Exploration of Novel Small Molecule Phenelzine Analogue LSD1 Inhibitors

by

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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. We designed and synthesized analogues of a monoamine oxidase antidepressant, phenelzine, and tested for 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 homolog, 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. Additionally, treatment of cancer cell lines 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. Ultimately, this work produced a potent and selective phenelzine analogue that is a useful probe in the continuing study of LSD1’s demethylase activity in both physiologic and pathophysiologic conditions.

Advisor: Philip A. Cole, M.D., Ph.D.

Thesis Reader: Sean D. Taverna, Ph.D.
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# Table of Contents

Abstract........................................................................................................................................................................... ii

Acknowledgements ........................................................................................................................................................ iii

Table of Contents ........................................................................................................................................................ iv

List of Tables ............................................................................................................................................................... v

List of Figures ............................................................................................................................................................... vi

List of Abbreviations ...................................................................................................................................................... xiii

Chapter 1. Introduction ...................................................................................................................................................... 1

Chapter 2. Synthesis of Phenelzine Analogues ............................................................................................................... 53

Chapter 3. Testing Phenelzine Analogues In Vitro for LSD1 Inhibition ................................................................. 74

Chapter 4. Bizine Effects in Cells .................................................................................................................................. 98

Appendices ...................................................................................................................................................................... 153

  Appendix I: Additional Phenelzine Analogue Synthetic Schemes and Synthesis ................................................. 153

  Appendix II: Compound 9-15 \(^1\)H NMR and \(^{13}\)C NMR Spectra .................................................................................. 185

  Appendix III: LSD1 and MAO A/B Kinetic Parameter Data Tables; Compound 9-15

    LSD1 Kinetic Plots of \(k_{\text{obs}}\) Versus Inhibitor Concentration ........................................................................... 211

References ........................................................................................................................................................................ 243

Disclosure of Potential Conflicts of Interest .................................................................................................................. 257

Curriculum Vitae ........................................................................................................................................................... 258
List of Tables

Table 1. Histone post-translational modifications (PTMs) and their associated residues. . 6
Table 2. Kinetics of phenelzine analogue LSD1 inhibitors (compounds 9-15)............. 83
Table 3. Phenelzine and 12d (bizine) selectivity profile for LSD1 vs. MAO A, MAO B,
and LSD2. ......................................................................................................................... 87
Table 4. 26 tumor suppressor genes (p-value = 5.80E-9), with original p-values, of the
146 genes that overlapped in the chemical inhibition and LSD1 knockout ChIP-seq
experiments. Tumor suppressor genes were identified using two data sets; one data
set used was from Vanderbilt University
(http://bioinfo.mc.vanderbilt.edu/TSGene/Human_716_TSGs.txt), and the other data
set used was from Memorial Sloan-Kettering Cancer Center
(http://cbio.mskcc.org/CancerGenes/). ................................................................. 123
Table 5. 20 of the 40 Johns Hopkins Drug Library hits that were confirmed to be false
positives through validation experiments. ............................................................... 138
List of Figures

Figure 1. This figure is taken from Friso et al\textsuperscript{1}. Main mechanisms of epigenetics in eukaryotic cells. ................................................................. 2

Figure 2. This figure is taken from Tonna et al\textsuperscript{7}. DNA is packaged into chromatin. ........ 4

Figure 3. This figure is taken from Kato et al\textsuperscript{11}. Examples of different histone modification marks. ................................................................. 7

Figure 4. This figure is taken from Tarakhovsky et al\textsuperscript{18}. Writers, erasers, and readers in epigenetics................................................................. 10

Figure 5. This figure is taken from Bannister et al\textsuperscript{9}. Cross-talk occurring between various histone tail residues................................................................. 14

Figure 6. LSD1 demethylation of di-methylated Lys4 of histone H3 (H3K4) mechanism of action. ................................................................. 18

Figure 7. This figure was modified from Klose et al\textsuperscript{44}. The crystal structure of LSD1 modeled onto a crystal structure of a nucleosome........................................... 21

Figure 8. Complexes in which LSD1 can be found in....................................................... 24

Figure 9. (A) This figure was taken from Forneris et al\textsuperscript{30}. One model of the di-methylated H3K4 peptide-binding site in LSD1, based on the co-crystal structure of LSD1 with a peptide analog containing Met in place of methylated Lys4 at the fourth position of the H3 tail peptide. (B) This figure was taken from Yang et al\textsuperscript{29}. Another model of the di-methylated H3K4 peptide-binding site in LSD1, based on the co-crystal structure of LSD1 with a suicide inhibitor warhead moiety in place of methylated Lys4 at the fourth position of the H3 tail peptide. ........................................... 25
Figure 10. This figure was modified from Zhang et al and Anand et al\textsuperscript{41,67}. The domain arrangements of both LSD2 and LSD1................................................................. 30

Figure 11. Previously published LSD1 inhibitors............................................................. 40

Figure 12. This figure was taken from Khan et al\textsuperscript{124}. Proposed mechanisms by which tranylcypromine can modify FAD to form atropaldehyde-FAD, cinnamaldehyde-FAD, and/or tranylcypromine-FAD adducts to inhibit MAOs........................................... 43

Figure 13. This figure was taken from Khan et al\textsuperscript{124}. Proposed mechanism by which tranylcypromine inactivates MAOs by generation of N(5) adducts. ......................... 44

Figure 14. Proposed LSD1 inhibition mechanism by phenelzine..................................... 47

Figure 15. This figure was taken from Rotili et al\textsuperscript{146}. Structures of dual drug inhibitors. 52

Figure 16. Smaller phenelzine analogues synthesized and characterized..................... 56

Figure 17. Larger phenelzine analogs synthesized and characterized............................ 58

Figure 18. Synthesis of compound 12a............................................................................. 60

Figure 19. Synthesis of compounds 12b and 12d............................................................ 62

Figure 20. Synthesis of compound 14........................................................................... 64

Figure 21. Suicide inhibition............................................................................................ 75

Figure 22. Spectrophotometric assay for the determination of LSD1 inhibition............. 78

Figure 23. Inhibition of LSD1 by phenelzine. (A) Steady-state progress curve of LSD1 inactivation by phenelzine ranging from 0 to 100 µM. (B) $k_{\text{obs}}$ values obtained from steady-state data plotted against inhibitor concentration to determine $k_{\text{inact}}$ and $K_{i(\text{inact})}$ values. ............................................................................................................................... 81

Figure 24. 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. ........................................................................................................ 85

Figure 25. Product concentration versus time of LSD2 with 20 µM of phenelzine, 12a, 12b, 12d (bizine), and 14 ........................................................... 88

Figure 26. Product concentration versus time of LSD1 with 10 µM phenelzine, 1 µM 12d (bizine), and 0.5 µM and 1 µM the hydrazino peptide inhibitor ....................... 90

Figure 27. Quantification of methylation states of H3K4 as a result of LSD1 inhibition by phenelzine or 12d (bizine) as determined by the MassSQUIRM technique. ........ 92

Figure 28. Rate versus substrate concentration of the GST-LSD1 batch used to test phenelzine and compounds 12a, 12b, 12d (bizine), and 14. ................................. 95

Figure 29. LSD1 inhibition by 12d (bizine) in LNCaP cells. (A) Cells were treated with 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 3 biological replicates. (C) Cells were treated with 12d (bizine) (0.4–10 µM) for 48 h and blotted against LSD1 and actin. ....................................................................................... 103

Figure 30. (A) In vitro kinetic data of phenelzine, 12d (bizine), and compound 12l. (B) Cells were treated with phenelzine (3–40 µM) for 48 h and blotted against H3K4me2 and Total H3. (C) Cells were treated with compound 4l (0.4–10 µM) for 48 h and blotted against H3K4me2 and Total H3. (D) Compound 12l H3K4me2 band density quantification plot as determined by 2 biological replicates. .......... 106

Figure 31. The HCT116 parental cell line was treated with 5-20 uM of 12d (bizine) for 48 h and blotted against H3K4me2 and Total H3................................................. 108
Figure 32. H3K4me2 Western blot after four different treatment conditions were utilized to treat LNCaP cells for 6 h with either DMSO or 10 µM 12d (bizine): (1) serum starving cells for 24 h prior to treatment in serum-free media (SFM), (2) serum starving cells for 24 h prior to treatment in serum-containing media, (3) treatment in SFM, and (4) treatment in serum-containing media. ................................. 110

Figure 33. Two biological replicates of cells treated with 10 µM of either phenelzine, tranylcypromine, or 12d (bizine) for 48 h and blotted against H3K4me2. Ponceau stains were used as loading controls. Numbers underneath each band indicate relative band density. ........................................................................................................... 112

Figure 34. (A) LNCaP Cells were treated with 10 µM 12d (bizine) and collected at various indicated time points and blotted against H3K4me2 and Total H3. (B) Two additional biological replicates of LNCaP cells treated with 10 µM 12d (bizine) for 30 min, 6 h, 12 h, and 24 h and blotted against H3K4me2 and Total H3. (C) 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 h, 24 h, 48 h, 72 h, and 96 h, but not at 12 h based on 3 biological replicates. ........................................................................................................... 114

Figure 35. H460, A549, and MDA-MB-231 cell lines were treated with 12d (bizine) (0.4–10 µM or 20 µM) for 48 h and blotted against H3K4me2 and Total H3.

* Determined using biological triplicates. ................................................................................ 116

Figure 36. A) ML-1, (B) KG1a, (C) HL-60, (D), HNT-34 cell lines were treated with 10 µM 12d (bizine) for either 6 h or 48 h and blotted against H3K4me2 and Total H3.
Quantification plots of H3K4me2 band density were determined using biological triplicates. Figure 37. Shown are representative examples of three genes’ Integrative Genomics Viewer (IGV)\textsuperscript{20,21} tracks from the list of 2,432 genes identified through the ChIP-seq experiment that showed an increase in H3K4me2 with LSD1 inhibition by \textit{12d} (bizine) (with two biological replicates): (A) RGMB (chr5:98,079,869-98,189,371); (B) SMARCA2 (chr9:1,999,116-2,177,398); (C) ERRFI1 (chr1:7,902,135-8,201,537). Red boxes mark statistically significant peak increases with \textit{12d} (bizine) treatment. Scale indicated by tick marks. Figure 38. Comparing the 1,767 gene dataset from the \textit{12d} (bizine) inhibition ChIP-seq experiment to the 1,587 gene dataset from a ChIP-seq experiment that used an LSD1\textemdash/\textemdash hematopoietic cell line showed that there were 146 genes in common at a statistically significant p-value (0.0028). Figure 39. q-rtPCR results. (A) Relative mRNA changes of 10 select tumor suppressor genes and Rest in response to either DMSO or 10 µM \textit{12d} (bizine) treated LNCaP cells for 48 h. (B) Relative mRNA change of the gene, LRBA, in response to either DMSO or 10 µM \textit{12d} (bizine) treated LNCaP cells for 6 h, 12 h, 24 h, and 72 h. (C) Relative mRNA changes of 10 select tumor suppressor genes and Rest in response to either DMSO or 10 µM \textit{12d} (bizine) treated LNCaP cells for 6 h. Figure 40. MTS assay on four solid-tumor cell lines, (A) LNCaP, (B) H460, (C) A549, (D) MDA-MB-231, treated with 5-80 µM \textit{12d} (bizine) for 96 h. Red box indicates that there is no effect on cell viability at \textit{12d} (bizine) concentrations of 10 µM or less in any of the cell lines.
Figure 41. DNA replication dose response curves using a $^3$H thymidine assay in H460 cells and LNCaP cells after 48 h treatment with 12d (bizine) in either (A) SFM or (B) serum-containing media. ................................................................. 129

Figure 42. DNA replication dose response curves using a $^3$H thymidine assay in H460 cells after 48 h treatment with phenelzine. ................................................................. 131

Figure 43. DNA replication dose response curves using a $^3$H thymidine assay in H460 cells after 48 h treatment with compound 12l. ........................................................ 132

Figure 44. DNA replication dose response curves using a $^3$H thymidine assay in (A) ML-1 (B) KG1a (C) HL-60 (D) HNT-34 cells after 24 h, 48 h, 72 h, and 96 h treatment with compound 12d (bizine). .................................................................................. 134

Figure 45. The H460 cell line was treated simultaneously with 12d (bizine) and (A) azacytidine, (B) SAHA, (C) TSA, (D) MGCD0103, (E) MS-275, (F) LBH-589 for 48 h and DNA replication was monitored using the $[^3]$H thymidine assay. Synergy was determined by CompuSyn using a non-constant ratio approach. CI > 1, CI = 1, or CI < 1 indicates antagonism, additivity, or synergy, respectively. For example, points above, on, or under the red line indicate antagonism, additivity, or synergy, respectively. Fa indicates the fraction of cells affected by given doses of each drug. ................................................................................................................................. 136

Figure 46. Scratch assays were performed on H460 cells treated with either DMSO, 10 $\mu$M phenelzine, or 10 $\mu$M 12d (bizine) and imaged after either 0, 1 (24 h), 2 (48 h), or 3 days (72 h). ......................................................................................................................... 140

Figure 47. Figure provided by Manuela Basso (Dr. Rajiv Ratan’s laboratory). Neurons were exposed to 5 mM HCA and treated with either (A) 12d (bizine) or (B)
phenelzine for 48 h, after which cell cytotoxicity was measured using a MTT assay. (Two-way ANOVA, Bonferroni post hoc test; **p < 0.01; ***p < 0.0001 compared to no HCA).

Figure 48. Figure provided by Sifei Xin (Dr. Robert Silicano’s laboratory). Bcl-2 transduced cells as models of HIV-1 latency were treated for 72 h with 1-20 µM 12d (bizine) either alone or with either 1 µM SAHA or 30 nM LBH-589 and then analyzed for their level of re-activation as a percentage of co-stimulation.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>O-GlcNAc</td>
<td>β-N-acetylglucosamine</td>
</tr>
<tr>
<td>miRNAs</td>
<td>MicroRNAs</td>
</tr>
<tr>
<td>DNMTs</td>
<td>DNA methyltransferases</td>
</tr>
<tr>
<td>PTMs</td>
<td>Post-translational modifications</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>HATs</td>
<td>Histone acetyltransferases</td>
</tr>
<tr>
<td>HDACs</td>
<td>Histone deacetylases</td>
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<td>RISC</td>
<td>RNA-induced silencer complex</td>
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<tr>
<td>LSD1</td>
<td>Lysine-specific demethylase 1</td>
</tr>
<tr>
<td>H3K4</td>
<td>Lys4 of histone H3</td>
</tr>
<tr>
<td>H3K9</td>
<td>Lys9 of histone H3</td>
</tr>
<tr>
<td>DNMT1</td>
<td>DNA methyltransferase 1</td>
</tr>
<tr>
<td>SWIRM</td>
<td>Swi3p/Rsc8p/Moira</td>
</tr>
<tr>
<td>AO</td>
<td>Amine oxidase</td>
</tr>
<tr>
<td>CoREST</td>
<td>RE1-silencing transcription factor corepressor 1</td>
</tr>
<tr>
<td>RCOR1</td>
<td>RE1-silencing transcription factor corepressor 1</td>
</tr>
<tr>
<td>NuRD</td>
<td>Nucleosome remodeling and deacetylation</td>
</tr>
<tr>
<td>MTA</td>
<td>Metastasis tumor antigen</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem</td>
</tr>
<tr>
<td>PELP1</td>
<td>Proline glutamic acid and leucine-rich protein 1</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>MAO A/B</td>
<td>Monoamine oxidase A/B</td>
</tr>
<tr>
<td>PAO</td>
<td>Polyamine oxidase</td>
</tr>
<tr>
<td>LAO</td>
<td>L-amino acid oxidases</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemic stem cells</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate-early</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>Prox1</td>
<td>Prospero-related homeobox</td>
</tr>
<tr>
<td>MAOIs</td>
<td>MAO A/B inhibitors</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>Di-methylated Lys4 of histone H3</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>Fa</td>
<td>Fraction affected</td>
</tr>
<tr>
<td>CI</td>
<td>Combination index</td>
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Chapter 1. Introduction

Epigenetics

The DNA sequence alone is insufficient to control the regulation of gene expression and resulting phenotypes. Every cell in the human body has the same DNA, therefore the DNA sequence cannot explain several phenomena, including that a stem cell divides and produces one differentiated cell and another undifferentiated stem cell, that a differentiated cell is able to divide and keep its phenotype, and that one random X chromosome in females is inactivated permanently in every cell early in development. Such phenomena allow for different types of cells to be present in the body and facilitate the repair and regeneration of tissues. The main molecular mechanisms that take place in every eukaryotic cell that result in such phenomena include histone modifications, DNA methylation, and non-coding RNA interactions (Figure 1). Such mechanisms fall under the field of epigenetics, a term coined by Waddington in 1942 to bring together both genetics and developmental biology, which is defined as the study of changes in gene expression that do not result from differences in the DNA sequence.
Figure 1. This figure is taken from Friso et al\textsuperscript{1}. Main mechanisms of epigenetics in eukaryotic cells.

Mechanisms that change gene expression that are not caused by differences in the DNA sequence include DNA methylation, histone modifications, and non-coding RNA interactions. DNA methylation on cytosine residues in nucleosomes can result in gene silencing. Histone modifications, such as methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deamination, or $\beta$-N-acetylglucosamine (O-GlcNAc) addition, can alter the structure of chromatin and result in either gene activation or repression. Non-coding RNAs, such as microRNAs (miRNAs), can target mRNAs to downregulate their protein expression.
DNA is packaged to allow the human genome to be of a reasonable width and length so that it can easily fit into the nucleus of each cell (Figure 2). About 146 base pairs of DNA are usually wrapped around an octamer of two copies each of histone proteins H2A, H2B, H3, and H4, making up what is called a nucleosome. Each nucleosome is connected to other nucleosomes, tethered by about 20 base pairs of DNA with the linker histone H1. The nucleosomes are then packaged into condensed chromatin, which ultimately forms chromosomes.
Figure 2. This figure is taken from Tonna et al\textsuperscript{2}. DNA is packaged into chromatin.

The DNA double helix, which is 2 nm in width, is wrapped around an octamer of histone proteins to form a nucleosome. The nucleosomes are then packaged to form a chromatin fiber of 30 nm in width. Consequently, the chromatin fiber is condensed to ultimately form a chromosome of approximately 1,400 nm in width.
The structure of chromatin is determined by several factors, including histone modifications, one of the main epigenetic mechanisms. Histone modifications are abundant on histone tails, which are N-terminal segments containing up to 40 amino acid residues that protrude from the nucleosomes. The histone modifications, or histone post-translational modifications (PTMs), alter chromatin structure by either directly altering chromatin intramolecular contacts or by recruiting various proteins, such as remodeling enzymes that can hydrolyze ATP and use the energy to reposition nucleosomes. Directly altering chromatin structural contacts may occur through a change in the charge of the histones that can alter electrostatic interactions inducing either a relaxed or compact chromatin state. The recruitment of various chromatin factors, such as remodeling enzymes, to change chromatin structure can occur through domains present in these recruited proteins that have the ability to interact with specific modifications. Ultimately, the chromatin structure alteration as a result of histone modification marks influences gene expression, in addition to DNA replication, repair, and recombination.

Histone PTMs take place on various histone protein tail residues including lysines, arginines, serines, threonines, tyrosines, and glutamates, and include methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deamination, or β-N-acetylglucosamine (O-GlcNAc) addition (Table 1 and Figure 3). These histone PTMs either individually or in combination influence the structure of chromatin and result in the activation or repression of various genes involved in many processes. Thus, it is of great importance to fully understand the complex dynamics of the various histone PTMs.
Table 1. Histone post-translational modifications (PTMs) and their associated residues.

<table>
<thead>
<tr>
<th>Histone Post-Translational Modification (PTM)</th>
<th>Associated Residue(s)</th>
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<tbody>
<tr>
<td>Methylation</td>
<td>Lysine and Arginine</td>
</tr>
<tr>
<td>Acetylation</td>
<td>Lysine</td>
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<tr>
<td>Phosphorylation</td>
<td>Serine, Threonine, and Tyrosine</td>
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<tr>
<td>Ubiquitination</td>
<td>Lysine</td>
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<tr>
<td>Sumoylation</td>
<td>Lysine</td>
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<td>ADP Ribosylation</td>
<td>Glutamate and Arginine</td>
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<td>Deamination</td>
<td>Arginine</td>
</tr>
<tr>
<td>β-N-Acetylglucosamine (O-GlcNAc)</td>
<td>Serine and Threonine</td>
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</table>
Figure 3. This figure is taken from Kato et al. Examples of different histone modification marks.

Histone tails of histones H2A, H2B, H3, and H4 may undergo many different histone PTMs including, but not limited to, acetylation, methylation, phosphorylation, and ubiquitination on various residues.
Histone methylation mainly occurs on lysine and arginine residues. As methylation does not alter the charge of the histone protein, transcriptional activation or repression depends on the specific lysine or arginine residue involved in the methylation. For example, methylated Lys4 of histone H3 has been shown to be a transcription activating chromatin mark linked to promoters of active genes\textsuperscript{7,8}, whereas the methylation of Lys9 of histone H3 has been shown to be a repressive mark\textsuperscript{9}. Additionally, this modification takes on further complexity by the various degrees of methylation on each residue that can take place. Lysine residues can be mono-, di-, or tri-methylated, whereas arginine residues can be mono-, as well as symmetrically or asymmetrically di-methylated.

