 0.044	N/A
13	0.10 ± 0.039	0.17 ± 0.21	1.7 ± 0.68	N/A
14	0.90 ± 0.45	0.18 ± 0.038	0.20 ± 0.11	N/A
15a	0.21 ± 0.076	0.21 ± 0.030	1.0 ± 0.41	N/A
15b	0.10 ± 0.035	0.17 ± 0.019	1.7 ± 0.60	N/A

recombinantly purified GST-LSD1 using a dimethyl-Lys4 histone H3-21mer peptide substrate by monitoring peroxide formation via a colorimetric peroxidase assay.[49] These results (Table 1) showed that adjusting the alkyl chain length (**9c**, **9h**, **10a,b**) could lead to modest increases or decreases in LSD1 inhibitory potency compared with that of phenelzine ($K_{i(inact)}$ = 5.6 μM; k_{inact} = 0.35 min^{-1}) (Supplementary Figure 5), whereas methyl or acetyl substitutions on the hydrazine (**9a,b,d,f,g**) negated LSD1 inhibitory action. Additionally, morpholine replacement of the phenyl ring (**11**) was not compatible with LSD1 inhibition. Furthermore, incorporation of a methoxy substituent at the 4-position of the phenyl ring of phenelzine made little difference (**9e**). Important LSD1 inhibitory potency enhancements were achieved by linking aryl groups through various tethers to the phenelzine core (**12**–**15**). This trend was loosely related to the previously reported results with tranylcypromine analogue **5**.[32]

Compounds **12a**–**e** showed that amino-phenelzine fused to phenyl-alkanoic acids via an amide spacer were improved LSD1 inhibitors compared to phenelzine itself. Of this set, compound **12d** containing the propanyl spacer was the most potent LSD1 inhibitor with a $K_{i(inact)}$ of 59 nM and a k_{inact} of 0.15 min^{-1} (Figure 4A,B). This $K_{i(inact)}$ for **12d** compares favorably to one of the most potent tranylcypromine analogues ($K_{i(inact)}$ = 0.61 μM) reported in the literature.[33] Alternatives to the alkanoic spacers in **12** including an alkenoic acid spacer (**13**) and an alkyl ether spacer (**14**) led to reduced LSD1 inhibitory potency. However, replacing the ethanyl tether with a *trans*-ethenyl group resulted in improved inhibitor potency as can be seen by comparing **12c** with **13**. Terminal aryl substitutions in the

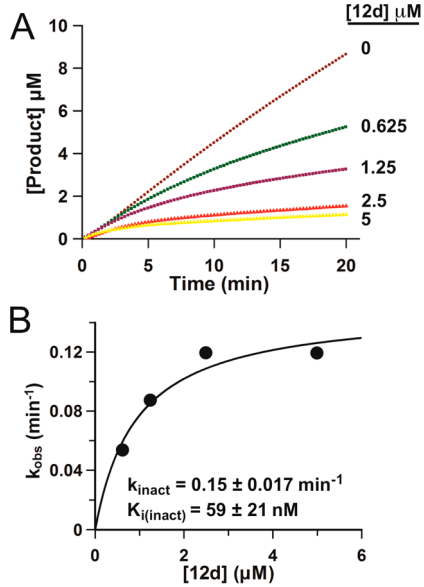

Figure 4. Inhibition of LSD1 by compound **12d** (bizine). (A) Steady-state progress curve of LSD1 inactivation by compound **12d** (bizine) ranging from 0 to 5 μM. (B) k_{obs} values obtained from steady-state data plotted against inhibitor concentration to determine k_{inact} and $K_{i(inact)}$ values.

context of the ethanyl and the propanyl spacers represented in **12f**–**k** generally had similar LSD1 potency as that of **12d**, suggesting that substitutions at this position are well tolerated. Of note, *N*-substitution of the amide linker attachment present

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in **12l,m** greatly attenuated LSD1 inhibition relative to **12d**, potentially highlighting the importance of the amide NH group in hydrogen bonding to the LSD1 active site. Interestingly, replacement of the terminal phenyl group in **12c,d** with an indole group to generate **15a,b** largely preserved LSD1 inhibitory potency.