There are many enzymes that add methyl groups (writers), remove methyl groups (erasers), or bind to methyl groups (readers) that participate in the dynamic process of reversible histone lysine methylation (Figure 4). Lysine methyltransferases, or writers, include enzymes that are members of the SET domain containing family, as well as the DOT1 family. All the lysine methyltransferases depend on S-adenosyl-L-methionine (SAM) as their methyl source. The SET domain histone lysine methyltransferases catalyze mono-, di-, and tri-methylation of lysines, with the active-site residues of the enzyme determining its target lysine residue\textsuperscript{10}. The DOT1 histone lysine methyltransferases methylate a histone core residue solely, Lys79 of histone H3\textsuperscript{10}. Ultimately, the histone lysine residues that are subject to mono-, di- and tri-methylation include H2AK118, H2BK23, H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20.

Demethylases, or erasers, that remove methyl groups from lysine residues include lysine-specific demethylase 1 (LSD1, also known as BHC110, KDM1, and AOF2), the homolog
of LSD1, LSD2, and iron-dependent Jmj demethylases, such as JARID1A-1D\textsuperscript{11,12}. LSD1 and LSD2, both belonging to the amine oxidase superfamily and utilizing FAD as their cofactor, catalyze the demethylation of mono- and di-methylated substrates only. The Jmj demethylases are able to demethylate all three methylated lysine states, mono-, di-, and tri-, and each demethylase possesses a high level of substrate specificity. Proteins that bind each particular methylated histone residue, otherwise known as readers, contain various domains, such as chromo, PHD, Tudor, WD40, ADD, MBT, and Zf-CW. Thus, there is an abundance of writers, erasers, and readers that determine the dynamics of histone methylation and subsequently its effects on various processes.
There are many enzymes that participate in adding, removing, and recognizing various PTMs, respectively called writers, erasers and readers. Enzymes that participate in writing include acetylases (acetyltransferases), methylases (methyltransferases), and phosphorylases (kinases). Enzymes that involve erasing PTMs include deacetylases, demethylases, and phosphatases. Lastly, domains involved in reading PTMs include domains such as bromo, chromo, PHD, and WD40.

Figure 4. This figure is taken from Tarakhovsky et al. Writers, erasers, and readers in epigenetics.
There are two classes of arginine methyltransferases that add methyl groups to arginine, type-I and type-II, which together form an 11-member protein family. Just like lysine methyltransferases, arginine methyltransferases use SAM as a methyl source. One protein, JMJD6, has been reported to remove methyl groups from arginine residues, demethylating Arg2 of histone H3 and Arg3 of histone H4, although this has not been reproduced in other labs\textsuperscript{14}. As for the demethylation of other arginine residues, a deimination reaction may convert methylated arginine to citrulline, which is in fact considered a separate histone PTM, although this is controversial\textsuperscript{15}.

Histone acetylation on lysine residues was first reported in 1964 by Allfrey et al\textsuperscript{16}. We now know that it is a highly dynamic process regulated by both histone acetyltransferases (HATs) and histone deacetylases (HDACs). There are several families that most HATs fall under based on sequence homology, structural similarities, and functional roles, including Gcn5-related N-acetyltransferases, MYST, p300/CBP, and nuclear receptor coactivators. Nevertheless, all HATs utilize acetyl-CoA as their co-substrate to transfer acetyl groups to lysine residues, neutralizing lysines’ positive charge, which can ultimately lead to a relaxed chromatin state, or euchromatin, and therefore gene activation. On the other hand, HDACs are classified in four classes based on sequence homology and domain organization, including Class I, IIA, IIB, III, and IV. HDACs remove acetyl groups from lysine residues, restoring the positive charge, and thus leading to a more compact chromatin state, or heterochromatin, which can repress transcription.

Histone phosphorylation on residues such as serines, threonines, and tyrosines is also a highly dynamic process that is regulated by kinases and phosphatases. Protein
kinases, classified into groups based on substrates they act upon, transfer phosphoryl
groups from ATP to the hydroxyl groups of the residues, which add negative charges.
This ultimately can lead to significant changes in chromatin structure, and consequently
has been shown to play roles in processes such as mitosis, apoptosis, and
gametogenesis\textsuperscript{17}. Phosphatases, also subdivided based upon their substrate specificity,
remove phosphate groups, and as a consequence also influence chromatin structure.

Other less well-characterized PTMs, such as deimination, ubiquitylation,
sumoylation, ADP ribosylation, and O-GlcNAc, have been detected on histone residues.
Deimination, as mentioned previously, is a reaction catalyzed by PADI4 that converts
arginine, or perhaps mono-methyl arginine, to a citrulline, neutralizing the positive
charge of arginine. Ubiquitylation, occurring on lysine residues, is quite a large histone
PTM as ubiquitin is a 76-amino acid polypeptide. The PTM is added via the sequential
action of three enzymes, E1, E2, and E3, and is removed via a de-ubiquitin isopeptidase\textsuperscript{4}.
It is involved in gene silencing, as well as transcriptional initiation and elongation\textsuperscript{18}.
Sumoylation, related to ubiquitination, involves the addition of a ubiquitin-like modifier
molecule also to a histone lysine residue via a specialized trio of E1, E2, and E3
enzymes. This PTM can prevent the acetylation and ubiquitylation at specific lysine
residues, and has been linked to transcriptional repression\textsuperscript{19}. Mono- and poly-ADP
ribosylation has been known to occur on histone glutamate and arginine residues. The
addition of poly-ADP ribosylation occurs via the poly-ADP-ribose polymerase (PARP)
family of enzymes, and the poly-ADP-ribose-glycohydrolase family of enzymes
catalyzes the removal of the mark\textsuperscript{20}. As this PTM confers a negative charge, it has been
generally linked to a relaxed chromatin state, and therefore to transcriptional activation\textsuperscript{20}. 
Histone mono-ADP-ribosylation occurs via the mono-ADP-ribosyltransferases, and has been linked to the DNA damage response as this modification is increased upon DNA damage\textsuperscript{20}. Lastly, O-GlcNAc is a histone PTM that occurs on serine and threonine residues. This PTM is added via the O-GlcNAc transferase that utilizes the donor substrate UDP-GlcNAc, and is removed via the \( \beta \)-N-acetylglucosaminidase\textsuperscript{21}. All of these histone PTMs are considered to contribute to the histone code and demonstrate the complexity of gene transcription regulation.

The histone code, or different combinations of histone PTMs, is proposed to influence specific biochemical outputs (Figure 5). There are multiple ways by which cross-talk between modification marks are thought to occur\textsuperscript{4}. One way is that there could be competition for modifications at one particular residue. For example, mechanisms to either add an acetyl, methyl, or ubiquityl group to one particular lysine residue on a histone tail would be competitive towards one another. Another way cross-talk can occur is if the addition of one modification mark is dependent on the addition of another modification mark. Cross-talk can also occur if a modification mark disrupts or enhances the binding of a protein to another modification mark. Any particular histone modifying enzyme’s activity can also potentially be perturbed if a particular modification results in its gene activation or repression. Lastly, cross-talk occurs if modifications work in a cooperative manner in the recruitment of a specific factor. Such cross-talk between histone modifications can be further influenced by the effects of DNA methylation, underscoring the potential for complexity in epigenetics.
Figure 5. This figure is taken from Bannister et al. Cross-talk occurring between various histone tail residues.

Histone PTMs can either positively or negatively influence other modifications. Arrows indicate a positive effect, and flat heads indicate a negative effect.
Another important epigenetic mechanism is DNA methylation, which was first proposed in 1975 to play a role in gene expression, including in X chromosome inactivation\textsuperscript{22,23}. The addition of methyl groups to convert the DNA base cytosine to 5-methylcytosine by either \textit{de novo} or maintenance DNA methyltransferases (DNMTs) does in fact silence gene expression. Such methylation usually occurs within CpG dinucleotides that are either found sparsely but globally, or in large clusters called CpG islands. It is now known that DNA methylation plays a large role in many processes, including in the previously mentioned X chromosome inactivation, genomic imprinting, repetitive element suppression, embryonic development, and preservation of chromosome stability\textsuperscript{24,25}. Dysregulation of DNA methylation can therefore have detrimental effects, and result in several human diseases, including cancer, where aberrant DNA methylation patterns in promoter regions of genes can alter gene expression\textsuperscript{26}.

An additional epigenetic mechanism involves non-coding RNAs, such as microRNA (miRNAs). miRNAs range are up to 25 nucleotides in length and can regulate multiple biological functions, including gene expression post-transcriptionally, through the RNA interference (RNAi) machinery\textsuperscript{27}. RNAi processing first begins by pri-miRNA, measuring about 70-100 nucleotides in length and containing a stem-loop, being converted to pre-miRNA, shaped as a stem-loop. The pre-miRNA then forms a complex with exportin-5 for export out of the nucleus, after which the pre-miRNA is then further processed into a double-stranded miRNA duplex. The RNA-induced silencer complex (RISC)-loading complex then removes one strand of the miRNA and the other miRNA strand stays as part of the mature RISC, which serves as a template to capture target mRNAs and prevent their translation into proteins. Recently, both DNA methylation and
histone modification have been implicated in regulating the expression of miRNA, and conversely miRNAs have been shown to regulate enzymes such as DNA methyltransferases and histone deacetylases\textsuperscript{27}. This demonstrates an important interplay between different epigenetic mechanisms that together contribute to the alteration of gene expression.
**Discovery and Evolution of LSD1**

Discovered a decade ago, lysine-specific demethylase 1 (LSD1) was the first histone demethylase reported\(^1\). The discovery of LSD1 disproved the notion that histone methylation is a stable and static modification. Since then, other histone demethylases have been identified, such as LSD2 and the Jumanji C domain family of enzymes, all of which have specific histone substrates.

LSD1, a 90 kDa flavin-dependent enzyme belonging to the amine oxidase protein superfamily, is primarily responsible for oxidatively cleaving one or two methyl groups from Lys4 of histone H3 (H3K4)\(^{11,28–33}\). Three methyl groups from H3K4 cannot be cleaved by LSD1 due to a requirement of an electron pair on the lysine amino group in the first step of the LSD1 demethylation reaction, which involves an oxidation by FAD of the amine to an iminium cation intermediate (Figure 6)\(^{11,28,34,35}\). The second step of the LSD1 demethylation reaction involves the attack of the iminium cation by a water molecule, after which the formed hemiaminal collapses to release the demethylated substrate and a formaldehyde molecule. The reduced cofactor, FADH, is then re-oxidized by molecular oxygen, generating a molecule of hydrogen peroxide.
Figure 6. LSD1 demethylation of di-methylated Lys4 of histone H3 (H3K4) mechanism of action.

Di-methylated H3K4 is a substrate of LSD1, where FAD oxidizes the amine of the substrate to an iminium ion. The iminium ion is then attacked by a water molecule, after which the formed hemiaminal collapses and yields the demethylated substrate and a formaldehyde molecule. The reduced cofactor, FADH, is then re-oxidized back to FAD by molecular oxygen, releasing hydrogen peroxide as a byproduct.
Although not well-established, there are other reported substrates of LSD1, which include mono- or di-methylated Lys9 of histone H3 (H3K9) in prostate cell lines and in cells infected by herpes virus, as well as non-histone tail substrates such as p53, DNA methyltransferase 1 (DNMT1), and E2F1. Demethylating non-histone tail substrates by LSD1 has been suggested to impact various biological pathways. For example, the demethylation of p53 at the K370 residue by LSD1 has been stated to alter the interaction of p53 with its co-activator 53BP1, which ultimately alters the DNA damage response pathway. Demethylating DNMT1 by LSD1 has been proposed to increase global DNA methylation, providing a mechanism for LSD1 to change gene expression patterns through non-histone targets. The demethylation of E2F1 at the K185 residue by LSD1 has been reported to alter E2F1 stability by inhibiting other modifications that signal for E2F1 degradation. Such action might ultimately inhibit the DNA damage-induced cell death pathway in the absence of p53.

LSD1 contains three domains, the Swi3p/Rsc8p/Moira (SWIRM) domain, the amine oxidase (AO) domain, and the Tower domain. The SWIRM and the AO domains are tightly knit through hydrophobic interactions, forming the core structure of LSD1. The SWIRM domain is thought to bind LSD1 to the nucleosome through its interaction with DNA. The AO domain, separated into two lobes by a 105 aa Tower domain insert, is the catalytic domain. One lobe of the AO binds FAD, while the other lobe of the AO binds the substrate. The resulting active site has a span of 25 angstroms, which makes for a large and deep pocket. The Tower domain facilitates the interaction of LSD1 with other proteins, such as the RE1-silencing transcription factor corepressor 1 (CoREST or RCOR1), which has been shown to bind the LSD1 Tower domain through its SANT
domain (Figure 7). In addition to the three domains, LSD1’s N-terminal region has recently been found to also play an important role. The N-terminal region was found to contain a nuclear localization signal, $^{112}$RRKRAK$^{117}$, which can interact with three importin alpha proteins to localize LSD1 to the nucleus$^{43}$. 
Figure 7. This figure was modified from Klose et al\textsuperscript{44}. The crystal structure of LSD1 modeled onto a crystal structure of a nucleosome.

The histone H3 tail can be seen protruding from the nucleosome into the AO domain (pictured in orange and yellow) of LSD1. The SWIRM domain (pictured in blue) is adjacent to the AO domain. The Tower domain (pictured in pink and green) can be seen bound to CoREST’s SANT domain (pictured in red).
LSD1 in Complexes

Cellular LSD1 is usually found in complexes, either those that act as repressors or activators of transcription. Complexes containing LSD1 that act as repressors of transcription include CoREST and NuRD (Figure 8). In the CoREST complex, LSD1 is bound to CoREST, HDAC1/2, as well as a few other proteins. Recently, in hematopoietic cells, two other CoREST (RCOR1) paralogs have been found to bind LSD1 including RCOR2 and RCOR3. RCOR2 acts as CoREST where it facilitates LSD1-mediated demethylation of H3K4. On the other hand, RCOR3 competitively inhibits LSD1 demethylation of H3K4, ultimately inhibiting differentiation of the hematopoietic cells, indicating that LSD1 and RCOR1/3 levels play a role in differentiation (erythroid versus megakaryocytic)46. In the NuRD complex, or the nucleosome remodeling and deacetylation complex, LSD1 is clustered with HDAC1/2, a chromodomain containing DNA-binding helicase protein (CHD3 or CHD4), a metastasis tumor antigen (MTA) relative called MTA2, BRCA2, and a few other proteins. Such complexes have been shown to protect LSD1 from degradation, bind it to chromatin, and allow LSD1 to recognize nucleosomes as substrates, as in vitro, LSD1 is efficiently able to demethylate core histones but not nucleosomes48,49. In addition to those roles, it has been found that together with HDAC1, LSD1 can participate in the regulation of the proliferation of embryonic stem or embryonic carcinoma/teratocarcinoma cells by enhancing the action of HDAC1 in the deacetylation of Lys16 of histone H450.

Complexes containing LSD1 that act as activators of transcription include androgen or estrogen receptor complexes (Figure 8)36,51. In such complexes, LSD1 has been proposed to change its substrate specificity from H3K4me2 to H3K9me2, activating
gene transcription, as unmethylated H3K9 is usually associated with active transcriptional states. To change the substrate specificity of LSD1, the hormone receptors have been suggested to induce a change in the structure of the active site of LSD1. How this might happen is puzzling as structures of LSD1 and H3K4 substrate analogs show well-defined molecular recognition for the major H3K4 substrate (Figure 9A-B). One protein that has been hypothesized to influence LSD1’s substrate specificity is the proline glutamic acid and leucine-rich protein 1, PELP1, which was reported to bind concurrently to an estrogen receptor, ERα, and LSD1, facilitating LSD1-catalyzed demethylation of H3K952. Another mechanism that has been reported to switch LSD1 demethylation specificity from H3K4me2 to H3K9me2 involves the action of protein kinase C, which is recruited to the androgen nuclear receptor target promoters, and is then able to phosphorylate Thr6 of histone H3 after hormone treatment53. Additionally, it has been proposed that, after LSD1 reactions release hydrogen peroxide byproduct, DNA oxidative damage can occur. This in turn is suggested to drive the recruitment of base excision repair enzymes that can loop chromatin and allow LSD1 to access H3K9me254.
Figure 8. Complexes in which LSD1 can be found in.

The CoREST and the NuRD complexes containing LSD1 act as transcriptional repressors. It is proposed that the hormone repressor complexes, such as the AR or ERα complexes, that appear to contain LSD1, can act as transcriptional activators by switching LSD1’s substrate from H3K4me2 to H3K9me2.
Figure 9. (A) This figure was taken from Forneris et al\textsuperscript{30}. One model of the di-methylated H3K4 peptide-binding site in LSD1, based on the co-crystal structure of LSD1 with a peptide analog containing Met in place of methylated Lys4 at the fourth position of the H3 tail peptide. (B) This figure was taken from Yang et al\textsuperscript{29}. Another model of the di-methylated H3K4 peptide-binding site in LSD1, based on the co-crystal structure of LSD1 with a suicide inhibitor warhead moiety in place of methylated Lys4 at the fourth position of the H3 tail peptide.
Several other LSD1 complexes have been described. For example, in Y79 retinoblastoma cells, LSD1 was found to bind to TLX, which is an NR2E orphan nuclear receptor that is believed to act as a constitutive transcriptional repressor. Additionally, LSD1 has been found to be part of transcription elongation protein complexes that act as both transcriptional activators and repressors, including the MLL and the ELL complexes. This shows the diversity of different interactions LSD1 can have, all of which are in need of further study to decipher the full extent of LSD1’s role in each complex.

To either activate or repress gene transcription, complexes containing LSD1 must be recruited by certain proteins to various promoters of genes. An example of a protein that has shown to recruit complexes containing LSD1 to certain gene promoters is Snail, a transcription factor that is a member of the Snail family. Snail has been shown to recruit LSD1 complexes containing HDAC1/2 to promoters to repress BRCA1 in breast cancer leading to tumor metastasis. Additionally, Snail has been shown to associate with LSD1 in complex with the malignant brain tumor domain-containing protein, SFMBT1. This Snail/SFMBT1 complex can bind di- and tri-methyl H3K4 and recruit LSD1 to gene promoters, leading to the repression of epithelial markers such as TGFbeta. Such epithelial marker repression can induce the epithelial-to-mesenchymal (EMT) process, leading to the enhanced migratory capacity of tumor cells. These studies support the biomedical significance of understanding various interactions between LSD1 and Snail.

LSD1 itself has also been found to contain PTMs. In human neuroblastoma SKNBE cells, LSD1 can be phosphorylated by protein kinase CK2, a pleiotropic serine/threonine kinase, at three residues, Ser131, Ser137, and Ser166, in the N-terminal
region. Although, the phosphorylation events do not directly affect LSD1’s catalytic activity, they could possibly modulate LSD1’s interaction with binding partners, such as with CoREST and HDAC1/2\textsuperscript{62,63}. In addition to CK2, protein kinase C\(\alpha\) has been found to phosphorylate LSD1 in a circadian manner. Once phosphorylated LSD1 can form a complex with CLOCK:BMAL1, that together facilitate E-box-mediated transcriptional activation, ultimately impairing the rhythmicity and phase resetting of the circadian clock\textsuperscript{64}. 
Other Enzymes in the Amine Oxidase Superfamily

Enzymes that belong to the same amine oxidase superfamily and use a similar mechanism as LSD1 include LSD2, monoamine oxidase A/B (MAO A/B), polyamine oxidase (PAO), and the L-amino acid oxidases (LAO)\(^65\). These flavin-containing amine oxidases contribute to various biological functions, including gene expression regulation, neurotransmitter metabolism, polyamine metabolism, vitamin B5 biosynthesis, and amino acid metabolism. Like LSD1, these enzymes utilize FAD as their cofactor, which in animals is obtained from dietary sources, as animals cannot synthesize vitamin B2, the precursor of FAD. In all of the amine oxidases, the active site is located in close proximity to the flavin ring of the FAD and extends through the substrate-binding domain. The active sites are all quite different, as evolution has varied the size and shape of them to fit each enzyme’s function and type of substrate. Amine oxidase family members show low to moderate amino acid sequence identity (10% to 32%). However, in the context of inhibitor development, it is helpful to consider how specificity can be achieved for individual family members.

LSD2 (also known as AOF1 or KDM1B), the homolog of LSD1, has ~32% sequence identity to LSD1. It has been found to also demethylate mono- and di-methylated H3K4, as well as mono- and di-methylated H3K9\(^66\). Like LSD1, LSD2 contains the SWIRM and the C-terminal AO domain. However, LSD2 does not contain the Tower domain, nevertheless, it has been found to associate with proteins, such as transcriptional elongation factors and phosphorylated RNA polymerase II\(^67\). LSD2 also has a unique addition to its N-terminal region that includes two distinct zinc finger domains, a CW-type, responsible for binding methylated histones, and a C4H2C2-type
(Figure 10). The zinc finger domains have been shown to interact with the AO and the SWIRM domains, resulting in a change in LSD2’s active site, which may allow for enhanced inhibitor selectivity\textsuperscript{67}. 
Figure 10. This figure was modified from Zhang et al and Anand et al $^{41,67}$. The domain arrangements of both LSD2 and LSD1.

The LSD1 homolog LSD2 contains a zinc finger, SWIRM, and an AO domain. LSD1 contains a SWIRM, AO, and Tower domain.
MAO A/B enzymes have about 20% amino acid sequence identity compared to LSD1. Such enzymes are found in neurons and astroglia of the central nervous system, as well as in the liver, gastrointestinal tract, placenta, and platelets. Their principal role is to oxidatively deaminate monoamines, and their main substrates include epinephrine, norepinephrine, serotonin, melatonin, phenethylamine, benzylamine, dopamine, tyramine, and tryptamine. MAO metabolism of neurotransmitters explains their targeting by established antidepressant drugs such as phenelzine, pargyline, and tranylcypromine. These antidepressant drugs work as suicide, or mechanism-based inactivators of MAOs. Due to the similarity of LSD1 and MAOs, insights into MAOs and their inhibitors can be crucial in designing and discovering novel inhibitors of LSD1.
Role of LSD1 in Cancer

LSD1 plays a crucial role in higher eukaryotes as genetic deletion of LSD1 from mice has been shown to result in embryonic lethality, with the arrest of embryonic development at or before embryonic day 5.5\textsuperscript{39}. A variety of studies have indicated that LSD1 is important in EMT transition\textsuperscript{58,70}, proliferation\textsuperscript{71}, differentiation\textsuperscript{72}, haematopoiesis\textsuperscript{73–75}, adipogenesis\textsuperscript{76}, and maintenance of DNA methylation\textsuperscript{39}. Furthermore, dysregulation of LSD1, such as overexpression, has been suggested to play a role in tumorigenesis\textsuperscript{77}.

Several studies have found a direct link between LSD1 overexpression and cancer and have suggested how LSD1 can potentiate tumorigenesis through various pathways. For example, Lv et al found that overexpression of LSD1 increases tumor cell proliferation, migration, and invasion in non-small cell lung cancer\textsuperscript{78}. Lim et al found that LSD1 is highly expressed in ER-negative breast cancers, and that inhibiting LSD1 results in growth inhibition of breast cancer cells\textsuperscript{51}. Metzger et al found that high levels of LSD1 correlate with the severity of prostate cancer\textsuperscript{36}. In prostate cancer specimens, LSD1 expression was correlated with known mediators of prostate cancer progression such as VEGF-A. siRNA depletion of LSD1 in prostate cancer cells decreased VEGF-A, which blocked androgen induced PSDA and Tmprss2 expression. Additionally, chemically inhibiting LSD1 reduced proliferation of both androgen (LnCaP) and androgen-independent cells (LnCaP: C42, PC3)\textsuperscript{79}. Ding et al found that LSD1 was up-regulated in colon cancer tissue samples, and that LSD1 expression correlated with metastasis. Inhibiting LSD1 in colon cancer cell lines resulted in reduced proliferation and migration of the cells\textsuperscript{80}. The knockout of LSD1 in colorectal cancer cells (HCT116) resulted in an
increased population of cells in the G1-phase of the cell cycle\textsuperscript{81}, as well as reduced cell proliferation and tumorigenicity\textsuperscript{82}. Qin et al found pancreatic cancer patient tissue samples to have increased levels of LSD1 protein levels, and that knockdown of LSD1 slowed proliferation and tumorigenicity of the cancer cells. The growth of the pancreatic cancer cells was shown to be due to LSD1 synergizing with the Hypoxia Inducible Factor-1alpha (HIF1alpha), which together maintain the glycolytic process that contribute to increased proliferation\textsuperscript{83}. Ding et al found that LSD1 was overexpressed in ovarian endometriomas, and that inhibiting LSD1 with tranylcypromine reduced cellular proliferation, cell cycle progression, and invasiveness\textsuperscript{84}. Lan et al found LSD1 to be elevated in bladder cancer, and that knockdown of LSD1 suppressed bladder cancer cell line proliferation. The bladder cancer cell proliferation was proposed to be due to LSD1 co-localizing with bladder cancer stem cells in the basal layer of bladder carcinoma tissue, ultimately playing a role in maintaining the pluripotency\textsuperscript{85}. Konovalov et al found LSD1 to be overexpressed in stage IIIC and high-grade ovarian tumors, and that inhibiting LSD1 in ovarian cancer cell lines induced cytotoxicity\textsuperscript{86}. Yu et al found high expression of LSD1 to correlate with the severity of esophageal squamous cell carcinoma (ESCC) in patients, as well as dose dependent attenuation in migration of ESCC cells in vitro after LSD1 inhibition\textsuperscript{87}. Zhao et al found LSD1 expression to correlate with human hepatocellular carcinoma (HCC) severity in HCC tissues, and that knockdown of LSD1 expression in HCC cells (SMMC-7721) resulted in decreased cell proliferation\textsuperscript{88}. Yuan et al found that LSD1 was up-regulated in tongue cancer cells, as well as in tongue squamous cell carcinoma samples. It was shown that LSD1 expression associated with tumor size, pathological grade, and reduced survival of patients\textsuperscript{89}. Bennani-Baiti et al
found LSD1 to play a role in sarcoma pathology. Specifically, LSD1 was found to be overexpressed in several sarcoma types, including rhabdomyosarcoma, synovial sarcoma, chondrosarcoma, Ewing’s sarcoma, and osteosarcoma\textsuperscript{90}. Thus, LSD1 overexpression has been implicated in numerous solid-tumors, and therefore LSD1 is an intriguing target for novel solid-tumor therapeutics.

Epigenetic changes are linked to many hematopoietic malignancies, such as acute leukemias, which are disorders where there is an uncontrolled self-renewal, proliferation, and impaired differentiation of leukemic stem cells (LSC)\textsuperscript{91}. Targeting LSD1 with inhibition has thus been looked at for therapeutic potential in non-solid tumors, such as in leukemia\textsuperscript{92–95}. LSD1 was shown to be essential in regulating LSC by activating LSC associated oncogenic target genes. This was further verified by showing that knockdown of LSD1 resulted in AML cell impairment of differentiation and apoptosis, as well as the inability to form colonies, which is consistent with LSC potential loss\textsuperscript{93,95}. Thus, LSD1 inhibition may have promise in combating AML.

The re-expression of genes as a result of LSD1 inhibition in cancer cell lines may antagonize LSD1’s role in tumorigenesis, and several studies have investigated these mechanisms. For example, in the Calu-6 (anaplastic epithelial lung carcinoma) cell line, tumor suppressor genes such as SFRP2, HCAD (CDH13), and GATA4 have been shown to be modulated by LSD1\textsuperscript{3}. Additionally, in the HCT116 (colon carcinoma) cell line, tumor suppressors SFRPs and GATA have been implicated\textsuperscript{96}. In both pancreas and breast cancer cells, mRNA levels of LSD1 have been found to correlate inversely with levels of the tumor suppressor Lefty1\textsuperscript{97}. E-cadherin has also been shown to be reduced under conditions when LSD1 expression is elevated in colon cancer specimens\textsuperscript{98}. Additionally,
E-cadherin has also been shown to re-expressed by chemically inhibiting LSD1 in several human acute myeloid leukemia cell lines\textsuperscript{92}. Such changes in gene expression show that inhibiting LSD1 may result in the re-expression of important genes that may have the ability to inhibit cancer cell growth.
Other Roles of LSD1

LSD1 has also been implicated in brain function and dysfunction, indicating its potential as a target for therapeutics against neurological diseases. It has been shown that LSD1 may regulate long-term memory formation\textsuperscript{99}. Additionally, LSD1’s distribution in the brain is up-regulated under ischemic conditions\textsuperscript{100}. A splice variant of LSD1, called neuroLSD1, has been identified in mammalian brain. Phosphorylation of neuroLSD1 can induce dissociation of the CoREST complex, reactivating repressed target genes in the brain. The ratio between LSD1 and neuroLSD1 has been observed to change in response to neuronal activation, and this may be important in various pathogenic events associated with neurological disorders\textsuperscript{101}. Exploring the possibility of LSD1 inhibition to confer neuroprotection is therefore of some interest.

LSD1 has also been investigated as a target for antiviral therapies. Inhibiting LSD1 has been found to result in methylation across viral lytic immediate-early (IE) gene promoters, resulting in the suppression of viral IE gene expression\textsuperscript{37}. This suggests that LSD1 can play a role in blocking viruses, such as herpesvirus, from reactivation from latency\textsuperscript{37}. This was further confirmed when Liang et al also found that LSD1 inhibition in cells with herpes simplex virus (HSV) can potently block IE gene expression and suppress viral reactivation from latency, confirming LSD1’s potential as a target in novel antiviral therapies\textsuperscript{102}. Furthermore, Bag et al. has shown that LSD1 chemical inhibition can prevent LSD1 and the IE complex from binding IE gene promoters, which ultimately results in potent anti-HSV-1 activity\textsuperscript{103}.

Regarding metabolic control, inhibiting LSD1 has been shown to stimulate de novo glucose synthesis and reduce intracellular glycogen regulation\textsuperscript{104}. In lipid
homeostasis, Prospero-related homeobox (Prox1) can interact with LSD1 to recruit LSD1 to repress CYP7A1 gene expression. As CYP7A1 is responsible for catalyzing the first step in bile acid synthesis in the liver, targeting LSD1 can impact lipid regulation\textsuperscript{105}. In addition, blood pressure sensitivity to dietary salt intake was found to be affected by LSD1 by the alteration of renal Na(+) handling\textsuperscript{106}. LSD1 chemical inhibition during a high-salt diet was shown to increase blood pressure, with increased vascular contraction and reduced relaxation, signifying LSD1’s role in the regulation of arterial hypertension\textsuperscript{107}.

Additionally, other miscellaneous roles of LSD1 in various pathways have been reported. For example, LSD1 was found to play a role in the DNA damage response pathway, where it is recruited directly to sites of DNA damage along with E3 ubiquitin ligase RNF168. Knockdown of LSD1 was shown to result in hypersensitivity to gamma-irradiation and increased homologous recombination\textsuperscript{108}. LSD1 has also been shown to modulate osteogenic differentiation of human adipose-derived stem cells by regulating osteogenesis-associated gene expression. LSD1 inhibitors were found to boost osteogenic differentiation, which could ultimately lead to the use of LSD1 inhibitors for the development of bone by tissue engineering\textsuperscript{109}. Additionally, it was found that feeding mice a high-fat diet increased levels of LSD1 in the white adipose tissue and induced mitochondrial activity to regulate metabolism, ultimately limiting weight gain. This has linked LSD1 to metabolic adaptation in white adipose tissues as there was LSD1-dependent expression of genes, such as those involved in oxidative phosphorylation, which promoted the formation of islets of metabolically active brown-like adipocytes in
white adipose tissue\textsuperscript{110}. Such studies show the extensive range of roles LSD1 can participate in, as well as potential use of LSD1 inhibitors against various serious diseases.
**LSD1 Inhibitors**

Synthetic inhibitors of LSD1’s catalytic activity are predicted to reactivate gene expression of silenced genes, such as tumor suppressors, and thus to be beneficial in the treatment of diseases, including cancer. Several laboratories have pursued development of LSD1 inhibitors, and have reported inhibitors including peptides (1,2), MAO A/B inhibitors (MAOIs) and derivatives thereof (3–6), polyamines (7), and guanidine containing compounds (8) (Figure 11). Such compounds show great potential to be used as novel therapeutics against various diseases.
Figure 11. Previously published LSD1 inhibitors.

LSD1 inhibitors include: (1) Histone H3-21mer peptides with various modified lysine residues, X; (2) N-terminal SNAIL1 20-mer peptide; (3) Phenelzine; (4) Tranlycypromine; (5), (6) Tranlycypromine analogs; (7) Polyamine analog; (8) Guanidinium containing compound.
Peptide inhibitors of LSD1 have shown to be quite potent and effective against LSD1. Peptide 1 is an example from a series of LSD1 peptide inhibitors, where warhead groups were placed on Lys4 of the N-terminal H3 tail 21-mer, yielding $K_{i\text{(inact)}}$ values in the nanomolar range\textsuperscript{111}. Peptide 2 is a 20-mer N-terminal derivative of SNAIL1, a transcription factor that recruits and directs LSD1 to specific genes involved in regulating EMT, which is required for cancer cell invasion\textsuperscript{122}. The SNAIL1 derived peptides, including peptide 2, were found to bind to LSD1’s active site and compete with the H3 substrate as an endogenous inhibitor. Further development of such peptidomimetic small molecule inhibitors show potential as the first six amino acids of peptide 2 have been found to be sufficient to bind and inhibit LSD1/CoREST at low micromolar concentrations\textsuperscript{113}. In addition, Ogasawara et al explored binding tranylepyromine, and analogs thereof, to the Lys4 of the N-terminal H3 tail 21-mer for an LSD1-targeted delivery of the inhibitor\textsuperscript{123}.

One strategy in developing LSD1 inhibitors that has shown great promise has been the development of tranylepyromine analogs, such as compounds 5 and 6\textsuperscript{119,120}. Tranylepyromine is a mechanism-based inactivator of MAO enzymes. As an irreversible inhibitor it is oxidized by the MAOs to a more chemically reactive species, which can subsequently covalently modify and kill the MAOs. One proposed mechanism of inactivation of MAOs by tranylepyromine involves a one-electron oxidation and facile cyclpropyl ring opening, forming two possible benzylic carbon radicals\textsuperscript{124–126}. One of the benzylic radicals can then react with C(4a) of FAD to form atropaldehyde-FAD. Another one of the benzylic radicals can also then react with C(4a) of FAD to form cinnamaldehyde-FAD, which can then further undergo cyclization to produce a five-
membered ring linking both the C(4a) and N(5) positions of FAD (Figure 12). Another potential mechanism of inactivation of MAOs by tranylcypromine leads to the formation of N(5) adduct A and N(5) adduct B (Figure 13)\textsuperscript{114,124,127}. 

Figure 12. This figure was taken from Khan et al. Proposed mechanisms by which tranylecypromine can modify FAD to form atropaldehyde-FAD, cinnamaldehyde-FAD, and/or tranylecypromine-FAD adducts to inhibit MAOs.
Figure 13. This figure was taken from Khan et al\textsuperscript{124}. Proposed mechanism by which tranylcyromine inactivates MAOs by generation of N(5) adducts.
As MAOs and LSD1 have many similarities, tranylcypromine has in fact been tested for LSD1 inhibition, and has been found to be a weakly potent LSD1 mechanism-based inactivator with a \( K_{i\text{inact}} \) of 0.5 mM and a \( k_{i\text{inact}} \) of 0.67 min\(^{-1}\)\(^{125,126,128}\). There has since been many different tranylcypromine analogs developed as LSD1 inhibitors. Tranylcypromine analogs modified by aryl attachments have been shown to produce more selective LSD1 inhibitors with enhanced potency\(^{99,114,115,129–132}\). In 2009, Ueda et al first synthesized tranylcypromine analogs that linked a homoserine with the phenyl ring of tranylcypromine through an ether bond. Such compounds gave IC\(_{50}\) values of 1.9 to 22 \( \mu \)M and showed various cancer cell line growth inhibitory effects with a GI\(_{50}\) value as low as 9 \( \mu \)M\(^{129}\). In 2010, Binda et al also synthesized tranylcypromine analogs with additional phenyl rings added to the tranylcypromine phenyl ring through an amide bond. The compounds displayed \( K_i \) values as low as 1.1 \( \mu \)M, and showed growth inhibition of acute promyelocytic leukemia cells that acted synergistically with retinoic acid\(^{114}\). Later in 2010, Mimasu et al reported 2-pentafluorphenylcyclopropylamine compounds as well as other derivatives with bulky hydrophobic groups added to tranylcypromine, which gave \( K_i \) values as low as 0.61 \( \mu \)M\(^{115}\). In both 2010 and 2011, several N-alkylated tranylcypromine analogs were reported that inhibited LSD1 with \( K_i \) values of as low as 5 nM, although full characterization of the compounds has not yet been disclosed\(^{130–132}\). In 2012, more N-alkylated derivatives of tranylcypromine also showed highly potent brain-penetrant LSD1 inhibitory activities with IC\(_{50}\) values as low as 30 nM, although full enzymologic details have not yet been reported\(^{99}\). Nevertheless, no LSD1 inhibitor has yet demonstrated success in clinical trials.
MAO inhibitors other than tranylcypromine have also been tested for LSD1 inhibition. Culhane et al tested phenelzine and pargyline, both widely used MAO inhibitors, for LSD1 inhibition, as well as examined multiple novel H3 tail peptide analogues containing various warhead groups for LSD1 inhibition\textsuperscript{111 118}. Interestingly, it was found that phenelzine was a more potent small molecule LSD1 inhibitor than previously suggested, and that the hydrazino moiety was a highly potent time-dependent inhibitor of LSD1\textsuperscript{111}. The mechanism of inactivation of LSD1 by phenelzine was proposed to be initiated by a two electron oxidation by flavin that yields a diazene that may be converted to the diazonium species, which can be attacked by the flavin, thus yielding a FAD-inhibitor adduct and N\textsubscript{2}\textsuperscript{111} (Figure 14).
Figure 14. Proposed LSD1 inhibition mechanism by phenelzine.

FAD can oxidize the hydrazine moiety of phenelzine to a diazonium species, which can be attacked by the flavin to yield a FAD-inhibitor adduct and N₂, resulting in the irreversible inhibition of LSD1.
Various other LSD1 inhibitors have been studied, and show varying potencies and effects on cancer cells. Polyamine analogs, including compound 7, display low µM potencies against LSD1⁹⁶,¹³³. Guanidine compounds, such as compound 8, also show low µM potency and have been found to inhibit the growth of pluripotent cancer cells that express stem cell markers Oct4 and Sox2, such as teratocarcoma, embryonic carcinoma, and seminoma or embryonic stem cells¹¹⁷. Low molecular weight amidoximes exhibit IC₅₀ values as low as 15.6 µM, and increase levels of H3K4me2 in Calu-6 lung carcinoma cells³. Namoline, a reversible LSD1 inhibitor, blocks androgen-induced LNCaP cell proliferation and xenograft tumor growth and shows an IC₅₀ value of 51 uM¹³⁴. 3-amino/guanidine substituted phenyl oxazoles have also been studied as reversible LSD1 inhibitors, and impede the viability of both cervical and breast cancer cells¹²¹. Desferrioxamine, a model iron chelator, has been shown to cause the down-regulation of LSD1, linking abnormalities in iron metabolism during cancer and LSD1¹³⁵. Natural polyphenols, such as resveratrol, curcumin, and quercetin, have also been studied as inhibitors of LSD1. Independent of their antioxidant properties, they inhibit LSD1 and reduce myogenic gene expression and differentiation of C2C12 fibroblasts¹³⁶. Cryptotanshinone was also shown to inhibit growth of the androgen receptor (AR)-positive prostate cancer cell line, LNCaP, by disrupting the interaction between AR and LSD1¹³⁷. Geranylgeranoic acid and its derivatives have also been observed to inhibit LSD1, and in human neuroblastoma SH-SY5Y cells have been shown to induce NTRK2 gene expression¹³⁸. Novel N’-(1-phenylethylidene)-benzohydrazides inhibit LSD1 in a potent, specific, and reversible manner, and block proliferation of several cancer cell lines including breast and colorectal¹³⁹. Lastly, cytotoxic triazole-dithiocarbamate based
inhibitors of LSD1 antagonize cell migration and invasion, of LSD1 overexpressing gastric cancer cell lines MGC-803 and HDG-27140. Thus, there is a great deal of interest in the development of LSD1 inhibitors.
LSD1’s Potential as a Drug Combination Target

Drug combination therapies have many advantages, including lowering doses of toxic agents, reducing the potential for drug resistance, and inhibiting multiple independent pathways that converge on a single essential molecular process\textsuperscript{141}. Histone modifying enzymes are great candidates for drug combinations as they work close together in changing chromatin structure, and consequently gene expression. Additionally, it has been previously shown that affecting one histone modifying enzyme can have effects on others. For example, Meng et al showed that treating ovarian cancer cells (SKOV3) with trichostatin A (TSA) and decitabine, HDAC and DNMT1 inhibitors respectively, lowered the expression levels of LSD1 and increased H3K4me2 levels\textsuperscript{142}. Vasilatos et al also showed that knockdown of LSD1 expression in breast cancer cells decreased mRNA levels of HDAC isozymes\textsuperscript{143}. Thus, targeting LSD1 and other epigenetic enzymes simultaneously offers therapeutic promise.

As mentioned, LSD1 and HDAC1/2 are found in cellular complexes, thus inhibiting both LSD1 and HDACs simultaneously has potential to yield additive or synergistic pharmacologic impact. In fact, previous studies have shown that targeting both LSD1 and HDAC1/2 in cells results in synergistic cell proliferation inhibition. For example, it was found that treating triple-negative breast cancer cells with both pargyline, a potential LSD1 inhibitor, and SAHA, an established HDAC inhibitor, lead to synergistic growth inhibition and apoptosis\textsuperscript{143}. Additionally, co-treatment with LBH-589, an HDAC inhibitor, and SP2509, an LSD1 inhibitor, SP2509, showed synergy in blocking the growth of cultured and primary AML blasts, and improved survival of mice engrafted with human AML cells with low toxicity\textsuperscript{144}. Other combination therapies
targeting LSD1 have also been shown to have promise. For example, Xu et al looked at
the combined effect of retinoic acid and LSD1 siRNA inhibition on cell death in a human
neuroblastoma cell line, SH-SY5Y. It was found that combined treatment led to higher
rates of cell death than single gene targeting, implying that neuroblastoma can be better
treated with the addition of an epigenetic drug inhibitor, such as an LSD1 inhibitor\textsuperscript{145}.