To confirm the LSD1 inhibition peroxidase assay results obtained with **12d**, we turned to a recently developed isotope-based mass spectrometric assay, MassSQUIRM, to directly and quantitatively assess **12d** effects on Lys4-methylation.[50] This assay is conducted for an extended time period utilizing a high LSD1 concentration, with conditions where LSD1-catalyzed demethylation of the H3-21-K4Me2 substrate nears completion, resulting in extensive conversion of the substrate to mono- and unmethylated H3-21. As reported previously, greater than 10 mM phenelzine is needed to extinguish LSD1 activity under MassSQUIRM conditions.[50] Thus, we compared 50 μM each of phenelzine and analogue **12d** in an identical LSD1 inhibition MassSQUIRM assay. Results showed that 50 μM phenelzine had a negligible impact on LSD1 inhibition, whereas the same concentration of **12d** led to very substantial LSD1 inhibition, with the unreacted dimethyl-peptide remaining as the major species at the conclusion of the experiment (Supplementary Figure 6). These experiments corroborate the findings with the spectrophotometric peroxidase assay that showed that **12d** was a far more potent LSD1 inhibitor than phenelzine.

To assess the relative selectivity of our potent LSD1 phenelzine analogue **12d**, we carried out counter screen enzyme assays versus MAO A, MAO B, and LSD2. As shown in Supplementary Table 1, based on $k_{inact}/K_{i(inact)}$ measure of inactivation efficiency, **12d** is 23-fold selective for inhibiting LSD1 versus MAO A, 63-fold selective versus MAO B, and >100-fold versus LSD2. In contrast, phenelzine preferentially inhibits MAO A and is equipotent in blocking MAO B compared with LSD1. These results support the potential utility of **12d** as a selective pharmacologic probe for cellular LSD1 histone demethylase activity.

Compound 12d Effects on Cellular H3K4 Methylation. The ability of compound **12d**, that we hereafter call bizine, to induce bulk histone H3-Lys4 methylation was assessed using Western blots in the prostate cancer LNCaP cell line, which has been used successfully in previous LSD1 inhibitor studies,[51,52] with histone H3 methylation-state-specific antibodies. As can be seen, after 48 h treatment with bizine, there was a dose-dependent increase in H3K4Me2 signal (Figure 5A,B). The EC_{50} of this bizine effect was ~2 μM. There were no significant reproducible changes in H3K4Me1, H3K4Me3, unmethylated H3K4, or other histone H3 marks examined including H3K9Me2, H3K9Ac, and H3K36Me3 (Figure 5A). Furthermore, there was no discernible effect of bizine on LSD1 protein levels (Figure 5C). The increase in cellular global H3K4Me2 levels after treatment with bizine is a primary effect that is consistent with prior studies with less selective LSD1 inhibitors and genetic LSD1 alterations.[14,32,37] However, in our hands with LNCaP cells, the MAO inhibitor phenelzine, which is an ~100-fold weaker LSD1 inhibitor than bizine, did not induce H3K4Me2 changes at concentrations up to 40 μM (Figure 5D). Additionally, the ~30-fold weaker *N*-methyl bizine analogue **12l** did not show changes in H3K4Me2 at concentrations up to 10 μM (Supplementary Figure 7A,B). Taken together, these results are consistent with the hypothesis that the Western blot effects related to bizine are mediated through LSD1 inhibition.

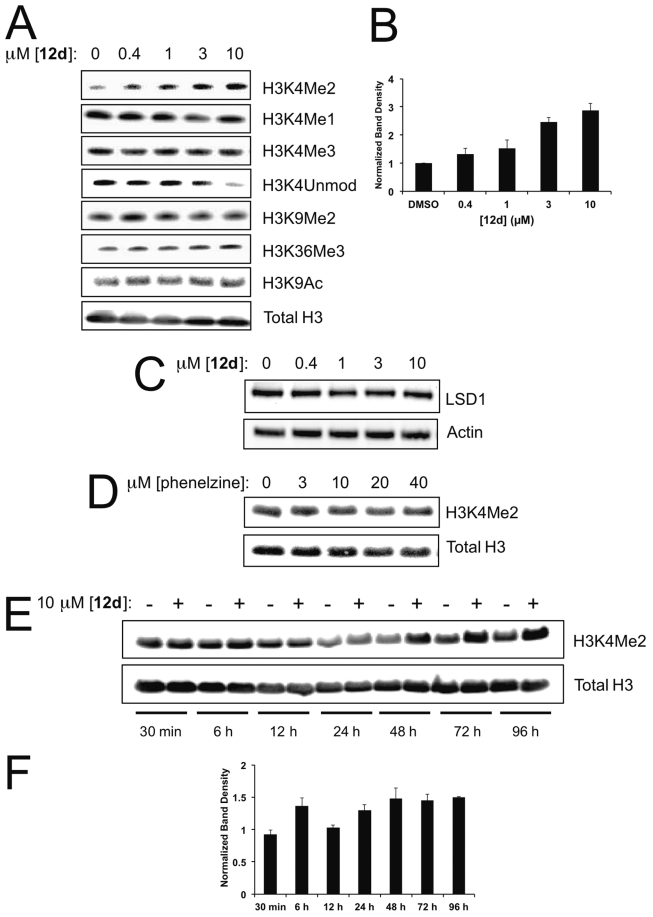

Figure 5. LSD1 inhibition by compound **12d** (bizine) in LNCaP cells. (A) Cells were treated with compound **12d** (bizine) (0.4−10 μM) for 48 h and blotted against indicated proteins. (B) H3K4Me2 band density quantification plot. Statistically significant increases were observed at 3 μM and 10 μM **12d** (bizine) treatment as determined by three biological replicates. (C) Cells were treated with compound **12d** (bizine) (0.4−10 μM) for 48 h and blotted against LSD1 and actin. (D) Cells were treated with phenelzine (3−40 μM) for 48 h and blotted against H3K4Me2 and total H3. (E) Cells were treated with 10 μM compound **12d** (bizine) and collected at various indicated time points and blotted against H3K4Me2 and total H3. (F) H3K4Me2 band density quantification plot normalized to vehicle at each indicated time point after 10 μM **12d** (bizine) treatment. Statistically significant increases were observed at 6, 24, 48, 72, and 96 h but not at 30 min and 12 h based on 3 biological replicates.

We further examined the effects of bizine on histone H3K4 methylation by assaying additional cancer cell lines (Supplementary Figure 8), H460, A549, and MDA-MB-231, each of which has been used in previous LSD1 inhibitor studies.[10,53] With the lung cancer line H460, there were comparable dose−response effects of bizine on H3K4Me2 levels. The lung cancer line A549 and the breast cancer line MDA-MB-231 also showed increases in H3K4Me2 in response to bizine, but a higher concentration (20 μM) was required for reproducible effects.

We also measured the kinetics of bizine's effect on H3 methylation in the LNCaP cell line. This time course experiment revealed that changes in H3K4Me2 could be detected within 6 h of compound exposure and effects can be observed up to 96 h (Figure 5E,F). However, there was a reproducible drop in H3K4Me2 at 12 h, which suggests a somewhat complex dynamic process involving competing

waves of lysine methyltransferase and demethylase action (Supplementary Figure 9). However, it seems that cellular turnover of H3K4-methylation can be a relatively rapid process, on a time scale that is commensurate with many dynamic protein acetylation and phosphorylation events.[54,55]

To examine the effect of LSD1 inhibition on chromatin H3 Lys-methylation with individual gene resolution, we carried out a ChIP-seq experiment in LNCaP cells treated for 48 h with bizine. Differential peaks between samples with two biological replicates were identified by diffReps.[56] In total, we obtained 17,542 differential H3K4Me2 peaks between cells treated with 10 μM bizine versus vehicle (Supplementary Table 2). Among those, 10,874 peaks were found to be upregulated (cut off p-value: $p < 0.0001$) with LSD1 inhibition. Out of those peaks, there were 2,432 genes identified that showed an increase in H3K4Me2 with LSD1 inhibition near the genes' promoter regions (Supplementary Table 3 and Supplementary Figure 10). Furthermore, gene ontology (GO) analysis of these 2,432 genes revealed many processes related to LSD1 function (Supplementary Table 4).

After culling the list to exclude microRNA and nonstandard gene names from the 2,432 gene list, we compared the remaining 1,767 genes to the 1,587 genes identified in a ChIP-seq experiment that used a LSD1−/− hematopoietic cell line, which also analyzed H3K4Me2 increases at gene promoters.[57] There were 146 genes (p-value = 0.0028) that overlapped in the chemical inhibition and LSD1 knockout experiments (Supplementary Table 5). This indicated the presence of a statistically significant overlap in genes affected despite the different LSD1 inhibition methods and cell lines used. GO analysis performed on the 146 genes showed that gene regulation was one of the top five statistically significant processes affected (Supplementary Table 6). Of note, many (26) (p-value = 5.80×10^{-9}) of the 146 overlapped genes (Supplementary Table 7) are established or proposed to be tumor suppressors, including CDH1 and CDKN2A, which have been validated to be affected by LSD1 inhibitors in prior studies.[13,58] This is consistent with the proposal that LSD1 inhibitors might have anticancer applications.