Targeting two different enzymes with one compound is another interesting
strategy that has shown promise. Rotili et al synthesized compounds that combine the
moiety of an LSD1 inhibitor, tranylcypromine, with that of JMJD2 inhibitor 2-
oxoglutarate templates (Figure 15)\textsuperscript{146}. The compounds were able to in fact target both
LSD1 and JMJD2 enzymes in vitro, and showed significant inhibition of both LNCaP
and HCT116 cell proliferation. Such a strategy has potential to be applied to other drug
combinations, such as by combining moieties of LSD1 inhibitors with those of various
HDAC inhibitors.
Figure 15. This figure was taken from Rotili et al\textsuperscript{146}. Structures of dual drug inhibitors.

An LSD1 inhibitor, tranylcypromine, can be combined with various moieties of JmjC inhibitors to form compounds that serve as dual drug inhibitors.
Chapter 2. Synthesis of Phenelzine Analogue s

Part 2A: Introduction

As discussed in Chapter 1, LSD1 dysregulation can lead to the disruption of many crucial biological processes. Accordingly, the potential use of LSD1 inhibitors against various serious diseases has become an important focus of study. Recently, there has been an emergence of many novel LSD1 inhibitors in the literature (Figure 11). Peptide 1, a histone H3-21 peptide, and peptide 2, a SNAIL1 20-mer peptide, are one class of LSD1 inhibitors. Additionally, MAOIs, such as compounds 3-4, have been tested for LSD1 inhibition due to similarities in the structural and mechanistic features of the MAOs and LSD1. Many novel compound 4 analogs have in fact been developed, such as compounds 5-6. Polyamine analogs, such as compound 7, and guanidinium group containing small molecules, such as compound 8, have also been studied as novel LSD1 inhibitors. There is, however, room for improvement in terms of small molecule LSD1 inhibitor potency and selectivity. More potent and selective LSD1 inhibitors could enhance their clinical potential as well as reduce off-target effects, allowing for a more precise understanding of LSD1’s role in biology.

As mentioned previously, multiple MAOIs have been tested for LSD1 inhibition, including phenelzine (compound 3), tranylcypromine (compound 4), and pargyline. The aforementioned MAOIs are all non-selective, irreversible inhibitors used clinically to treat psychiatric diseases such as major depressive disorder, panic, and anxiety. As MAOs metabolize monoamine neurotransmitters, such as epinephrine and norepinephrine, the MAOIs yield their therapeutic benefits by increasing the synaptic
concentrations of such catecholamines to enhance stimulation of the neuroadrenergic synapse. But, such MAOIs are generally not first line agents used as they can cause a number of side effects, such as hypertension. This is due to MAOs being found in many tissues throughout the body, as well as MAOIs being commonly involved in drug-drug interactions and dietary substance interactions. For example, high blood pressure can occur when taking MAOIs and eating foods that contain high amounts of tyramine, as tyramine is a substrate of MAOs and a compound that increases blood pressure. Although pargyline was taken off the market in 2007, both phenelzine and tranylcypromine have continued to be important agents used for the treatment of patients that are resistant to other antidepressant medications.

Lee et al originally tested MAOIs, such as tranylcypromine and phenelzine, for LSD1 inhibition\textsuperscript{147}. The results showed that tranylcypromine was more potent than phenelzine in inhibiting LSD1, with phenelzine showing inhibition of H3K4 demethylation by LSD1 at 200 µM\textsuperscript{147}. This study paved the way for multiple tranylcypromine analogs to be developed as LSD1 inhibitors as described in Chapter 1. But, phenelzine was later shown by a previous Cole lab member, Jeff Culhane, to be a better LSD1 inhibitor than previously thought\textsuperscript{111}. In fact, tranylcypromine was found to be less potent than phenelzine for LSD1 inhibition. In addition, a histone H3 21-mer peptide with Lys4 modified to contain a hydrazine moiety was found to be one of the most potent suicide inactivators known for LSD1 with a $K_{\text{inact}}$ of 4.35 nM\textsuperscript{111}. Thus, an array of novel small molecule phenelzine analogs was synthesized to build upon the discovery that small molecule arylalkyl hydrazines have the potential to be potent and selective LSD1 inhibitors.
The first phenelzine analogs to be synthesized were compounds 9-11 (Figure 16), which were synthesized by previous Cole lab members, Shonoi Ming, Robert Hsiao, Rong Huang, and Lindsay Avery. The synthesis of compounds 9-11, including a detailed scheme (Appendix I Figure 1), are described in Appendix I. Compounds 9-11 varied by having variations in the alky chain length between the phenyl ring and the hydrazine moiety, an ether substitution in the alkyl chain, methoxy substitutions to the para position of the phenyl ring, as well as small substitutions, such as methyl and acetyl, to the hydrazine moiety. This study expanded the array of phenelzine analogs synthesized.
Figure 16. Smaller phenelzine analogues synthesized and characterized.

Compounds were synthesized and characterized by previous Cole lab members, Shonoi Ming, Robert Hsiao, Rong Huang, and Lindsay Avery.
Part 2B: Results and Discussion

Based on prior findings that phenelzine was a moderately potent, mechanism-based inactivator of LSD1, and to further expand the structure-activity relationship (SAR) study of the previously synthesized compounds, phenelzine analogs were synthesized that had various larger substitutions on the para position of the phenyl ring of phenelzine. Compounds 12a, 12b, 12d, and 14, were first synthesized in this study, and later other phenelzine analogs were synthesized by Jay Kalin to complete the SAR study (Figure 17). Compounds 12a, 12b, 12d, and 14 had various larger hydrophobic groups attached to the para position of the phenyl ring on phenelzine. The rest of the compounds, including 12c, 12e-m, 13, and 15a-b, had various substitutions on the distal phenyl ring of compound 12d, as well as additional variations in the alkyl linker.
Figure 17. Larger phenelzine analogs synthesized and characterized.

Compounds 12a, 12b, 12d, and 14, were first synthesized in this study, and later other phenelzine analogs, including 12c, 12e-m, 13, and 15a-b, were synthesized by Jay Kalin to complete the SAR study.
Compound 12a was synthesized using the starting material, 2-(4-aminophenyl)ethanol, which was reacted with excess benzoic anhydride in the presence of the base, N,N-diisopropylethylamine, to acylate both the aryl amine and the aliphatic alcohol. The diacylated intermediate compound was then exposed to sodium hydroxide to saponify the ester bond formed. The resulting N-[4-(2-Hydroxyethyl)phenyl]benzamide (16a) was then reacted with carbon tetrabromide and triphenylphosphine to yield N-[4-(2-Bromoethyl)phenyl]benzamide (17a) via the Appel reaction. The last step of the synthesis involved reacting the alkyl bromide, 17a, with excess anhydrous hydrazine, after which HCl was added to yield the salt form of the final product, compound 12a (N-[4-(2-Hydrazinylethyl)phenyl]benzamide dihydrochloride) (Figure 18).
Figure 18. Synthesis of compound 12a.
Compounds 12b and 12d were synthesized using the starting materials, phenylacetic acid and 4-phenylbutyric acid, respectively, which were reacted with thionyl chloride to form the acid chloride intermediates. The compounds were then reacted with 2-(4-aminophenyl)ethanol in the presence of the base, N,N-diisopropylethylamine, to yield diacylated products. The esters were then saponified using sodium hydroxide to form appropriate alcohols. The resulting compounds, N-[4-(2-Hydroxyethyl)phenyl]-2-phenylacetamide (16b) and N-[4-(2-Hydroxyethyl)phenyl]-4-phenylbutanamide (16d), were then reacted with carbon tetrabromide and triphenylphosphine to yield N-[4-(2-Bromoethyl)phenyl]-2-phenylacetamide (17b) and N-[4-(2-Bromoethyl)phenyl]-4-phenylbutanamide (17c), respectively. Lastly, the alkyl bromides, 17b and 17c, were treated with excess anhydrous hydrazine, after which HCl was added to yield the salt form of the final products, compound 12b (N-[4-(2-Hydrazinylethyl)phenyl]-2-phenylacetamide dihydrochloride) and 12d (N-[4-(2-Hydrazinylethyl)phenyl]-4-phenylbutanamide dihydrochloride), respectively (Figure 19).
Figure 19. Synthesis of compounds 12b and 12d.
Compound 14 was synthesized using the starting material, 1-(3-bromopropyl)-4-(phenylmethoxy)-benzene, which was reacted with excess anhydrous hydrazine, after which HCl was added to yield the salt form of the final product, compound 14 {3-[4-(Benzyloxy)phenyl]propyl}hydrazine (Figure 20).
Figure 20. Synthesis of compound 14.
The synthesis of compounds 12c, 12e-m, 13, and 15a-b, including detailed schemes (Appendix I Figures 3-4), are described in Appendix I. After synthesis, each compound was characterized via $^1$H NMR, $^{13}$C NMR, and high-resolution mass spectrometry. All final compounds were estimated to be >97% pure as determined by NMR. (See Appendix II for compound 9-15 $^1$H NMR and $^{13}$C NMR spectra.)
Part 2C: Materials and Methods

General. NMR spectra were recorded on either a Bruker 400 MHz (1H, 400 MHz; 13C, 101 MHz), a Varian 400 MHz (1H, 400 MHz), or a Bruker 500 MHz (1H, 500 MHz; 13C, 125 MHz) spectrometer. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (J) are in hertz (Hz). The following abbreviations were used to describe multiplicity: br (broad), s (singlet), d (doublet), t (triplet), quin (quintet), m (multiplet), dd (double doublet), td (triple doublet), dt (double triplet). NMR spectra were processed using ACD/NMR Processor Academic Addition, version 12.01 (Advanced Chemistry Development, Inc., Toronto, On, Canada, www.acdlabs.com, 2013). When DMSO-d6 was used as the sole NMR solvent, the hydrazine protons were visible; however, the peaks were very broad and could not be accurately integrated. High resolution ESI/APCI spectra were recorded on either an Agilent LCTOF instrument at the Mass Spectrometry Facility of the University of California, Riverside (NSF grant CHE-0541848) or a Shimadzu IT-TOF instrument at the Research Resources Center Mass Spectrometry Facility of the University of Illinois at Chicago. Solvents were purchased from Aldrich as anhydrous and used as received. Starting materials and reagents were purchased from commercial sources and were also used as received. Reaction progress was monitored by thin layer chromatography (TLC) using pre-coated, glass supported silica gel plates (Sigma-Aldrich F254, 60 Å pore size, 250 μM thickness). All final compounds are estimated to be >97% pure as determined by NMR. (See additional supplementary file for all NMR spectra.)
**General Procedure C for hydrazine displacement reactions.** Under nitrogen, the appropriate alkyl bromide (1 mol equiv) was dissolved in EtOH (3 mL/mmol) in a round-bottomed flask. To this stirred solution was added anhydrous hydrazine (20 mol equiv) dropwise. The solution was then heated to reflux for 0.5-1.75 h with monitoring by TLC. After cooling, EtOH was removed in vacuo and 1 N NaOH (80 mL) was added. The aqueous layer was extracted with DCM (3 x 80 mL) and dried in vacuo. The base was then dissolved in MeOH (10 mL) and 6 N HCl (2.5-3.5 mL/mmol) was added dropwise while stirring the solution on ice. The solution was left to stir on ice for 10-15 min after which the precipitate was filtered and washed with cold Et₂O to yield the desired product.

**General Procedure D for amide coupling.** The appropriate acid (1 mol equiv) was dissolved in DCM (10 mL, 0.25 mL/mmol). The stirred solution was then placed in an ice bath and thionyl chloride (5 mol equiv) was slowly added. After the addition was complete, the resulting solution was stirred on ice for 10 min and then transferred to an oil bath and heated to 55 °C. The solution was then stirred for 7.25-7.50 h and monitored by TLC. The solution was then cooled to RT and dried to furnish the appropriate acid chloride. While drying the acid chloride, 2-(4-aminophenyl)ethanol (2.00 g, 14.6 mmol) was placed under nitrogen and dissolved in DCM (20 mL). The stirred solution was placed on ice and N,N-diisopropylethylamine (18 mL, 102.1 mmol) was slowly added followed by the slow addition of the solid dried acid chloride. After the addition was complete, the resulting solution was stirred overnight and allowed to warm to RT. DCM (100 mL) was added and the organic phase was washed with 1 N HCl (100 mL), saturated sodium bicarbonate (100 mL), brine (100 mL), and dried in vacuo. The solid
was then dissolved in MeOH (100 mL) at RT and to this stirred solution, 1 N NaOH (20-50 mL) was added in portions. Stirring was continued for 6 h and the reaction was monitored by TLC. After completion, the solution was concentrated in vacuo and EtOAc (100 mL) was added. The organic layer was washed with 1 N HCl (100 mL), saturated sodium bicarbonate (2 x 100 mL), brine (100 mL), and then dried to furnish the crude product, which was further purified by flash chromatography (SiO₂, 50% hexanes/EtOAc) to yield the desired product.

**General Procedure E for bromination.** Under nitrogen, the appropriate alcohol (1 mol equiv) was dissolved in DCM (8-20 mL/mmol) in a round-bottomed flask. To this stirred solution was added triphenylphosphine (2 mol equiv) and carbon tetrabromide (2 mol equiv). The resulting solution was stirred for 6 h and monitored by TLC. Upon completion, the solution was concentrated in vacuo to give the crude product, which was further purified by flash chromatography (SiO₂, 20-25% hexanes/EtOAc) to furnish the desired product.

**N-[4-(2-Hydroxyethyl)phenyl]benzamide (16a):** Under nitrogen, 2-(4-aminophenyl)ethanol (2.00 g, 15.0 mmol) was dissolved in DCM (20 mL). The stirred solution was placed on ice and N,N-diisopropylethylamine (22.9 mL, 131 mmol) was slowly added followed by the slow addition of benzoic anhydride (14.8 g, 66.0 mmol). After the addition was complete, the solution was stirred overnight and allowed to warm to RT. DCM (100 mL) was added to this solution and the organic phase was washed with 1 N HCl (100 mL), saturated sodium bicarbonate (100 mL), brine (100 mL), and dried in
vacuo. The intermediate was then dissolved in MeOH (100 mL). To this stirred solution, 1 N NaOH (50 mL) was added in portions. The resulting solution was stirred at RT for 6 h and monitored by TLC. The solution was then concentrated in vacuo and EtOAc (100 mL) was added. The organic layer was washed with 1 N HCl (100 mL), saturated sodium bicarbonate (2 x 100 mL), brine (100 mL), and then dried to furnish the crude product, which was further purified via flash chromatography (SiO2, 50% hexanes/EtOAc) to yield the title compound as an off-white solid (0.600 g, 17%). \(^1\)H NMR (400 MHz, MeOD): \(\delta 7.92 (m, 2H), 7.54 (m, 5H), 7.24 (m, 2H), 3.75 (t, J = 7.1 Hz, 2H), 2.82 (t, J = 7.1 Hz, 2H).

\(N\)-[4-(2-Hydroxyethyl)phenyl]-2-phenylacetamide (16b): The title compound was synthesized from phenylacetic acid (5.95 g, 43.7 mmol) according to general procedure D and isolated as an off-white solid (3.20 g, 86%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 7.41 (m, 2H), 7.35 (m, 5H), 7.15 (d, J = 8.6 Hz, 2H), 7.09 (s, 1H), 3.81 (t, J = 6.6 Hz, 2H), 3.74 (s, 2H), 2.81 (t, J = 6.4 Hz, 2H).

\(N\)-[4-(2-Hydroxyethyl)phenyl]-4-phenylbutanamide (16d): The title compound was synthesized from 4-phenylbutyric acid (7.18 g, 43.7 mmol) according to general procedure D and isolated to furnish the pure product as an off-white solid (6.20 g, 49%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 7.61 (s, 1H), 7.41 (d, J = 8.6 Hz, 2H), 7.29 (m, 2H), 7.21 (m, 3H), 7.13 (d, J = 8.3 Hz, 2H), 3.79 (t, J = 6.6 Hz, 2H), 2.80 (t, J = 6.6 Hz, 2H), 2.69 (t, J = 7.5 Hz, 2H), 2.32 (t, J = 7.5 Hz, 2H), 2.04 (quin, J = 7.5 Hz, 2H).
**N-[4-(2-Bromoethyl)phenyl]benzamide (17a):** The title compound was synthesized from **N-[4-(2-Hydroxyethyl)phenyl]benzamide (16a)** (0.600 g, 2.49 mmol) according to general procedure E and isolated to furnish the final product as an off-white solid (0.600 g, 79%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.98 (s, 1H), 7.86 (dd, $J_1 = 8.2$ Hz, $J_2 = 1.1$ Hz, 2H), 7.61 (d, $J = 8.6$ Hz, 2H), 7.50 (m, 3H), 7.21 (d, $J = 8.3$ Hz, 2H), 3.56 (t, $J = 7.5$ Hz, 2H), 3.15 (t, $J = 7.5$ Hz, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$): δ 165.76, 136.69, 135.06, 134.82, 131.81, 129.26, 128.72, 126.98, 120.44, 38.74, 33.04.

**N-[4-(2-Bromoethyl)phenyl]-2-phenylacetamide (17b):** The title compound was synthesized from **N-[4-(2-Hydroxyethyl)phenyl]-2-phenylacetamide (16b)** (0.750 g, 2.93 mmol) according to general procedure E and isolated to furnish the desired product as a an off-white solid (0.500 g, 49%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.36 (m, 8H), 7.12 (d, $J = 8.3$ Hz, 2H), 3.72 (s, 2H), 3.52 (t, $J = 7.5$ Hz, 2H), 3.11 (t, $J = 7.6$ Hz, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$): δ 169.12, 136.38, 134.93, 134.34, 129.44, 129.15, 129.10, 127.61, 120.04, 44.68, 38.63, 33.03. ESI-HRMS: calcd. for C$_{16}$H$_{16}$NOBr: [M+H]$^+$ = m/z 318.0497, found: [M+H]$^+$ = m/z 318.0488.

**N-[4-(2-Bromoethyl)phenyl]-4-phenylbutanamide (17c):** The title compound was synthesized from **N-[4-(2-Hydroxyethyl)phenyl]-4-phenylbutanamide (16d)** (0.700 g, 2.47 mmol) according to general procedure E and isolated to furnish the pure product as a off-white solid (0.500 g, 58%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.46 (d, $J = 8.3$ Hz, 2H), 7.31 (m, 3H), 7.20 (m, 5H), 3.54 (t, $J = 7.6$ Hz, 2H), 3.12 (t, $J = 7.6$ Hz, 2H), 2.71 (t, $J = 7.5$ Hz, 2H), 2.34 (t, $J = 7.6$ Hz, 2H), 2.07 (quin, $J = 7.5$ Hz, 2H). $^{13}$C NMR (101 MHz,
$\text{CDCl}_3$: $\delta$ 170.98, 141.26, 136.63, 134.68, 129.16, 128.46, 128.40, 126.01, 119.99, 38.69, 36.66, 34.99, 33.04, 26.81. ESI-HRMS: calcd. for $\text{C}_{18}\text{H}_{20}\text{NOBr}$: $[\text{M}+\text{H}]^+ = m/z$ 346.0808, found: $[\text{M}+\text{H}]^+ = m/z$ 346.0801.

$\text{N-[4-(2-Hydrazinylethyl)phenyl]benzamide dihydrochloride (12a)}$: The title compound was synthesized from $\text{N-[4-(2-Bromoethyl)phenyl]benzamide 17a}$ (0.400 g, 1.31 mmol) according to general procedure C and isolated to yield the product as a white powder (0.370 g, 91%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 10.32, (s, 1H), 7.97 (dd, $J_1 = 8.5$ Hz, $J_2 = 1.1$ Hz, 2H), 7.74 (d, $J = 8.6$ Hz, 2H), 7.54 (m, 3H), 7.20 (d, $J = 8.3$ Hz, 2H), 3.13 (t, $J = 7.6$ Hz, 2H), 2.85 (t, $J = 7.3$ Hz, 2H). $^{13}$C NMR (101 MHz, DMSO-$d_6$): $\delta$ 165.53, 137.74, 134.95, 133.26, 131.65, 128.80, 128.46, 127.78, 120.68, 51.37, 30.85. ESI-HRMS: calcd. for $\text{C}_{15}\text{H}_{17}\text{N}_3\text{O}$: $[\text{M}+\text{H}]^+ = m/z$ 256.1447, found: $[\text{M}+\text{H}]^+ = m/z$ 256.1444.