Bizine Antiproliferation Effects. We next pursued the effects of bizine on cell proliferation using a ^{3}H-thymidine incorporation assay as a measure of the rate of DNA synthesis. These studies revealed that bizine can slow the rate of cellular proliferation with an IC_{50} of 14 and 16 μM in treated H460 and LNCaP cancer cell lines, respectively (Figure 6A,B). These IC_{50}'s are considerably higher than the EC_{50}'s for Western blot changes in H3K4Me2. These findings raised concerns about the mechanistic basis of the cancer cell antiproliferative effects by bizine. To further address this issue, we tested the impact of phenelzine on H460 cell proliferation. There was less than a 50% reduction in ^{3}H-thymidine incorporation in H460 cells after 48 h with 80 μM phenelzine (Supplementary Figure 11), indicating that MAO inhibition by bizine does not primarily contribute to its cell growth inhibitory effects. These experiments suggest that LSD1 inhibition by bizine likely contributes to cancer cell growth inhibition and perhaps that nearly complete LSD1 inhibition at concentrations well above the bizine Western blot EC_{50} are necessary for reducing cell growth.

As LSD1 is an enzyme implicated in gene silencing, it is plausible that LSD1 inhibitors combined with histone deacetylase (HDAC) or DNA methyltransferase (DNMT) inhibitors might result in additive or synergistic effects. This

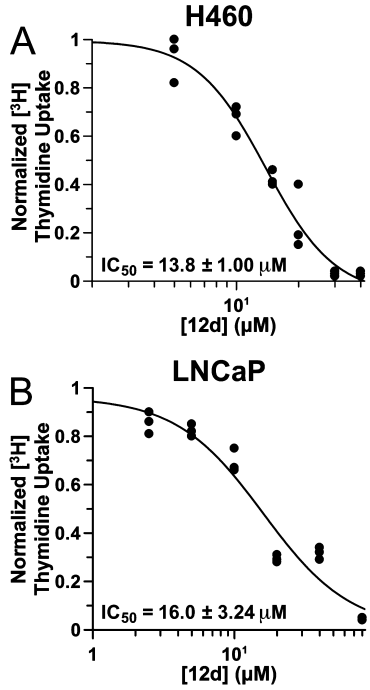

Figure 6. DNA replication dose response curves using a [^{3}H] thymidine assay in (A) H460 cells and (B) LNCaP cells after 48 h treatment with compound **12d** (bizine).

concept has been evaluated previously with LSD1 inhibitors that have low selectivity and potency but nevertheless show additivity and synergy with various HDAC and DNMT inhibitors.[53,59] Here we examine bizine in binary combinations with one DNMT inhibitor, azacytidine, as well as five HDAC inhibitors, SAHA, TSA, MGCD0103, MS-275, and LBH-589, using ^{3}H-thymidine incorporation in H460 cells after 48 h treatment. The combination index (CI) was calculated for each inhibitor pair.[60] Unexpectedly, four of the agents, azacytidine, SAHA, TSA, and MGCD0103, when combined with bizine, exhibited moderate antagonism, CI > 1, on inhibition of H460 cell proliferation at all ratios of the two agents examined (Supplementary Figure 12A−F). The basis of this antagonism is uncertain but may be related to various factors including changes in bizine uptake by cells or metabolism as well as complex pathway effects. MS-275 and LBH-589, in combination with bizine, showed additive to synergistic effects on inhibition of H460 cell proliferation, with the most synergy observed at the highest concentrations of compounds employed. These results reveal that in H460 cells, dual LSD1/HDAC inhibition may be promising, provided a suitable combination of inhibitors is identified that may reflect the precise specificities of the compounds involved.

LSD1 Inhibition and Neuroprotection. HDAC inhibition has previously been reported to protect against oxidative stress in neurons subjected to homocysteic acid (HCA) treatment, which induces glutathione depletion.[61,62] We thus explored whether bizine might confer neuroprotection against HCA-induced oxidative stress. Indeed, 0.5 μM bizine led to significantly enhanced survival of neurons after HCA treatment in a dose-dependent fashion (Figure 7A,B). This level of neuroprotection was comparable to the effect of 10 μM phenelzine, consistent with the greater potency of bizine versus phenelzine as an LSD1 inhibitor. These results suggest that LSD1 might serve as an attractive target to treat or protect