$\text{N-[4-(2-Hydrazinylethyl)phenyl]-2-phenylacetamide dihydrochloride (12b)}$: The title compound was synthesized from $\text{N-[4-(2-Bromoethyl)phenyl]-2-phenylacetamide 17b}$ (0.400 g, 1.16 mmol) according to general procedure C and isolated to yield the product as a white powder (0.188 g, 51%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 10.37 (s, 1H), 7.57 (d, $J = 8.3$ Hz, 2H), 7.32 (m, 4H), 7.24 (m, 1H), 7.14 (d, $J = 8.6$ Hz, 2H), 3.64 (s, 2H), 3.07 (t, $J = 7.6$ Hz, 2H), 2.79 (t, $J = 7.6$ Hz, 2H). $^{13}$C NMR (101 MHz, MeOD): $\delta$ 172.37, 138.22, 137.00, 136.84, 130.26, 130.15, 129.72, 128.08, 121.69, 57.22, 44.79, 34.39. ESI-HRMS: calcd. for $\text{C}_{16}\text{H}_{19}\text{N}_3\text{O}$: $[\text{M}+\text{H}]^+ = m/z$ 270.1605, found: $[\text{M}+\text{H}]^+ = m/z$ 270.1601.
\textit{N-[4-(2-Hydrazinylethyl)phenyl]-4-phenylbutanamide dihydrochloride (12d):} Under nitrogen, \textit{N-[4-(2-Bromoethyl)phenyl]-4-phenylbutanamide 17c (0.400 g, 1.15 mmol)} was dissolved in EtOH (4 mL). To this stirred solution was added anhydrous hydrazine (0.720 mL, 23.1 mmol) dropwise. The solution was then refluxed for 1 h and monitored by TLC. After cooling, EtOH was removed and 1 N NaOH (80 mL) was added. The aqueous layer was extracted with DCM (3 x 80 mL) and dried in vacuo. The hydrazine free base was then dissolved in MeOH (10 mL) and 6 M HCl (2 mL) was added dropwise while stirring the solution on ice. The solution was left to stir on ice for 10 min after which Et₂O (5 mL) was added and the reaction was concentrated in vacuo to yield a precipitate that was filtered and washed with cold Et₂O. The precipitate was dried to yield the product as a light yellow powder (0.132 g, 33%). \(^1\text{H NMR (400 MHz, DMSO-}d_6\text{)}: \delta 9.89 (s, 1H), 7.53 (d, \textit{J} = 8.5 \text{ Hz, 2H}), 7.29 (m, 2H), 7.19 (m, 3H), 7.14 (d, \textit{J} = 8.5 \text{ Hz, 2H}), 3.07 (t, \textit{J} = 7.8 \text{ Hz, 2H}), 2.78 (t, \textit{J} = 7.9 \text{ Hz, 2H}), 2.61 (t, \textit{J} = 7.6 \text{ Hz, 2H}), 2.31 (t, \textit{J} = 7.5 \text{ Hz, 2H}), 1.88 (quin, \textit{J} = 7.5 \text{ Hz, 2H}). \(^{13}\text{C NMR (101 MHz, DMSO-}d_6\text{)}: \delta 170.94, 141.76, 137.90, 132.41, 128.78, 128.39, 128.38, 125.85, 119.29, 51.42, 35.79, 34.70, 30.79, 26.92. \text{ESI-HRMS: calcd. for C}_{18}\text{H}_{23}\text{N}_3\text{O: }[\text{M+H}]^+ = m/z \ 298.1913, \text{ found: }[\text{M+H}]^+ = m/z \ 298.1914.

{3-[4-(Benzyloxy)phenyl]propyl}hydrazine (14): The title compound was synthesized from 1-(3-bromopropyl)-4-(phenylmethoxy)-benzene (400 mg, 1.30 mmol) according to general procedure C and isolated as a white powder (0.152 g, 34%). \(^1\text{H NMR (400 MHz, DMSO-}d_6\text{)}: \delta 7.37 (m, 5H), 7.12 (d, \textit{J} = 8.3 \text{ Hz, 2H}), 6.93 (dd, \textit{J}_1 = 8.6 \text{ Hz, } \textit{J}_2 = 3.0 \text{ Hz, 2H})}
2H), 5.06 (s, 2H), 2.87 (t, $J = 7.3$ Hz, 2H), 2.56 (t, $J = 7.3$ Hz, 2H), 1.83 (quin, $J = 7.1$ Hz, 2H). $^{13}$C NMR (101 MHz, DMSO-$d_6$): $\delta$ 156.68, 137.26, 133.11, 129.31, 128.46, 127.81, 127.69, 114.72, 69.16, 49.83, 31.16, 26.63. ESI-HRMS: calcd. for C$_{16}$H$_{20}$N$_2$O: [M+H]$^+$ = m/z 257.1654, found: [M+H]$^+$ = m/z 257.1648.
Chapter 3. Testing Phenelzine Analogues In Vitro for LSD1 Inhibition

Part 3A: Introduction

In vitro enzyme assays are crucial in the study and development of enzyme inhibitors. In vitro assays can determine whether a compound is a reversible or an irreversible inhibitor of a particular enzyme, as well as determine its potency and selectivity in a controlled system. Additionally, as in vitro enzyme inhibition assays can be conducted quickly and are relatively inexpensive, they are great tools for the high-throughput screening of compounds in order to find potential drug candidates.

Many drugs are reversible enzyme inhibitors, interacting with enzymes in a non-covalent fashion, and thus the enzyme can readily reactivate from the binary enzyme-inhibitor complex. In contrast, MAOIs, such as phenelzine, as mentioned previously, are irreversible inhibitors, or inactivators, where they interact with the enzyme to permanently ablate its function. There are two types of irreversible enzyme inactivation, affinity labeling and mechanism-based inactivation. Phenelzine belongs to the mechanism-based inactivation category. Mechanism-based inactivators are compounds that are inert until they are processed by the enzyme, forming a highly reactive species that can covalently modify the enzyme prior to diffusion away from the active site. This covalent modification irreversibly abolishes the enzyme’s function, and for the activity to recover, new enzyme must be biosynthesized by the cell. Phenelzine is therefore considered a suicide inhibitor, a term used for mechanism-based inactivators (Figure 21).
Suicide inhibitors, such as phenelzine, are compounds that are inert until they are processed by an enzyme to unmask a reactive moiety, at which point they can then covalently modify the enzyme prior to diffusing away from the active site. This irreversibly inactivates the target enzyme. $k_{\text{inact}}$ is the theoretical maximum rate constant of inactivation that would be achieved at infinite concentration of inhibitor. $K_{i(\text{inact})}$ is the concentration of inhibitor at one half the $k_{\text{inact}}$.

**Figure 21. Suicide inhibition.**
Mechanism-based inactivators of enzymes show a signature product versus time curve, where the product production levels off with time to reach a steady state velocity that is equal to zero. To determine inhibitor potency, represented by $k_{\text{inact}}$, and $K_{i(\text{inact})}$ values, $k_{\text{obs}}$ values are extracted from the enzyme reaction progress curves in the presence of various concentrations of irreversible inhibitor. The rate constant, $k_{\text{obs}}$, characterizes the speed of inactivation at a particular inhibitor concentration, which effectively corresponds to how quickly the plot changes from an initial rate to a final rate. Plotting $k_{\text{obs}}$ values for each inhibitor concentration tested yields a plot that reaches a plateau at high values of inhibitor concentration. From this relationship, the $k_{\text{inact}}$ and $K_{i(\text{inact})}$ values are determined. The term $k_{\text{inact}}$ represents the theoretical maximum rate of inactivation that would occur at infinite inhibitor concentration. It is analogous to $k_{\text{cat}}$ for a standard substrate processing turnover number. The term $K_{i(\text{inact})}$ represents the amount of inhibitor at one half the $k_{\text{inact}}$, and is thus loosely associated with the binding strength of the inhibitor to the enzyme in the initial enzyme-inhibitor complex formation. It is analogous to the $K_m$ for a substrate-enzyme interaction. The $K_{i(\text{inact})}$ value is usually adjusted for via the Cheng-Prusoff equation, which takes into consideration the substrate concentration being used in the reaction, as well as the enzyme’s $K_m$ value. To quantify the overall efficiency of inactivation, the quotient $k_{\text{inact}}/K_{i(\text{inact})}$ is used.

Experimental protocols to test compounds for enzyme inhibition in vitro can involve the use of spectrophotometric, mass spectrometric, radiometric, HPLC, or electrochemical techniques. A common spectrophotometric assay used for the determination of LSD1 inhibition involves a peroxidase-coupled reaction (Figure 22)\textsuperscript{148}. This assay monitors the hydrogen peroxide generated by the LSD1 demethylation
reaction. The coupling enzyme, horseradish peroxidase (HRP), utilizes the hydrogen peroxide generated to oxidize two dye molecules, 4-amino-antipyrine (4AP) and 3,5-dichloro-2-hydroxybenzene sulphonic acid (DHBS), to produce a quinone-imine, which is a red chromophore that can be detected at 515 nm. To increase the sensitivity of the assay, it is possible to replace 4AP and DHBS with an Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine), in which case the chromophore produced is resorufin, detected at 571 nm. Regardless of the chromophore produced, the Beer-Lambert law is then used to convert the absorbance of each chromophore to concentration. Ultimately, the concentration of either chromophore produced corresponds to the LSD1 catalyzed-demethylation reaction, yielding a reaction progress curve, which can be used to characterize potential LSD1 inhibitors.
Figure 22. Spectrophotometric assay for the determination of LSD1 inhibition.

The LSD1 demethylation reaction produces hydrogen peroxide, which is detected via a horseradish peroxidase (HRP) coupling reaction. In the presence of hydrogen peroxide, HRP converts two dye molecules, 4-amino-antipyrine (4AP) and 3,5-dichloro-2-hydroxybenzene sulfonic acid (DHBS), into a quinone-imine chromophore, which is detected at 515 nm using a spectrophotometer.
Another in vitro assay used to determine LSD1 inhibition is called MassSQUIRM, or mass spectrometric quantitation using isotopic reductive methylation\textsuperscript{149}. MassSQUIRM, as its name implies, uses reductive methylation, isotopic labeling, and mass spectrometry to quantify different methylation levels, such as of LSD1’s substrate. The MassSQUIRM reactions, for this purpose, contain LSD1 and a dimethylated H3K4 21-mer peptide that is biotinylated as substrate in the presence or absence of an inhibitor. After the enzyme reaction is run, the mixture of substrate and product biotinylated peptides are isolated using streptavidin, and reductive methylation is performed with the use of deuterio-formaldehyde. Unreacted dimethyl substrate retains the protio isotope such that the mono- and di-demethylated substrate species pick up increased mass during reductive methylation allowing them to be readily distinguished. Ultimately, MassSQUIRM quantifies the amount of di-, mono-, or unmodified peptide forms in each reaction, leading to accurate analysis of LSD1 inhibition.

Compounds 9-11 were first assayed by previous Cole lab members, Shonoi Ming, Robert Hsiao, Rong Huang, and Lindsay Avery, for their ability to inhibit recombinantly purified GST-LSD1 using a di-methylated H3K4 21-mer peptide substrate by monitoring peroxide formation via the colorimetric peroxidase assay mentioned previously\textsuperscript{148}. The results (Table 2) of compounds 9-11 showed that phenelzine analogues with variations to their alkyl chain length, such as compounds 9c and 9h, had at most small enhancements in potency compared with phenelzine ($K_{i\text{(inact)}} = 5.6 \, \mu M; \, k_{\text{inact}} = 0.35 \, \text{min}^{-1}$) (Figure 23). Functionalization of the hydrazine moiety with methyl or acetyl groups, such as compounds 9a, 9b, 9d, 9f, and 9g, negated LSD1 inhibitory action, indicating the importance of the unsubstituted hydrazine moiety. Compound 9e, containing a methoxy
substitution at the *para* position of the phenyl ring on phenelzine, was able to inhibit LSD1 with similar potency to phenelzine's, indicating that substitutions to the *para* position of the phenyl ring on phenelzine are well-tolerated. Compounds 10a and 10b, possessing a heteroatom substitution in the alkyl chain and variations in the overall chain length before the hydrazine moiety, showed no improvement in potency over phenelzine. Lastly, morpholine replacement of the phenyl ring, as seen in compound 11, was not compatible with LSD1 inhibition, indicating the importance of the phenyl ring.

Compounds 9-11 kinetic graphs of $k_{obs}$ values versus inhibitor concentrations can be found in Appendix III. These results indicated that substitutions to the *para* position of the phenyl ring on phenelzine are tolerable in terms of LSD1 inhibition, as long as there are no added substitutions to the hydrazine moiety. This study’s aim was to assay the phenelzine analogues synthesized in Chapter 2 for LSD1 inhibition to expand the SAR study.
Figure 23. Inhibition of LSD1 by phenelzine. (A) Steady-state progress curve of LSD1 inactivation by phenelzine ranging from 0 to 100 µM. (B) $k_{obs}$ values obtained from steady-state data plotted against inhibitor concentration to determine $k_{inact}$ and $K_{i(inact)}$ values.
Part 3B: Results and Discussion

To expand on the SAR of compounds 9-11, compounds 12a, 12b, 12d, and 14, were tested for LSD1 inhibition also using the spectrophotometric peroxidase-coupled assay. Later, the rest of the larger phenelzine analogues synthesized, including compounds 12c, 12e-m, 13, and 15a-b, were assayed by Jay Kalin to complete the phenelzine analogue SAR study. The results (Table 2) showed that important LSD1 inhibitory potency enhancements were achieved by linking aryl groups through various tethers to the phenelzine core (12–15). (The second replicate of LSD1 inhibition kinetic parameters for phenelzine, 12a, 12b, 12d, and 14 can be found in Appendix III Table 1.) This trend was loosely related to the previously reported results with tranylcypromine analogue 5.
Table 2. Kinetics of phenelzine analogue LSD1 inhibitors (compounds 9-15).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_{i\text{(inact)}}$ ($\mu$M)</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$k_{\text{inact}}/K_{i\text{(inact)}}$ ($\mu$M$^{-1}$min$^{-1}$)</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenelzine</td>
<td>5.6±1.3</td>
<td>0.35±0.056</td>
<td>0.063±0.018</td>
<td>N/A</td>
</tr>
<tr>
<td>9a</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>85.00</td>
</tr>
<tr>
<td>9b</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td>9c</td>
<td>5.0±1.1</td>
<td>0.32±0.010</td>
<td>0.064±0.014</td>
<td>N/A</td>
</tr>
<tr>
<td>9d</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>46.74</td>
</tr>
<tr>
<td>9e</td>
<td>8.0±3.5</td>
<td>0.15±0.023</td>
<td>0.019±0.0087</td>
<td>N/A</td>
</tr>
<tr>
<td>9f</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td>9g</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>9h</td>
<td>22±3.0</td>
<td>0.12±0.01</td>
<td>0.0055±0.00087</td>
<td>N/A</td>
</tr>
<tr>
<td>10a</td>
<td>44±9.7</td>
<td>0.15±0.010</td>
<td>0.0034±0.00079</td>
<td>N/A</td>
</tr>
<tr>
<td>10b</td>
<td>12±2.1</td>
<td>0.22±0.020</td>
<td>0.018±0.0036</td>
<td>N/A</td>
</tr>
<tr>
<td>11</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td>12a</td>
<td>0.28±0.11</td>
<td>0.19±0.036</td>
<td>0.70±0.31</td>
<td>N/A</td>
</tr>
<tr>
<td>12b</td>
<td>0.37±0.033</td>
<td>0.20±0.0087</td>
<td>0.54±0.054</td>
<td>N/A</td>
</tr>
<tr>
<td>12c</td>
<td>0.26±0.058</td>
<td>0.24±0.022</td>
<td>0.92±0.22</td>
<td>N/A</td>
</tr>
<tr>
<td>12d</td>
<td>0.059±0.021</td>
<td>0.15±0.017</td>
<td>2.5±0.96</td>
<td>N/A</td>
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<tr>
<td>12e</td>
<td>0.26±0.11</td>
<td>0.22±0.038</td>
<td>0.86±0.39</td>
<td>N/A</td>
</tr>
<tr>
<td>12f</td>
<td>0.156±0.047</td>
<td>0.17±0.018</td>
<td>1.1±0.35</td>
<td>N/A</td>
</tr>
<tr>
<td>12g</td>
<td>0.138±0.0.48</td>
<td>0.17±0.020</td>
<td>1.2±0.44</td>
<td>N/A</td>
</tr>
<tr>
<td>12h</td>
<td>0.207±0.089</td>
<td>0.26±0.042</td>
<td>1.2±0.57</td>
<td>N/A</td>
</tr>
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<td>12i</td>
<td>0.282±0.076</td>
<td>0.21±0.024</td>
<td>0.74±0.22</td>
<td>N/A</td>
</tr>
<tr>
<td>12j</td>
<td>0.204±0.098</td>
<td>0.18±0.034</td>
<td>0.88±0.46</td>
<td>N/A</td>
</tr>
<tr>
<td>12k</td>
<td>0.223±0.064</td>
<td>0.17±0.020</td>
<td>0.76±0.24</td>
<td>N/A</td>
</tr>
<tr>
<td>12l</td>
<td>2.0±0.73</td>
<td>0.24±0.033</td>
<td>0.12±0.045</td>
<td>N/A</td>
</tr>
<tr>
<td>12m</td>
<td>1.6±0.49</td>
<td>0.22±0.025</td>
<td>0.14±0.044</td>
<td>N/A</td>
</tr>
<tr>
<td>13</td>
<td>0.10±0.039</td>
<td>0.17±0.21</td>
<td>1.7±0.68</td>
<td>N/A</td>
</tr>
<tr>
<td>14</td>
<td>0.90±0.45</td>
<td>0.18±0.038</td>
<td>0.20±0.11</td>
<td>N/A</td>
</tr>
<tr>
<td>15a</td>
<td>0.21±0.076</td>
<td>0.21±0.030</td>
<td>1.0±0.41</td>
<td>N/A</td>
</tr>
<tr>
<td>15b</td>
<td>0.10±0.035</td>
<td>0.17±0.019</td>
<td>1.7±0.60</td>
<td>N/A</td>
</tr>
</tbody>
</table>
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 one of the more potent LSD1 inhibitors with a $K_{i\text{(inact)}}$ of 59 nM and a $k_{\text{inact}}$ of 0.15 min$^{-1}$ (Figure 24). This $K_{i\text{(inact)}}$ for 12d compares favorably to one of the most potent tranylcypromine analogues ($K_{i\text{(inact)}} = 0.61 \mu$M) reported in the literature$^{115}$. Alternatives to the alkanoic spacers in 12, including an alkenoic acid spacer (13) and an alkyl ether spacer (14), led to slightly reduced LSD1 inhibitory potency. However, replacing the ethanyl tether with the trans-ethenyl group resulted in improved inhibitor potency as can be seen by comparing 12c with 13, indicating that potential importance of compound rigidity. Terminal aryl substitutions in the 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 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. (Compound 12-15 kinetic graphs of $k_{\text{obs}}$ values versus inhibitor concentrations can be found in Appendix III.)
Figure 24. 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) $k_{\text{obs}}$ values obtained from steady-state data plotted against inhibitor concentration to determine $k_{\text{inact}}$ and $K_{i(\text{inact})}$ values.
To assess the relative selectivity of one of our more potent and simple LSD1 phenelzine analogues, 12d (hereafter called bizine), as well as compounds 12a, 12b, and 14, counter screen enzyme assays were carried out versus MAO A, MAO B, and LSD2. As shown in Table 3, based on $k_{\text{inact}}/K_{\text{f(\text{inact})}}$ values, which measure inactivation efficiency, bizine showed to be 23-fold selective for inhibiting LSD1 versus MAO A, 63-fold selective versus MAO B, and $>100$-fold selective versus LSD2. In contrast, phenelzine preferentially inhibited MAO A and was equipotent in blocking MAO B compared with LSD1. Similar to bizine, compounds 12a, 12b, and 14, showed a decrease in MAO A/B potency by about 1.5 to 3.5 fold as compared to phenelzine. They were also found to be about 1.5 to 8.5-fold selective for inhibiting LSD1 versus MAO A, and 5.5 to 8.5-fold selective versus MAO B. Interestingly, as bizine, phenelzine and compounds 12a, 12b, and 14 did not show any LSD2 inhibition at concentrations up to 20 µM (Figure 25). First and second replicates of MAO A/B inhibition kinetics for compounds phenelzine, 12a, 12b, 12d (bizine), and 14 can be found in Appendix III Table 2-5.
Table 3. Phenelzine and 12d (bizine) selectivity profile for LSD1 vs. MAO A, MAO B, and LSD2.