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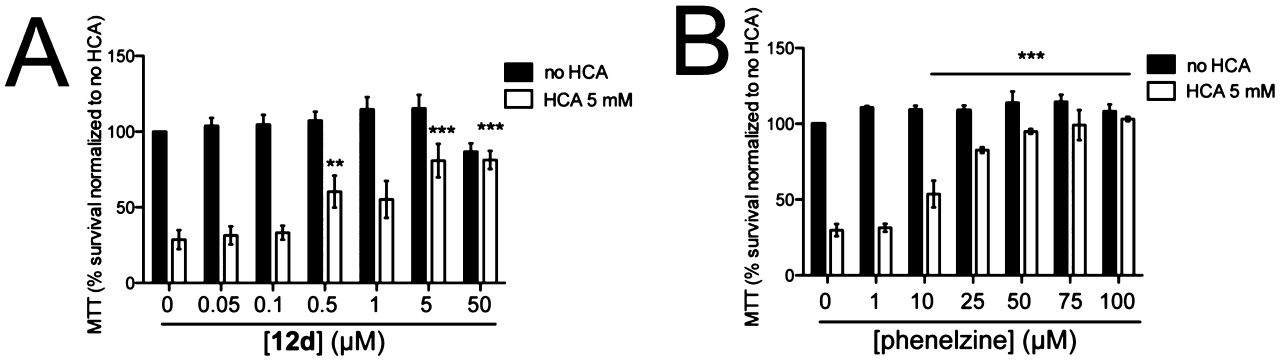

Figure 7. LSD1 inhibition protects neurons against oxidative stress-mediated cell death. (A) Compound **12d** (bizine) and (B) phenelzine halt neuronal cell death. (Two-way ANOVA, Bonferroni post hoc test; $^{**}p < 0.01$; $^{***}p < 0.0001$ compared to no HCA).

against neurologic disease, such as stroke, which can be placed in the context of prior work that investigated LSD1 functions in the brain.[48,63]

Conclusion. This study describes a potent and selective LSD1 inhibitor, bizine, derived from the MAO inhibitor phenelzine. Structure–activity relationships demonstrate the key roles of the hydrazine functionality, the secondary amide linker, and the second aryl group in achieving potent LSD1 inhibition. Bizine shows potent action in cancer cells as demonstrated by modulating histone H3K4 methylation and exhibiting moderate antiproliferative properties. Interestingly, some HDAC inhibitors show additive to synergistic effects in combination with bizine in reducing H460 cell growth, whereas other HDAC inhibitors and azacytidine did not. A potentially promising direction is the application of LSD1 inhibition in neuroprotection against oxidative stress. In conclusion, we believe that bizine should be a useful probe in the continuing functional evaluation of LSD1's demethylase activity in physiologic and pathophysiologic conditions.

METHODS

GST-LSD1 Enzymatic Assays. GST-LSD1 production from an *E. coli* expression system followed by purification using glutathione affinity chromatography were performed as previously described.[64] Rate measurements were performed using a peroxidase-coupled assay as previously described.[6] To determine LSD1 activity, 100 μL reactions were initiated by the addition of 2 μL of GST-LSD1 (to obtain 96–154 nM GST-LSD1 final concentration) to reaction mixtures consisting of 50 mM HEPES buffer (pH 7.5), 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 0.04 mg mL^{-1} horseradish peroxidase (Worthington Biochemical Corporation), and appropriate concentration of buffered substrate (dimethyl-Lys-4 H3-21, ARTKme2QTARKSTGGKAPRKQLA, synthesized and purified as described previously[36]). Absorbance changes were measured at 515 nm using a Beckman Instruments DU series 600 spectrophotometer equipped with a thermostatted cell holder (T = 25 °C), and product formation was calculated using the extinction coefficient of 26,000 M^{-1}. Under these conditions, GST-LSD1 displayed a k_{cat} of ~3 min^{-1} and a K_m for dimethyl-Lys-4 H3-21 of ~40 μM, but the specific parameters were measured for each batch and used for inhibitor parameter calculations. For inhibition studies, phenelzine analogue compounds were dissolved in dimethylsulfoxide (DMSO) to make 5 mM stock solutions that were diluted into reactions at appropriate concentrations. Reactions were run at similar conditions as previously stated with 60–300 μM dimethyl-Lys-4 H3-21 substrate. Progress curves conducted for 20 min were then fit to the following eq 1:

$$\text{product} = (v_o/k_{obs})(1 - e^{-k_{obs}t}) \quad (1)$$

The Kitz and Wilson method was then used to analyze the k_{obs} values to obtain k_{inact} and $K_{i(inact)}$ values with the following eq 2:

$$k_{obs} = (k_{inact}[I])/(K_{i(inact)} + [I]) \quad (2)$$

The following Cheng–Prusoff equation, eq 3, was then used to extrapolate the $K_{i(inact)}$ value to zero substrate:

$$K_i^{app} = K_i(1 + S/K_m) \quad (3)$$

Each experiment was repeated at least two independent times and repeat measured values were typically within 20% of each other.

MAO A/B Activity and Inhibition Assays. MAO A was purchased from Sigma (product number M 7316). MAO B was purchased from Sigma (product number M 7441). MAO A/B activity was measured spectrophotometrically using a peroxidase-coupled assay as previously described.[6] First, 100 μL reactions were initiated by the addition of 2 μL of MAO A/B (to obtain 100–200 nM final concentration for MAO A and 0.837 μM final concentration for MAO B) to reaction mixtures consisting of 50 mM HEPES buffer (pH 7.5), 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 0.04 mg mL^{-1} horseradish peroxidase (Worthington Biochemical Corporation), and appropriate concentration of buffered substrate (tyramine). Absorbance changes were measured at 515 nm using a Beckman Instruments DU series 600 spectrophotometer equipped with a thermostatted cell holder (T = 25 °C), and product formation was calculated using the extinction coefficient of 26,000 M^{-1}. Under these conditions, MAO A displayed a k_{cat} of 3 ± 0.1 min^{-1} and a K_m for tyramine of 26 ± 3 μM. MAO B displayed a k_{cat} of 0.2 ± 0.02 min^{-1} and a K_m for tyramine of 94 ± 26.0 μM. For inhibitor studies, phenelzine analogue compounds were dissolved in dimethylsulfoxide (DMSO) to make 5 mM stock solutions that were diluted into reactions at appropriate concentrations. Reactions were run at similar conditions as previously stated with 125 μM tyramine substrate for MAO A and with 125–1,000 μM tyramine substrate for MAO B. Progress curves were then fit accordingly to eqs 1–3 as previously stated. Each experiment was repeated at least two independent times, and repeat measured values were typically within 20% of each other.

Cell Culture. LNCaP, H460, and A549 cells were maintained in RPMI 1640 + GlutaMAX (Invitrogen 61870-036) supplemented with 10% fetal bovine serum (FBS, Gibco 10437-028) and 1 unit mL^{-1} penicillin, 1 μg mL^{-1} streptomycin (Gibco 15140-122). MDA-MB-231 cells were maintained in DMEM (Gibco 11965) supplemented with 10% FBS and 1 unit mL^{-1} penicillin, 1 μg mL^{-1} streptomycin, and 292 μg mL^{-1} L-glutamine (Corning 30-009-Cl). All cells were grown at 37 °C in 5%/95% CO_2/air.

Western Blot. Cells were seeded in 150 × 25 mm plastic tissue culture dishes (Corning 430599). Cells were treated at ~70% confluency with phenelzine analogues (>97% purity as determined by NMR) or vehicle in serum-free media for 48 h. Whole-cell extracts were isolated using RIPA buffer (Sigma R0278) and 1x protease inhibitor cocktail (Roche 11836170001). Histone extracts were isolated as described previously.[65] Concentration of whole cell lysates and histone extracts were determined using a Micro BCA Protein

Assay Kit (Thermo Scientific 23235). Proteins were resolved by 10−12% NuPAGE Novex Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen) by iBlot. Data are presented from one representative experiment. Each experiment was repeated at least three independent times with nearly identical results.

Oxidative Toxicity and Neuron Viability Assays. Immature primary cortical neurons were obtained from fetal Sprague−Dawley rats at embryonic day 17 (E17) as previously described[66] and plated at a density of 10^6 cells mL^{-1} in 96-well plates for the viability experiments. The next day cells were rinsed and then placed in medium containing 5 mM homocysteic acid (HCA). In the dose-curve experiments, increasing concentrations of **12d** (bizine) or phenelzine were added at the time of HCA treatment. The next day, cell viability was assessed by the MTT assay (Promega).[67] The 2-way ANOVA followed by the Bonferroni post-test was used to measure statistical significance. $p < 0.05$ was considered to be statistically significant. Each bar represents four different rat cultures. The use of animals and procedures were approved by the Institutional Animal Care and Use Committees of Weill Medical College of Cornell University.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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