<table>
<thead>
<tr>
<th>Enzyme Tested</th>
<th>Inhibitor</th>
<th>$K_{i\text{inact}}$ (µM)</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$k_{\text{inact}}/K_{i\text{inact}}$ (µM$^{-1}$ min$^{-1}$)</th>
<th>Selectivity for LSD1 vs. Enzyme Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAO A</td>
<td>phenelzine</td>
<td>0.82±0.47</td>
<td>0.24±0.057</td>
<td>0.29±0.18</td>
<td>0.217</td>
</tr>
<tr>
<td></td>
<td>12d</td>
<td>2.6±2.3</td>
<td>0.30±0.11</td>
<td>0.11±0.11</td>
<td>22.7</td>
</tr>
<tr>
<td>MAO B</td>
<td>phenelzine</td>
<td>3.9±1.7</td>
<td>0.20±0.040</td>
<td>0.051±0.025</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>12d</td>
<td>6.5±4.6</td>
<td>0.26±0.14</td>
<td>0.040±0.036</td>
<td>62.5</td>
</tr>
<tr>
<td>LSD2</td>
<td>phenelzine</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>12d</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
Figure 25. Product concentration versus time of LSD2 with 20 µM of phenelzine, 12a, 12b, 12d (bizine), and 14.
Bizine was also compared to another potent LSD1 inhibitor made in the Cole lab, the histone H3 21-mer peptide with a Lys4 modified to contain a hydrazine moiety (hydrazino peptide inhibitor), which was found to inhibit LSD1 with a $K_{i\text{(inact)}}$ of 4.35 nM. The hydrazino peptide inhibitor was a more potent LSD1 inhibitor than bizine, as expected, as it showed no product formation at 0.5 µM, whereas bizine still showed product formation at 1 µM (Figure 26). 10 µM of phenelzine was less effective as an LSD1 inhibitor than 1 µM of bizine, again indicating bizine’s significant LSD1 potency improvement over phenelzine.
Figure 26. Product concentration versus time of LSD1 with 10 µM phenelzine, 1 µM 12d (bazine), and 0.5 µM and 1 µM the hydrazino peptide inhibitor.
To confirm the LSD1 inhibition peroxidase assay results obtained with bizine, the previously mentioned isotope-based mass spectrometric assay, MassSQUIRM, was used to directly and quantitatively assess bizine effects on Lys4 methylation\textsuperscript{149}. The LSD1 demethylation reactions for this assay were run for 30 minutes utilizing a high LSD1 concentration, where the conditions allowed LSD1 to catalyze the demethylation of the di-methylated H3K4 substrate to a high level. This resulted in the extensive conversion of the di-methylated substrate to mono- and unmethylated H3K4 21-mer. As reported previously, greater than 10 mM of phenelzine was needed to extinguish LSD1 activity under MassSQUIRM conditions\textsuperscript{149}. Thus, 50 µM each of phenelzine and bizine were compared in an identical LSD1 inhibition MassSQUIRM assay. Results showed that 50 µM phenelzine had a negligible impact on LSD1 action, whereas the same concentration of bizine led to very substantial LSD1 inhibition, with the unreacted di-methylated peptide remaining as the major species at the conclusion of the experiment (Figure 27). These experiments corroborate the findings with the spectrophotometric peroxidase assay that showed that bizine was a far more potent LSD1 inhibitor than phenelzine. Thus, two separate in vitro assays, one using a spectrophotometric approach and another a mass spectrometry approach, establish that the phenelzine analogue inhibitor, bizine, is a potent inhibitor of LSD1. This shows the potential utility of bizine as a selective pharmacologic probe for cellular LSD1 histone demethylase activity.
Figure 27. Quantification of methylation states of H3K4 as a result of LSD1 inhibition by phenelzine or 12d (bizine) as determined by the MassSQUIRM technique.
Part 3C: Materials and Methods

**GST-LSD1 Activity and Inhibition Assays.** GST-LSD1 production from an *E. coli* expression system followed by purification using glutathione affinity chromatography were performed as previously described\(^{150}\). Rate measurements were performed using a peroxidase-coupled assay as previously described\(^{33}\). 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 (4AP), 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS), 0.04 mg mL\(^{-1}\) horseradish peroxidase (HRP, Worthington Biochemical Corporation), and appropriate concentration of buffered substrate (di-methylated H3K4 21-mer, ARTKme2QTARKSTGGKAPRKQLA, synthesized and purified as described previously\(^{118}\)). 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 \(k_{\text{cat}}\) of \(\sim 4.2 \text{ min}^{-1}\) and a \(K_m\) for di-methylated H3K4 21-mer of \(\sim 20 \text{ µM}\), but the specific parameters were measured for each batch and used for appropriate inhibitor parameter calculations. (See Figure 28 for the plot of rate versus substrate concentration of the GST-LSD1 batch used to test phenelzine and compounds 12a, 12b, 12d (bizine), and 14.)

For the inhibition studies, all of the 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 of the di-methylated H3K4 21-mer substrate. Progress curves conducted for 20 min were then fit to the following eq 1

\[
\text{Product} = \left(\frac{v_0}{k_{\text{obs}}}\right)(1-e^{-k_{\text{obs}}t})
\]  

eq. 1

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

\[
k_{\text{obs}} = \frac{(k_{\text{inact}}*[I])/(K_{i(\text{inact})}+[I])}{(K_{i(\text{inact})}+[I])}
\]  

eq. 2

The following Cheng-Prusoff equation, eq 3, was then used to a extrapolate the \(K_{i(\text{inact})}\) value to zero substrate

\[
K_{i,\text{app}} = K_i*(1+S/K_m)
\]  

eq 3

Each experiment was repeated at least two independent times and repeat measured values were typically within 20% of each other.
Figure 28. Rate versus substrate concentration of the GST-LSD1 batch used to test phenelzine and compounds 12a, 12b, 12d (bizine), and 14.
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\textsuperscript{33}. 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 (4AP), 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS), 0.04 mg mL\textsuperscript{-1} horseradish peroxidase (HRP, 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\textsuperscript{-1}. Under these conditions, MAO A displayed a $k_{cat}$ of $3\pm0.1 \text{ min}^{-1}$ and a $K_m$ for tyramine of $26\pm3.0 \text{ µM}$. MAO B displayed a $k_{cat}$ of $0.2\pm0.02 \text{ min}^{-1}$ and a $K_m$ for tyramine of $94\pm26 \text{ µM}$. For the inhibitor studies, all the 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.
**LSD2 Enzymatic Assays.** LSD2-His_{6} production from an *E. coli* expression system followed by purification using nickel affinity chromatography were performed as previously described\textsuperscript{151}. Initial velocity measurements were performed using a peroxidase-coupled assay, which monitors hydrogen peroxide production as previously described\textsuperscript{33}. The time courses of the reaction were measured under aerobic conditions using a Beckman Instruments DU series 600 spectrophotometer equipped with a thermostatted cell holder (T = 25 °C). The 100 µL reactions were initiated by addition of enzyme (430 nM LSD2) to reaction mixtures consisting of 50 mM HEPES buffer (pH 7.5), 0.1 mM 4-aminoantipyrine (4AP), 1 mM 3,5-dichloro-2-hydroxybenzene-sulfonic acid (DHBS), 0.76 µM horseradish peroxidase (HRP, Worthington Biochemical Corp.), 20 µM phenelzine analogue and 100 µM di-methylated H3K4 21-mer. Absorbance changes were monitored at 515 nm, and an extinction coefficient of 26,000 M\(^{-1}\) cm\(^{-1}\) was used to quantify product formation. Progress curves were then fit accordingly to eq 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.

**MassSQUIRM Assays.** MassSQUIRM inhibition experiments were performed in triplicate as described previously\textsuperscript{149}. The reaction mixtures containing 13.3 µM di-methylated H3K4 21-mer biotinylated peptide (^1ARTKme2QTARKSTGGKAPRKQLYKbio), 50 mM HEPES (pH 7.5), and 50 µM phenelzine or 12d (bizine), were incubated at 25 °C for 5 min, prior to initiation with 215 nM GST-LSD1. The demethylase reactions were run at 25 °C for 30 min and then analyzed as reported previously.
Chapter 4. Bizine Effects in Cells

Part 4A: Introduction

Cell-based assays can involve measuring a particular protein or product, cell proliferation, motility, toxicity, and morphology, all of which delve into the complexities of cellular systems. Such experiments can reveal many important characteristics and effects of any particular compound that otherwise could not have been identified in vitro.

There are a number of cell-based assays that may be used to detect LSD1 inhibition, including looking at histone modification mark effects by Western blot, observing demethylase activity of LSD1 using substrates fixed on solid platforms, and measuring various non-histone proteins linked to LSD1 inhibition, such as the cell surface marker CD86\textsuperscript{152}. When LSD1 activity is inhibited, its substrate, di-methylated Lys4 of histone H3 (H3K4me2), should increase. The activity of LSD1 can therefore be correlated to changes in the level of bulk H3K4me2 by utilizing Western blot. This can be done by isolating histones from LSD1 inhibitor treated cells, running the samples on a gel to separate out histone proteins H2A, H2B, H3, and H4, and then transferring the proteins to an appropriate membrane that can then be blotted for with an antibody against H3K4me2. There are several disadvantages to this technique. For example, observed changes in bulk H3K4me2 may not necessarily be a result of LSD1 inhibition as it has been found that the inhibition of other enzymes, such as HDACs, can affect H3K4me2 levels\textsuperscript{153}. Additionally, the change in bulk H3K4me2 due to LSD1 inhibition may not be significant as LSD1 demethylation of histones may be specific to only a small subset of genes. Moreover, compensation mechanisms, such as the up- or down-regulation or
various methyltransferases or demethylases, may occur after LSD1 inhibition that can blunt the H3K4me2 changes. Furthermore, cell conditions, such as confluency and passage number, have potential to impact the activity of LSD1, which can lead to fluctuations in levels of H3K4me2 between samples. Nevertheless, monitoring H3K4me2 has been the most widely used technique to detect LSD1 inhibition in cells.

The second assay to detect LSD1 inhibition is commercially available through Abnova. This assay involves applying nuclear extracts from treated cells onto plates that contain fixed peptide substrate, H3K4me2. An antibody is then added to measure the amount of methylated H3K4, which should negatively correlate with LSD1 activity. A disadvantage of this technique is that other enzymes in the nuclear extracts could act upon the peptide substrate, H3K4me2, such as the JmjC demethylases and SET domain methyltransferases. Additionally, cell conditions and the nuclear extraction process may influence LSD1 activity.

Monitoring proteins, other than histones, can also serve to detect LSD1 activity. For example, Lynch et al identified a cell surface marker, CD86, that has been shown to be up-regulated in response to LSD1 inhibition. Levels of CD86 can be detected using the quantitative reverse transcriptase-polymerase chain reaction method (qRT-PCR), flow cytometry, or enzyme-linked immunosorbent assay. A disadvantage of relying on this measurement is that cell conditions, and other proteins, such as MYB and FFI1, can alter CD86 expression. Additionally, this technique has only been verified in THP1, a myeloid leukemia cell line. All of the aforementioned assays do not directly measure LSD1 inhibition in cells and contain numerous disadvantages, all of which should be kept in mind when interpreting results using such techniques.
ChIP-seq is a cell-based assay that combines chromatin immunoprecipitation with DNA sequencing for genome wide profiling of proteins, including histone modifications such as H3K4me2, associated with DNA\textsuperscript{155}. The assay involves crosslinking proteins to DNA with formaldehyde, and then shearing the chromatin via sonication. The fractionated chromatin is then pulled down with specific antibodies in order to obtain DNA bound to the specific protein of interest. Lastly, the pulled down DNA is separated from proteins and sequenced. Inhibiting LSD1 can lead to an increase in H3K4me2 at gene promoters, which can lead to gene re-expression. Thus, ChIP-seq can be used to identify genes that have an increase in H3K4me2 at their promoters regions in response to LSD1 inhibition.

There are several different assays employed to measure cell proliferation rates, including those that use colorimetry, such as MTT, MTS, and alamarBlue, and those that use radioactivity, such as $^3$H thymidine. Colorimetric assays determine cell cytotoxicity indirectly. In the MTS assay, the reagent, MTS, is added to cells, and only viable cells are able to reduce the reagent, via a mitochondrial dehydrogenase, to a colored formazan product, which is detected at 490 nm\textsuperscript{156}. On the other hand, the $^3$H thymidine assay more directly correlates with DNA replication by the measurement of the incorporation of radioactive $^3$H thymidine into cellular DNA. Thus, the MTS assay is typically better at detecting apoptosis, whereas the $^3$H thymidine assay is useful in looking at DNA replication inhibition in response to drug treatment.

Cell proliferation assays are also commonly used to detect synergy between two different drugs. For such an assay, separate IC$_{50}$ curves are run for each separate drug. Then, either a constant or a non-constant ratio approach is chosen. A constant ratio
approach is used when the two drugs have similar IC\textsubscript{50} values\textsuperscript{157,158}. In such a case, the concentrations of each drug chosen for the drug combination treatments are kept at constant ratios to one another. A non-constant ratio approach is used when drugs have a difference in their IC\textsubscript{50} ‘s by 10-fold or more\textsuperscript{157,158}. In this case, the first drug’s concentration is kept constant while varying the second drug’s concentration. Fraction affected (Fa) values are then calculated for each drug combination, which signify the fraction of cells that show an effect from drug treatment. Those Fa values are then plotted against either drug alone or against drug combinations. Lastly, the Chou-Talalay method is used to generate combination index (CI) values for each drug combination, which ultimately determines antagonism, additivity, or synergy present for each Fa value\textsuperscript{157}. This present study not only looks at bizine in various drug combinations, but also at bizine effects on various histone modification marks, H3K4me2 on gene promoters, cell proliferation, and migration.
Part 4B: Results and Discussion

LSD1 Inhibition Effects on Cellular H3K4 Methylation

The ability of bizine to induce bulk methylation of H3K4 was assessed using Western blots with histone H3 methylation state specific antibodies in the prostate cancer LNCaP cell line, which has been used successfully in previous LSD1 inhibitor studies\textsuperscript{134,159}. This was done to correlate the activity of LSD1 in cells to changes in the level of bulk substrate upon LSD1 inhibition. As can be seen, after 48 h treatment with 0.4-10 µM bizine, there was a dose-dependent increase in H3K4me2 signal (Figure 29A,B). This increase in the cellular global H3K4me2 level after treatment with bizine is a primary effect that is consistent with prior studies with less selective LSD1 inhibitors and genetic LSD1 alterations\textsuperscript{96,114,119}. Ultimately, the EC\textsubscript{50} of this bizine effect was estimated to be \(~2 \) µM. This value is significantly higher than the $K_{i\,(\text{inact})}$ value of 59 nM observed in vitro. The higher EC\textsubscript{50} value observed in cells can be due to cell permeability of the compound, metabolic enzymes acting on the compound that are present in cells, or change in potency of the compound to LSD1 when it is complexed with other proteins, such as CoREST and HDAC1/2, in the cell.
Figure 29. LSD1 inhibition by 12d (bizine) in LNCaP cells. (A) Cells were treated with 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 3 biological replicates. (C) Cells were treated with 12d (bizine) (0.4–10 µM) for 48 h and blotted against LSD1 and actin.
There were no significant reproducible changes in H3K4me1, H3K4me3, unmethylated H3K4 or other histone H3 marks examined including H3K9me2, H3K9ac, and H3K36me3 (Figure 29A). H3K4me1 is an intermediate state between unmethylated H3K4 and H3K4me2, where H3K4me2 shows an increase, and, theoretically, unmethylated H3K4 would be expected to decrease. Therefore, it is plausible that H3K4me1 levels would not show any change. However, H3K4me3 would be expected to increase due to H3K4me2 increasing. No H3K4me3 changes were observed upon bizine treatment. This is perhaps due to compensation mechanisms occurring, such as the up-regulation of Jmj domain containing demethylases. Additionally, it is possible that levels of H3K4me3 are already high in the cell so that the change in this mark in response to the H3K4me2 increase is not large enough to be seen by Western blot. Lastly, the antibody may not be ideal in terms of selectivity to observe H3K4me3 changes. As mentioned previously, unmodified H3K4 should theoretically decrease in response to an H3K4me2 increase. In fact, unmodified H3K4 was slightly decreased at high bizine concentrations in a few Western blot replicates, although not enough to call it statistically significant. But, again, an explanation for not seeing a change in this mark could be due to compensation mechanisms or levels of unmodified H3K4 being high in the cell so as to not show a bulk change in response to the H3K4me2 increase triggered by LSD1 inhibition. H3K9me2 levels were not changed, and this could be attributed to it not being a substrate of LSD1 in this cell line. The lack of change in H3K9ac and H3K36me3 observed could be attributed to H3K4me2 not affecting those modification marks, or at least the extent of the changes caused by H3K4me2 to those modification marks is not large enough to be seen by Western blot.
Furthermore, Western blots for LSD1 protein levels were performed to see whether the increases in H3K4me2 observed upon bizine treatment could be due to changes in LSD1 protein expression. There was no discernible effect of bizine on LSD1 protein levels up to bizine concentrations of 10 µM (Figure 29C). This indicates that bizine does not likely affect the expression of LSD1 in the LNCaP cell line.

To assess bizine specificity for LSD1 inhibition, the cellular potency of bizine was compared to that of the MAO inhibitor phenelzine, a ~100-fold weaker LSD1 inhibitor than bizine in vitro, and the phenelzine analogue 12l, a ~30-fold weaker LSD1 inhibitor than bizine in vitro (Figure 30A). Cellular potency of the compounds was determined by H3K4me2 modification mark changes, and thus H3K4me2 was blotted for after 48 h of phenelzine treatment up to 40 µM, and phenelzine analogue 12l treatment up to 10 µM. No H3K4me2 changes were observed in either the phenelzine or compound 12l treated cells, indicating that the Western blot H3K4me2 effects observed after bizine treatment were most likely mediated through LSD1 inhibition (Figure 30B-D).

Additionally, the results indicated that not only is bizine more potent than phenelzine and compound 12l in vitro, but it is also more potent than either of the compounds in the LNCaP cell line.
Figure 30. (A) In vitro kinetic data of phenelzine, 12d (bizine), and compound 12l.

(B) Cells were treated with phenelzine (3–40 μM) for 48 h and blotted against H3K4me2 and Total H3. (C) Cells were treated with compound 4l (0.4–10 μM) for 48 h and blotted against H3K4me2 and Total H3. (D) Compound 12l H3K4me2 band density quantification plot as determined by 2 biological replicates.
Testing bizine in an LSD1 KO cell line is another way to look at bizine specificity. In an LSD1 KO cell line, levels of H3K4me2 would not be expected to increase after bizine treatment, whereas in the parental cell line they would be. This would ultimately indicate bizine’s specificity for LSD1 inhibition in cells. For this purpose, HCT116 colon cancer cells were obtained from Dr. Robert Casero’s laboratory that had both heterozygous and homozygous LSD1 knockouts. The HCT116 LSD1 knockout cells grow at a reduced rate and show gene expression changes as compared to the HCT116 parental cell line. After treating the HCT116 parental cell line with 5-20 µM of bizine for 48 h, it was found that there were no changes in the H3K4me2 levels with bizine treatment up to 20 µM (Figure 31). As bizine was unable to change H3K4me2 levels in the HCT116 parental cell line, the LSD1 knockout cells could not be used to assess bizine specificity. There are several reasons that may explain the lack of change in the H3K4me2 level in the HCT116 parental cell line upon bizine treatment. These include: bizine may not enter HCT116 cells efficiently, bizine is metabolized rapidly in these cells, or bizine inhibition of LSD1 may lead to rapid compensation in histone methylation.
Figure 31. The HCT116 parental cell line was treated with 5-20 μM of 12d (bizine) for 48 h and blotted against H3K4me2 and Total H3.
Different cell growth conditions were then tested to see if they have an influence on the magnitude of change occurring in H3K4me2 levels after bizine treatment. Four conditions were tested, including: serum starved cells for 24 h prior to either DMSO or 10 µM bizine treatment in serum-free media (SFM), serum starved cells for 24 h prior to either DMSO or 10 µM bizine treatment in serum-containing media, either DMSO or 10 µM bizine treatment in SFM, and either DMSO or 10 µM bizine treatment in serum-containing media. Results showed that each of these four conditions result in an increase in H3K4me2 in LNCaP cells in response to 6 h bizine treatment, although some conditions led to larger increases in H3K4me2 than others, particularly the conditions where serum was added to the media (Figure 32). This increase in H3K4me2 with serum addition may be attributable to the cells growing faster in serum, increasing the activity of LSD1. Ultimately, these results showed that any of the four treatment conditions could be used to observe bizine effects on H3K4me2 in LNCaP cells.
Figure 32. H3K4me2 Western blot after four different treatment conditions were utilized to treat LNCaP cells for 6 h with either DMSO or 10 µM bizine: (1) serum starving cells for 24 h prior to treatment in serum-free media (SFM), (2) serum starving cells for 24 h prior to treatment in serum-containing media, (3) treatment in SFM, and (4) treatment in serum-containing media.
Although the MAO inhibitor, tranylcypromine, is less potent than phenelzine for LSD1 inhibition in vitro, it has been shown in previous studies to increase levels of H3K4me2 in cells quite well\textsuperscript{147}. Therefore, we compared the H3K4me2 levels of LNCaP cells treated for 48 h with 10 µM phenelzine, 10 µM tranylcypromine, or 10 µM bizine. Interestingly, bizine and tranylcypromine showed similar effects on H3K4me2 levels, with each having about a 2-fold increase as compared to DMSO vehicle treated cells, whereas phenelzine did not show any effects on H3K4me2 levels, as mentioned previously (Figure 33). Bizine and tranylcypromine showing similar increases in cellular bulk H3K4me2 could be due to multiple factors, including that tranylcypromine may have a longer half-life in cells than bizine, that tranylcypromine is simply more cell permeable, or that tranylcypromine is hitting off-targets that are causing H3K4me2 increases. Nevertheless, it has to be kept in mind that there is a possibility that bizine could be acting on LSD1 more potently than tranylcypromine in cells despite not showing a bulk H3K4me2 increase. Ultimately, further experiments are needed to thoroughly compare bizine and tranylcypromine effects in cells.
Figure 33. Two biological replicates of cells treated with 10 µM of either phenelzine, tranyletyramine, or 12d (bizine) for 48 h and blotted against H3K4me2. Ponceau stains were used as loading controls. Numbers underneath each band indicate relative band density.
Kinetics of bizine effects on H3 methylation in the LNCaP cell line were also measured by collecting cells at seven different time points, 30 min, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, after 10 µM of bizine treatment. This time course experiment revealed that changes in H3K4me2 could be detected within 6 h of bizine exposure and effects can be observed up to 96 h (Figure 34A-C). However, there was a reproducible drop in H3K4me2 observed at 12 h, suggesting a somewhat complex dynamic process involving competing waves of lysine methyltransferase and demethylase action, further investigation of which is needed. Nevertheless, 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\textsuperscript{160,161}. 
Figure 34. (A) LNCaP Cells were treated with 10 µM 12d (bizine) and collected at various indicated time points and blotted against H3K4me2 and Total H3. (B) Two additional biological replicates of LNCaP cells treated with 10 µM 12d (bizine) for 30 min, 6 h, 12 h, and 24 h and blotted against H3K4me2 and Total H3. (C) 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 h, 24 h, 48 h, 72 h, and 96 h, but not at 12 h based on 3 biological replicates.
We further examined the effects of bizine on histone K4 methylation by assaying additional solid tumor cancer cell lines that have been used in previous LSD1 inhibitor studies\textsuperscript{78,162}. Cell lines, H460, A549, and MDA-MB-231, were all treated with varying concentrations of bizine and blotted against H3K4me2. With the lung cancer cell line, H460, there was a comparable dose-response effect of bizine on H3K4me2 levels as was seen in the LNCaP cell line (Figure 35). The lung cancer cell line, A549, and the breast cancer cell line, MDA-MB-231, also showed increases in H3K4me2 in response to bizine, but a higher concentration (20 µM) was required for reproducible effects (Figure 35). These results underscore the diversity of cellular responses to bizine treatment, as discussed regarding the HCT116 cell line.
Figure 35. H460, A549, and MDA-MB-231 cell lines were treated with 12d (bizine) (0.4–10 µM or 20 µM) for 48 h and blotted against H3K4me2 and Total H3.

*Determined using biological triplicates.
Bizine effects on the acute myeloid leukemia (AML) cell lines, ML-1, KG1a, HL-60, and HNT-34, were also investigated. All cells were treated with 10 µM of bizine and collected after either 6 h or 48 h, after which they were blotted for H3K4me2. All cell lines, except HL-60, showed statistically significant increases in H3K4me2 at both 6 h and 48 h (Figure 36A-D). The HL-60 cell line only showed a statistically significant increase in H3K4me2 at 48 h, which may be attributable to the slow growth of these cells (Figure 36C). These studies exemplify that non-solid tumors can be responsive to bizine.
Figure 36. A) ML-1, (B) KG1a, (C) HL-60, (D) HNT-34 cell lines were treated with 10 µM 12d (bizine) for either 6 h or 48 h and blotted against H3K4me2 and Total H3. Quantification plots of H3K4me2 band density were determined using biological triplicates.
To examine the effect of LSD1 inhibition on the methylation of H3K4 with individual gene resolution, ChIP-seq experiments, or chromatin immunoprecipitation followed by sequencing, were carried out on LNCaP cells treated for 48 hours with either vehicle (DMSO) or bicine. This identified specific genes that showed an increase in H3K4me2 at or near their promoter regions as a result of LSD1 inhibition. Our collaborator Xin Li in Dr. Andrew Feinberg’s laboratory at Johns Hopkins School of Medicine analyzed the data, where differential peaks between samples with two biological replicates were identified by diffReps<sup>163</sup>. In total, there were 17,542 differential H3K4me2 peaks identified between cells treated with 10 µM bicine versus vehicle (DMSO) (see ChIP-seq data accession number). Among those, 10,874 peaks were found to be up-regulated (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 (see ChIP-seq data accession number and Figure 37). Furthermore, gene ontology (GO) analysis of these 2,432 genes, done by our collaborator Jianfei Hu in Dr. Jiang Qian’s laboratory at Johns Hopkins School of Medicine, revealed that the top five processes present were metabolism, protein modification, regulation of gene expression, regulation of biosynthesis, and chromatin binding, most of which are related to LSD1 function.
Figure 37. Shown are representative examples of three genes’ Integrative Genomics Viewer (IGV)\textsuperscript{164,165} tracks from the list of 2,432 genes identified through the ChIP-seq experiment that showed an increase in H3K4me2 with LSD1 inhibition by 12d (bazine) (with two biological replicates): (A) RGMB (chr5:98,079,869-98,189,371); (B) SMARCA2 (chr9:1,999,116-2,177,398); (C) ERRFI1 (chr1:7,902,135-8,201,537). Red boxes mark statistically significant peak increases with 12d (bazine) treatment. Scale indicated by tick marks.
After culling the list to exclude microRNA and non-standard gene names from the 2,432 gene list, our collaborator Jianfei Hu compared the remaining 1,767 genes to the 1,587 genes identified in a ChIP-seq experiment that used an LSD1-/- hematopoietic cell line, Gr1\textsuperscript{dim} Mac1\textsuperscript{+}, which also analyzed H3K4me2 increases at gene promoters\textsuperscript{74}. There were 146 genes (p-value = 0.0028) that overlapped in the chemical inhibition and LSD1 knockout experiments (Figure 38). 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, again done by our collaborator Jianfei Hu, showed that the top five statistically significant processes were development, metabolism, regulation of gene expression, regulation of protein kinase activity, and regulation of transcription from RNA polymerase II promoters. Of note, many (26) (p-value = 5.80E-9) of the 146 overlapped genes (Table 4) were established or proposed to be tumor suppressors, including CDH1 and CDKN2A, which have been validated to be affected by LSD1 inhibitors in prior studies\textsuperscript{92,166}. This is consistent with the proposal that LSD1 inhibitors might have anti-cancer applications.
Figure 38. Comparing the 1,767 gene dataset from the 12d (bizine) inhibition ChIP-seq experiment to the 1,587 gene dataset from a ChIP-seq experiment that used an LSD1-/- hematopoietic cell line showed that there were 146 genes in common at a statistically significant p-value (0.0028).
Table 4. 26 tumor suppressor genes (p-value = 5.80E-9), with original p-values, of the 146 genes that overlapped in the chemical inhibition and LSD1 knockout ChIP-seq experiments. Tumor suppressor genes were identified using two data sets; one data set used was from Vanderbilt University (http://bioinfo.mc.vanderbilt.edu/TSGene/Human_716_TSGs.txt), and the other data set used was from Memorial Sloan-Kettering Cancer Center (http://cbio.mskcc.org/CancerGenes/).

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<th>Symbol</th>
<th>Full Name</th>
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</tr>
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<td>ERBB receptor feedback inhibitor 1</td>
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<td>1.08E-05</td>
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<td>c-abl oncogene 1, non-receptor tyrosine kinase</td>
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<td>ets variant 6</td>
<td>8.14E-05</td>
</tr>
<tr>
<td>Lox</td>
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<td>8.86E-05</td>
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There was also an attempt to probe the effects of the increase of H3K4me2 at the various genes’ promoters identified in the ChIP-seq experiment on gene expression levels. To do this, preliminary q-rtPCR experiments on select ChIP-seq gene hits were preformed in LNCaP cells. Results showed after 48 h of 10 µM bizine treatment, the 10 select tumor suppressor genes (Cdkn2a, Errfi1, Ptpn18, Cdhl, Phlpp2, Pkn3, Rgmb, Sash1, Trim14, Smarca2) and Rest hits from the ChIP-seq experiment did not show any changes in gene expression levels (Figure 39A). But, when looking at relative mRNA changes of select genes, such as LRBA, at four different time points, 6 h, 12 h, 24 h, and 72 h, after 10 µM bizine treatment, increases in gene expression levels at 6 h only were observed (Figure 39B). Consequently, after looking at gene expression level changes after 6 h of 10 µM bizine treatment, 6 of the 10 select tumor suppressor genes and the gene, Rest, showed changes in gene expression levels (Figure 39C). Unfortunately, these preliminary q-rtPCR results were not able to be reproduced, indicating that perhaps cell conditions, such as passage number, are important in obtaining consistent gene expression changes in response to bizine treatment. Future experiments are needed to confirm the tumor suppressor genes and Rest affected after 6 h of bizine treatment in LNCaP cells.
Figure 39. q-rtPCR results. (A) Relative mRNA changes of 10 select tumor suppressor genes and Rest in response to either DMSO or 10 µM 12d (bizine) treated LNCaP cells for 48 h. (B) Relative mRNA change of the gene, LRBA, in response to either DMSO or 10 µM 12d (bizine) treated LNCaP cells for 6 h, 12 h, 24 h, and 72 h. (C) Relative mRNA changes of 10 select tumor suppressor genes and Rest in response to either DMSO or 10 µM 12d (bizine) treated LNCaP cells for 6 h.
Bizine Anti-Proliferation Effects

Increasing H3K4me2 can cause gene re-expression to occur that can ultimately lead to cell proliferation changes. Thus, the effects of bizine on cell cytotoxicity using the MTS assay were investigated. The MTS was performed on four solid tumor cell lines, LNCaP, H460, A549, MDA-MB-231, treated with 5-80 µM bizine for 24 h, 48 h, 72 h, and 96 h. The results from all of the time points tested showed that there was no cell cytotoxicity with bizine treatment up to 10 µM. The 96 h time point showed moderate cell cytotoxicity effects at 20 µM bizine, particularly in the H460 and the MDA-MB-231 cell lines (Figure 40A-D). These results suggest that bizine does not initiate apoptosis in the LNCaP, H460, A549, or MDA-MB-231 cell lines at concentrations below 10 µM. Although, future experiments could be done to look at longer time points as histone modification mark effects on apoptosis could take longer than four days to take place.
Figure 40. MTS assay on four solid-tumor cell lines, (A) LNCaP, (B) H460, (C) A549, (D) MDA-MB-231, treated with 5-80 µM 12d (bizine) for 96 h. Red box indicates that there is no effect on cell viability at 12d (bizine) concentrations of 10 µM or less in any of the cell lines.
Although bizine showed low potency cell cytotoxicity effects in the LNCaP, H460, A549, or MDA-MB-231 cell lines as determined by the MTS assay, exploring cell proliferation by investigating the DNA replication rate was still of interest. Thus, a $^3$H thymidine incorporation assay was done to measure the rate of DNA synthesis in the LNCaP and H460 cell lines in response to a 48 h bizine treatment. These studies revealed that bizine slows cellular proliferation rate with an IC$_{50}$ of 14-42 µM and 16-34 µM in H460 and LNCaP cancer cell lines, respectively (Figure 41A-B). These IC$_{50}$’s are considerably higher than the EC$_{50}$’s for Western blot changes in H3K4me2, and raises concerns about the mechanistic basis of the cell anti-proliferative effects by bizine. But, perhaps the increase in concentration of bizine necessary for cell growth inhibition to occur could be due to the need for complete LSD1 inhibition in cells at bizine concentrations well above the EC$_{50}$, or perhaps due to a necessity of a longer bizine treatment time period.
Figure 41. DNA replication dose response curves using a $^3$H thymidine assay in H460 cells and LNCaP cells after 48 h treatment with 12d (bizine) in either (A) SFM or (B) serum-containing media.
To further address whether it is the LSD1 inhibition by bizine that is inhibiting cell growth, the impact of phenelzine on H460 cell proliferation was tested. There was less than a 50% reduction in \(^3\)H thymidine incorporation in H460 cells after 48 h with 80 \(\mu\)M phenelzine (Figure 42). This indicated that MAO inhibition by bizine may not primarily contribute to its cell growth inhibitory effects. Furthermore, we tested cell proliferation effects with the phenelzine analogue 12l, a \(\sim\)30-fold weaker LSD1 inhibitor than bizine in vitro, that showed no effects on H3K4me2 levels in cells with concentrations up to 10 \(\mu\)M. After 48 h of treatment, compound 12l slowed the cellular proliferation rate with an IC\(_{50}\) of 17 \(\mu\)M in H460 cells (Figure 43). This result indicated that there might be off-targets that both bizine and compound 12l are hitting, which are ultimately contributing to the attenuation of cancer cell growth. Future experiments are need to treat cells at lower bizine doses for extended periods of time to see if off target effects could be limited. Nevertheless, taken together, the cell proliferation studies indicate that bizine can be potentially used as an anti-cancer therapeutic to attenuate cancer cell proliferation, although the mechanism by which this effect occurs is in need of further study.
Figure 42. DNA replication dose response curves using a $^3$H thymidine assay in H460 cells after 48 h treatment with phenelzine.
Figure 43. DNA replication dose response curves using a $^3$H thymidine assay in H460 cells after 48 h treatment with compound 12l.

IC$_{50}$ = 16.9 ± 2.91 µM
AML cell lines, ML-1, KG1a, HL-60, and HNT-34, were also tested for cell proliferation inhibition by bizine using the $^3$H thymidine assay. Cells were treated with 5-80 µM bizine at either 24 h, 48 h, 72 h, or 96 h. The results revealed that bizine slows the cellular proliferation rate with IC$_{50}$’s ranging from 8-40 µM for the AML cell lines, similar to the IC$_{50}$ values observed with the solid-tumor cancer cell lines (Figure 44A-D). This again indicates bizine’s potential as an anti-cancer therapeutic for either solid or non-solid tumors.
Figure 44. DNA replication dose response curves using a $^3$H thymidine assay in (A) ML-1 (B) KG1a (C) HL-60 (D) HNT-34 cells after 24 h, 48 h, 72 h, and 96 h treatment with compound 12d (bazine).
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 concept has in fact been evaluated previously with LSD1 inhibitors that have low selectivity and potency, but nevertheless show additivity and synergy with various HDAC and DNMT inhibitors.\textsuperscript{162,167} Therefore, bizine in binary combinations with one DNMT inhibitor, azacytidine, as well as five HDAC inhibitors, SAHA, TSA, MGCD0103, MS-275, and LBH-589, using \textsuperscript{3}H thymidine incorporation in H460 cells after 48 h treatment were explored. Synergy was identified using combination index (CI) values, which were determined by the CalcuSyn program using a constant ratio approach.\textsuperscript{157} Unexpectedly, four of the agents, azacytidine, SAHA, TSA, and MGCD0103, when combined with bizine, exhibited moderate antagonism, CI > 1, on H460 cell inhibition at all ratios of the two agents examined (Figure 45A-D). 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. On the other hand, MS-275 and LBH-589, in combination with bizine, showed additive to synergistic effects on H460 cell inhibition, with the most synergy observed at the highest concentrations of compounds employed (Figure 45E-F). 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.
Figure 45. The H460 cell line was treated simultaneously with 12d (bizine) and (A) azacytidine, (B) SAHA, (C) TSA, (D) MGCD0103, (E) MS-275, (F) LBH-589 for 48 h and DNA replication was monitored using the $[^3]$H thymidine assay. Synergy was determined by CompuSyn using a non-constant ratio approach. CI > 1, CI = 1, or CI < 1 indicates antagonism, additivity, or synergy, respectively. For example, points above, on, or under the red line indicate antagonism, additivity, or synergy, respectively. Fa indicates the fraction of cells affected by given doses of each drug.
To further investigate bizine’s potential synergy with other compounds, bizine was screened in combination with ~2,800 FDA approved drugs in Dr. Jin Liu’s Johns Hopkins Drug Library (JHDL). To do this, H460 cancer cells were incubated with 10 μM bizine and 2 μM of each JHDL compound for 48 h, and the $^3$H thymidine assay was then used to measure DNA replication rates. Hits were called those that gave >35% increase in cell proliferation inhibition as compared to either drug alone. In total, 40 drug combination hits were found that gave a >35% increase in cell proliferation inhibition. Interestingly, amongst those hits, six were steroid or steroid related drugs, one was believed to weakly inhibit MAO A/B, three were kinase inhibitors, one was a phosphatase inhibitor, ten were antimicrobial or antibacterial drugs, three were blood pressure drugs, and two were reductase inhibitors. Four drug combination hits showed an opposite effect (>35% decrease). 20 of the 40 hits were later followed up for verification, although unfortunately none of these validation experiments confirmed the screening data (Table 5).
Table 5. 20 of the 40 Johns Hopkins Drug Library hits that were confirmed to be false positives through validation experiments.

<table>
<thead>
<tr>
<th>False Positive Hits from Johns Hopkins Drug Library</th>
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<tr>
<td>Succinylsulfathiazole Sulfonamide</td>
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<tr>
<td>Nikethamide</td>
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<tr>
<td>Propafenone HCl</td>
</tr>
<tr>
<td>Lovastatin</td>
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<tr>
<td>Dicumarol</td>
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<tr>
<td>Diethylstilbestrol</td>
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<tr>
<td>Prazosin HCl</td>
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<tr>
<td>Tolbutamide</td>
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<tr>
<td>Fosfomycin Calcium</td>
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<td>Raloxifene HCl</td>
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<td>Candesartan Cilextil</td>
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<tr>
<td>Hesperertin</td>
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<td>Nelfinavir Mesylate</td>
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Other Bizine Effects in Cells

Inhibiting migration, in addition to inhibiting cell growth, is an important characteristic of a compound that has potential to be used as an anticancer therapeutic. Thus, to test for the potential of migration attenuation by bizine, scratch assays were performed in both LNCaP and H460 cell lines, and images were taken of the scratches at 0 h, 24 h, 48 h, and 72 h to look for “wound” closure as a representation of cell migration. Results showed that 10 µM of bizine was able to inhibit cell migration starting at about 48 h after treatment in H460 cells (Figure 46). As LNCaP cells double every 36 hours, a conclusive cell migration experiment could not be performed using the LNCaP cell line. Future experiments are necessary to decipher the mechanism by which bizine effects cell migration. Additionally, migration effects of bizine in other cell lines or at longer time points could be explored further.
Figure 46. Scratch assays were performed on H460 cells treated with either DMSO, 10 µM phenelzine, or 10 µM 12d (bizine) and imaged after either 0, 1 (24 h), 2 (48 h), or 3 days (72 h).
Bizine Collaborations

HDAC inhibition has previously been reported to protect neurons against oxidative stress induced by homocysteic acid (HCA) treatment, which depletes glutathione\textsuperscript{168,169}. Thus, our collaborator Manuela Basso in Dr. Rajiv Ratan’s laboratory at Weil Cornell Medical Institute explored whether bizine might confer neuroprotection against HCA-induced oxidative stress. It was found that 0.5 µM bizine led to a significantly enhanced survival of neurons after HCA treatment in a dose-dependent fashion (Figure 47A-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 against neurologic disease, such as stroke, which can be placed in the context of prior work that investigated LSD1 functions in the brain\textsuperscript{99,100}.
Neurons were exposed to 5 mM HCA and treated with either (A) 12d (bizine) or (B) phenelzine for 48 h, after which cell cytotoxicity was measured using a MTT assay. (Two-way ANOVA, Bonferroni post hoc test; **p < 0.01; ***p < 0.0001 compared to no HCA).

Figure 47. Figure provided by Manuela Basso (Dr. Rajiv Ratan’s laboratory).
As LSD1 inhibitors have been implicated in blocking viruses from reactivating from latency, our collaborator Sifei Xin in Dr. Robert Silicano’s laboratory at Johns Hopkins School of Medicine looked at bizine effects on the reactivation of HIV-1. A bcl-2 transduced model of HIV-1 latency was used, where cells were treated for 72 h with 1-20 µM bizine either alone or with either 1 µM SAHA or 30 nM LBH-589. Results showed that bizine inhibited the reactivation of HIV-1 latency when combined with either SAHA or LBH-589 (Figure 48). The mechanism by which this inhibition occurs is in need of further study. Such a study indicates the wide range of applications bizine could be used in.
Figure 48. Figure provided by Sifei Xin (Dr. Robert Silicano’s laboratory). Bcl-2 transduced cells as models of HIV-1 latency were treated for 72 h with 1-20 µM 12d (bizine) either alone or with either 1 µM SAHA or 30 nM LBH-589 and then analyzed for their level of re-activation as a percentage of co-stimulation.
Part 4C: Materials and Methods

Cell Culture. LNCaP, H460, A549, ML-1, KG1a, HL-60, and HNT-34 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). 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). HCT116 cells were maintained in McCoy’s 5A (Gibco 16600-082) supplemented with 10% FBS. All cells were grown at 37 °C in 5%/95% CO₂/air.

Western Blot. Cells were seeded in 150 x 25 mm plastic tissue culture dishes (Corning 430599). Cells were treated at ~70% confluency with vehicle or compounds in serum-free or serum-containing media for various time periods. Whole-cell extracts were isolated using RIPA buffer (Sigma R0278) and 1x protease inhibitor cocktail (Roche, 1183617001). Histone extracts were isolated as described previously. 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.

Antibodies. H3K4me was detected using a polyclonal rabbit antibody (abcam ab8895). H3K4me2 was detected using a monoclonal rabbit antibody (abcam ab32356). H3K4me3
was detected using a polyclonal rabbit antibody (abcam ab8580). H3K4-Unmodified was detected using a monoclonal mouse antibody (Active Motif 39763). H3K9me2 was detected using a monoclonal mouse antibody (abcam ab1220). H3K36me3 was detected using a polyclonal rabbit antibody (abcam ab9050). H3K9ac was detected using a polyclonal rabbit antibody (abcam ab4441). Total H3 was detected using a polyclonal rabbit antibody (abcam ab1791). LSD1 was detected using a polyclonal rabbit antibody (abcam ab17721). Actin was detected using a monoclonal mouse antibody (Sigma A1978).

**ChIP-seq Assay.** LNCaP cells were seeded in 2, 150 x 25 mm tissue culture dishes (Corning 430599) per condition. Cells were grown to ~70% confluency, and after washing with phosphate-buffered saline (2 x 10 mL) (PBS, Gibco 10010-023), the cells were treated with either vehicle (DMSO) or 10 µM 12d (bizine) (>97% purity as determined by NMR) and grown in serum-free media for 48 h. Cells were then cross-linked with 1% formaldehyde for 10 min at 37 °C. Cells were then placed on ice and washed with ice cold PBS (2 x 10 mL), scraped and pelleted. Pellets were then resuspended in PIPES buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP-40, 1x cOmplete, EDTA-free, Protease Inhibitor Cocktail Tablets (Roche)), lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, 1x cOmplete, EDTA-free, Protease Inhibitor Cocktail Tablets (Roche)), and sonicated to shear cross-linked DNA. Samples were kept in an ice bath at all times. Nucleic acid concentration was then measured using a Nanodrop (Thermo Scientific). The nucleic acid (20-100 µg) was then resuspended in 450-1,000 µL ChIP dilution buffer (0.01% SDS, 1.1% Triton-X 100, 1.2 mM EDTA,
16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, 1x cOmplete, EDTA-free, Protease Inhibitor Cocktail Tablets (Roche)), and pre-cleared by adding 30 µL Protein A Dynabeads (Invitrogen) and rotated for 30 minutes at 4°C. Samples were then incubated overnight at 4 °C with 5 µg of polyclonal rabbit H3K4me2 (milipore 07-030) (a no antibody control sample was included). 65 µL Dynabeads were then added to the samples and rotated for 2 h at 4°C. Dynabeads were then washed 2x with a low salt wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl); 1x with LiCl wash (0.25 M LiCl, 0.5% NP-40, 0.5% Na Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1); and 2x with TE pH 8.0. Elution buffer was then added to the beads (1% SDS, 0.1 M NaHCO₃) and samples were vortexed and rotated at RT for 15 minutes and sample transferred to a new tube. This step was repeated 2x. Cross-linking was reversed by the addition of 20 µL 5 M NaCl and heating at 65°C for 4 h. 10 mM EDTA, 40 mM Tris-HCl pH 6.5, and 40 µg Proteinase K (Thermo Scientific #EO0491) were then added and samples were incubated for 1 h at 45 °C. 500 µL phenol:chloroform was then added to the samples and they were rotated overnight at 4°C. Samples were then spun and the top layer (aqueous) was placed in a new tube. An equal volume of chloroform was added and vortexed and spun and the bottom layer discarded again. 50 µg mL⁻¹ of GlycoBlue (Life Technologies AM9515), 0.5 M NaOAc pH 5.2, and 2 volumes of 100% ethanol was added and samples were placed on ice for 15 minutes. Samples were then spun down and pellet was washed with 1 volume 70% EtOH and let dry. The pellets were then resuspended in TE and DNA concentrations were quantified by Qubit assay HS kit (Invitrogen Q32851).
Next Generation Sequencing/Library Generation (Done by Dr. Andrew Feinberg’s Laboratory at Johns Hopkins School of Medicine). Libraries were prepared from 10-20 ng of IP ChIP DNA and 100 ng of input DNA according to Illumina’s instructions along with the ChIP-seq DNA Sample Prep Kit (IP-102-1001). Briefly, samples were checked for quality and concentration from 150-250 bp on a bioanalyzer. DNA was end-repaired using Klenow polymerase in 58 µL of reaction buffer. For IP DNA, Klenow was diluted 1:5. Samples were incubated at 20°C for 30 minutes and subsequently purified on QIAquick PCR purification columns. A-tails were then added to the DNA with Klenow and dATP in NEB buffer 2 at 37°C for 30 minutes and cleaned with Qiagen MiniElute PCR purification columns. Sequencing adapters were then ligated onto the DNA for 15 minutes at room temperature followed by cleaning with MiniElute columns. Samples were then run on 2% agarose gels and DNA from 216-366 bp (DNA plus adapters) were cut from the gel and purified with a Qiagen QIAquickGel Extraction kit. Concentrations were then checked on a bioanalyzer and 8 ng were PCR amplified with Phusion polymerase (Fisher) for 15 cycles (10 sec 98°C, 30 sec 65°C, 30 sec 72°C) followed by 5 minutes at 72°C. Samples were then cleaned with Ampure kits (Illumina) and washed with 80% ethanol. DNA samples were resuspended at the end of the cleanup into 17.5 µL buffer EB (Qiagen) and subjected to next generation sequencing on Illumina HiSeq platform according to manufacturers instructions.

Peak Calling and Statistical Analysis of ChIP-seq Data (Done by Xin Li in Dr. Andrew Feinberg’s Laboratory and Jianfei Hu in Dr. Jiang Qian Laboratory at Johns Hopkins School of Medicine). 46 bp paired-end sequencing data were aligned to
the reference human genome (hg19) using BWA with default parameters\textsuperscript{171}. After alignment, duplicate reads were removed and only uniquely aligned reads were kept for further analysis. For narrow H3K4me2 peaks, MACS2 were used for peak calling with default parameters\textsuperscript{172}. Differential peaks between samples with two biological replicates were identified by diffReps\textsuperscript{163}. Ensemble human genome annotations were used to identify the human genes around identified peak regions. A gene is defined to be around a peak region if the closest distance between its Transcription Start Site (TSS) and the peak region is less than 2000 bp. In total 2432 Ensemble genes were found to be around the identified peak regions. Furthermore, to compare this ChIP-seq data set to the data set generated by Kerenyi et al., where target genes around LSD1(-/-)-specific and wt-specific histone modification peaks in Gr1dim Mac1+ cells were reported\textsuperscript{74}, we translated our Ensemble gene names into official symbol gene names. In this process, microRNA and genes represented by non-standard gene names were removed. A total of 1767 genes with official symbol names were identified. Utilizing all of the human genes identified with official symbol names for normalization, we computed the overlap significance by cumulative hypergeometric distribution. 146 of the 1587 Lsd1 KO-specific genes were recovered from our data set (p-val=0.0028). As a negative control, only 17 of the wt specific genes (TSG) were recovered (p-val=0.186). Additionally, to identify the number of tumor suppressor genes in the 146 genes identified to be in common, we used two TSG data sets. One data set used was from Vanderbilt University (http://bioinfo.mc.vanderbilt.edu/TSGene/Human_716_TSGs.txt), which contains 716 TSG genes. The other data set used was from Memorial Sloan-Kettering Cancer Center (http://cbio.mskcc.org/CancerGenes/), which contains 873 TSG genes. Utilizing all of the
human genes to normalize, we utilized a cumulative hypergeometric distribution to compute the number of TSG in our data set. From the two TSG datasets, 18 and 19 of the 146 recovered genes are TSG genes, with p-val of 3.72E-7 and 1.50E-6 respectively. Combining the two datasets together to define the total TSG genes (covering 1146 distinct TSG genes in total), we identified 26 of the 146 recovered genes as TSG genes, with a p-val of 5.80E-9.

**ChIP-seq Data Accession Number.** The GEO accession number for all ChIP-seq data is GSE55089.

**RNA Isolation and q-rtPCR.** RNA was isolated using the RNeasy Mini Kit (Qiagen #74104) according to the manufacturer’s instructions. cDNA was synthesized with the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen #18080-051) according to the manufacturer’s instructions. Quantitative real-time PCR (q-rtPCR) was done using the SYBR Green PCR Master Mix (Applied Biosystems #4309155) and analyzed by real-time PCR on a StepOnePlus PCR instrument (Applied Biosystems). Relative expression to GAPDH was quantified using the $\Delta\Delta$Ct method.

**MTS Assay.** Cells were seeded in 6 well plates (Corning 3516). Cells were treated at ~70% confluence with vehicle or compounds in either serum-free or serum-containing media for various time periods. The appropriate volume of CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay reagent (Promega G5421) was then added to cells
and incubated for 30-60 minutes, after which the absorbance of each well was taken at 490 nm using a plate reader.

3H Thymidine Assay. Cells were seeded in 96 well plates (Corning 3595). Cells were treated at ~70% confluency with vehicle or compounds in serum-free or serum-containing media for various time periods. 6 hours prior to harvesting cells, 10 µL of 0.1 mCi ml\(^{-1}\) Thymidine [methyl-\(^{3}\)H] (ARC ART0178) was added to each well. The cells were then harvested (PerkinElmer) and radioactivity was measured with a liquid scintillation counter (PerkinElmer MicroBeta).

Drug Combination Experiments. The H460 cell line was exposed to drugs alone or in combination. 12d (bizine) was added at three different fixed concentrations while the concentration of the other drug added was varied. After 48 h of treatment in serum-free media the 3H thymidine assay was performed as described above. The CPM of drug treated wells were compared to the CPM of control wells to calculate each fraction affected (Fa), where Fa = X means a decrease in growth of X%. Drug synergy was determined by isobologram analysis and derived from the median-effect principle of the Chou-Talalay method\(^{157}\). The combination index (CI) was calculated using CompuSyn\(^{TM}\) (ComboSyn Inc., Paramus, NJ) and the multiple drug effect equation to evaluate drug interactions\(^{157}\). A CI greater than, equal to, and less than one, respectively, indicates antagonistic activity, additivity, or synergy between two drugs. Data are presented from one representative experiment. Each experiment was repeated at least two independent times with nearly identical results.
**Scratch Assay:** Cells were seeded in 6 well plates (Corning 3516). Cells were treated at ~80-90% confluency with vehicle or compounds in serum-free media, and the cell monolayer was scraped in a straight line to create a “scratch/wound” with a p200 pipet tip\(^\text{173}\). Images were taken at time points 0 h, 24 h, 48 h, and 72 h. Scratch closure was monitored as a measure of cell migration.
Appendices

Appendix I: Additional Phenelzine Analogue Synthetic Schemes and Synthesis

Overview of the Synthetic Schemes. The compounds investigated in this study were synthesized from commercially available or readily prepared starting materials. A series of compounds containing substitutions to the hydrazine moiety was prepared via reductive amination with commercially available aldehydes and either substituted or protected hydrazines\textsuperscript{174}. Subsequent deprotection of the hydrazine was carried out in the presence of hydrochloric acid as necessary to yield compounds 9a-b, 9d, and 9f-g, which were isolated as free bases or as dihydrochloride salts (Appendix I Figure 1A). Additionally, phenelzine derivatives possessing heteroatom substitutions in the alkyl chain and variations in the overall chain length, as well as substitutions to the \textit{para} position of the phenyl ring, were easily prepared in one step from commercially available starting materials (Appendix I Figure 1B). Nucleophilic substitution of various alkyl bromides with excess anhydrous hydrazine resulted in the desired compounds 9c, 9e, 9h, 10a-b, and 14\textsuperscript{175,176}.

In addition, a series of compounds with larger hydrophobic groups attached to the \textit{para} position of the phenyl ring of phenelzine was prepared (Appendix I Figure 2). Excess benzoic anhydride was treated directly with 2-(4-aminophenyl)ethanol resulting in acylation of the aryl amine and aliphatic alcohol. Alternatively, an excess amount of
various phenyl alkyl substituted acids differing in alkyl linker length were converted to acid chlorides using thionyl chloride and then treated with 2-(4-aminophenyl)ethanol which yielded diacylated products similar to those obtained from the anhydride reaction. The esters were subsequently saponified with sodium hydroxide to provide the desired alcohols 16a-b and 16d. The Appel reaction was employed using triphenylphosphine and carbon tetrabromide to convert the alcohols to their respective alkyl bromides 17a-c. Then, the alkyl bromides were treated with excess anhydrous hydrazine to produce the desired final products 12a-b and 12d, which were isolated as hydrochloride salts as described in detail in the experimental section.

Additional variations in the alkyl linker and substitutions to the phenyl ring distal to the hydrazine of 12d were also explored. 4-(4-Chlorophenyl)butanoic acid and 4-(4-fluorophenyl)butanoic acid were obtained from their respective keto acids via a Wolff-Kishner reduction (Appendix I Figure 3). Amide bond formation was achieved using standard carbodiimide coupling conditions to generate intermediate alcohols 16c, 16e-k, 18a-b, and 19 from the respective acid and 2-(4-aminophenyl)ethanol. Subsequent conversion to the mesylate followed by nucleophilic substitution with excess anhydrous hydrazine yielded the desired products which were isolated as either sulfate or oxalate salts 12c, 12e-k, 15a-b, and 13 as indicated in the experimental section (Appendix I Figure 3).

Preparation of N-substituted amides was achieved by first protecting the alcohol of 16d as a silyl ether to generate common intermediate 20. Substitution of the amide nitrogen with methyl iodide or benzyl chloride using either sodium hydride or potassium tert-butoxide as the base, respectively, followed by deprotection in the
presence of TBAF\textsuperscript{181} resulted in the generation of intermediate alcohols \textbf{21a-b}. Alcohol to hydrazine conversion was carried out as previously described and the final products were isolated as oxalate salts \textbf{12l-m} (Appendix I Figure 4).
Appendix I Figure 1. Synthesis of LSD1 inhibitors with modifications to the alkyl chain and substitutions to the hydrazine moiety. (A) Reagents and conditions: a) AcOH, NaBH₃CN, MeCN, 0 °C to RT, 16 h; b) HCl, EtOAc, RT, 20 min - 2 h. (B) Reagents and conditions: a) N₂H₄, EtOH, 80 °C, 16 h.
Appendix I Figure 2. Synthesis of LSD1 inhibitors with variations in the length of the alkyl chain connecting the distal phenyl moiety to the phenelzine scaffold.

Reagents and conditions: a) SOCl₂, Et₃N, DCM, 0 °C to 55 °C, 8 h; b) i) 2-(4-aminophenyl)ethanol, DIPEA, DCM, 0 °C to RT, 16 h; ii) NaOH, MeOH, RT, 6 h; c) PPh₃, CBr₄, DCM, RT, 6 h; d) N₂H₄, EtOH, 80 °C, 1 h.
Appendix I Figure 3. Synthesis of LSD1 inhibitors possessing substitutions on the distal phenyl ring of 12d. Reagents and conditions: a) KOH, N₂H₄·H₂O, diethylene glycol, 120–130 °C, 2 h; b) 2-(4-aminophenyl)ethanol, EDC, DMAP, DCM, RT, 16 h; c) i) CH₃SO₂Cl, Et₃N, DCM 0 °C to RT, 1–3 h; ii) N₂H₄, EtOH, 80 °C, 2 h.
Appendix I Figure 4. Synthesis of N-substituted 12d derivatives. Reagents and conditions: a) TBDMSCl, Et$_3$N, DMAP, DCM, RT, 2 h; b) NaH, MeI, THF, 0 °C to RT, 4 h; c) KO'Bu, benzyl bromide, DCM/DMF, 0 °C to 60 °C, 16 h; d) TBAF, THF, RT, 24 h; e) i) CH$_3$SO$_2$Cl, Et$_3$N, DCM, 0 °C to RT, 1–3 h; ii) N$_2$H$_4$, EtOH, 80 °C, 2 h.
General Procedure A for hydrazine displacement reactions. Under argon, to a stirred solution of the appropriate alkyl bromide (1 mol equiv) in EtOH (1-3 mL/mmol) in a round-bottomed flask was added hydrazine (4-23 mol equiv). The mixture was refluxed overnight after which the volatiles were removed in vacuo and the residual product was dissolved in 1 N NaOH (10 mL). The aqueous layer was extracted with DCM (3 x 15 mL) and the combined organic layers were dried in vacuo. The residue was dissolved in MeOH (1-2 mL/mmol) and a 6 N HCl solution (0.3-0.4 mL/mmol) was added while stirring. After 20 min, the volatiles were removed in vacuo, and the desired product was purified via recrystallization from MeOH/Et₂O.

General Procedure B for reductive hydrazination. Under nitrogen and on ice, the appropriate aldehyde (1 mol equiv) was dissolved in MeOH (10 mL/mmol) in a round-bottomed flask. To this stirred solution was added 1-boc-1-methylhydrazine (1 mol equiv) dropwise. The ice bath was removed after 30 min, and the reaction was left to stir for 2 h. After cooling the reaction on ice, sodium cyanoborohydride (1.75 mol equiv) was slowly added along with acetic acid (150 µL/mmol, 1.5% v/v). EtOH was then removed in vacuo and either saturated sodium bicarbonate or 1 N NaOH (5 mL/mmol) was added. The aqueous layer was extracted with EtOAc (3 x 15 mL) and dried in vacuo. The product was then purified via flash chromatography (SiO₂, 75-90% hexanes/EtOAc). The base was dissolved in EtOAc (0.5 mL/mmol) and a 6 N HCl solution (0.5 mL/mmol) was added while stirring the solution on ice. After 2 h, the reaction was concentrated in vacuo and filtered. The resulting precipitate was washed with cold EtOAc to yield the desired product.
**General Procedure F for amide coupling.** The appropriate acid, 2-(4-aminophenyl)ethanol (1 mol equiv), EDC (1.2 mol equiv), and DMAP (0.1 mol equiv) were placed in a round-bottomed flask under argon at 0 °C and dissolved in anhydrous DCM (2 mL/mmol). The reaction mixture was allowed to warm to RT and stirred overnight (approximately 16 h). Then, the reaction was poured into H2O (20 mL) and the pH was adjusted to approximately 4 with an aqueous solution of 1 N HCl. The organic layer was isolated and the aqueous layer was further extracted with DCM (2 x 20 mL). The combined organic extracts were washed with 1 N HCl (15 mL) and brine (15 mL), dried over anhydrous Na2SO4, filtered, and concentrated in vacuo. The desired product was purified via recrystallization from EtOAc unless otherwise stated.

**General Procedure G for mesylate formation and hydrazine displacement reactions.**

The respective alcohol and triethylamine (1.2 mol equiv) were dissolved in anhydrous DCM (4 mL/mmol) under argon and cooled to 0 °C in an ice bath. Then, methanesulfonyl chloride (1.1 mol equiv) was dissolved in anhydrous DCM (1 mL/mmol) and added dropwise. The reaction was stirred for 1 h at 0 °C and then allowed to warm to RT and stirred for an additional 1-3 h or until complete as evidenced by TLC. The reaction was then slowly poured into an aqueous solution of 0.5 N HCl (approximately 10 mL), DCM was added (10 mL), and the organic layer isolated. The aqueous layer was further extracted with DCM (2 x 15 mL). The combined organic extracts were washed with brine (15 mL), dried with anhydrous Na2SO4, filtered, and concentrated in vacuo. The residue obtained was placed under argon, taken up in 95%
EtOH (4 mL), and cooled to 0 °C in an ice bath. Hydrazine (20 mol equiv) was dissolved in 95% EtOH (1 mL) and added dropwise to the reaction at 0 °C. The reaction was allowed to warm to RT and then heated at reflux (approximately 80 °C) for 2 h. After the reaction was complete as evidenced by TLC, it was cooled to RT and treated with a 1 N aqueous solution of NaOH (80 mL). DCM (15 mL) was added and the organic layer was isolated. The aqueous layer was further extracted with DCM (2 x 15 mL) and then the combined organic extracts were washed with brine (20 mL), dried with anhydrous Na₂SO₄, filtered, and concentrated in vacuo. See individual compounds for salt formation and purification.

**General Procedure H for sulfate salt formation.** The crude hydrazine was dissolved in MeOH (10 mL/mmol) and cooled to 0 °C in an ice bath. Concentrated H₂SO₄ (0.55 mL/mmol) was added dropwise to the solution and stirring was continued for 30 min at 0 °C. The resulting precipitate was isolated by filtration, washed with cold MeOH (2 mL), and dried under vacuum. Et₂O can be added dropwise to facilitate precipitation of the desired product.

**General procedure I for oxalate salt formation.** Oxalic acid (0.90 g, 10 mmol) was dissolved in MeOH (9 mL) and cooled to 0 °C in an ice bath. Then, the crude hydrazide was dissolved in MeOH (1 mL) and added dropwise to the solution of oxalic acid at 0 °C. Stirring was continued for 30 min after which Et₂O was added dropwise to facilitate precipitation of the desired product. The resulting precipitate was isolated by filtration, washed with cold MeOH (2 mL), and dried under vacuum.
1-Methyl-2-(2-phenylethyl)hydrazine dihydrochloride (9a): To a stirred solution of phenylacetaldehyde (200 µL, 1.7 mmol) in anhydrous CH₃CN (10 mL) in a round-bottomed flask at 0 °C was added t-buty1 1-methylcarboxylate (0.25 g, 1.7 mmol), followed by the addition of acetic acid (0.15 mL, 1.5% v/v). The reaction mixture was allowed to warm to RT and stirred for 2 h. Then, sodium cyanoborohydride (193 mg, 3.1 mmol) was added at 0 °C and stirring was continued overnight at RT. After completion, the volatiles were removed in vacuo and the desired compound was purified via flash chromatography (SiO₂, 75% hexanes/EtOAc) to yield a colorless oil (168 mg, 67%). ¹H NMR (400 MHz, CDCl₃): δ 7.30 (m, 5H), 3.10 (m, 5H), 2.82 (t, J = 8 Hz, 2H), 1.50 (s, 9H). This compound was taken up in EtOAc (1 mL) and to it was added a 6 M solution of aqueous HCl (1 mL) at RT. The reaction was stirred for 2 h and then the volatiles were removed in vacuo and the desired product was isolated as a white solid (137 mg, 92%). ¹H NMR (400 MHz, MeOD): δ 7.27 (m, 5H), 3.23 (m, 2H), 2.89 (t, J = 7.7 Hz, 2H), 2.79 (s, 3H). ¹³C NMR (125 MHz, DMSO-d₆): δ 138.55, 128.59, 128.40, 126.31, 48.62, 33.97, 32.41.

1,1-Dimethyl-2-(2-phenylethyl)hydrazine (9b): To a stirred solution of phenylacetaldehyde (0.20 mL, 1.7 mmol) in anhydrous CH₃CN (10 mL) in a round-bottomed flask at 0 °C was added N,N-dimethylhydrazine (143 µL, 1.88 mmol), followed by the addition of acetic acid (0.15 mL, 1.5% v/v). The reaction mixture was allowed to warm to RT and stirred for 2 h. Then, sodium cyanoborohydride (193 mg, 3.1 mmol) was added at 0 °C and stirring was continued overnight at RT. After completion, the volatiles
were removed in vacuo and the desired product was purified via flash chromatography (SiO2, 2:1 hexanes/EtOAc) and isolated as a colorless oil (100 mg, 36%). 1H NMR (500 MHz, MeOD): δ 7.27 (m, 4H), 7.20 (m, 1H), 3.17 (t, J = 7.5 Hz, 2H), 2.83 (s, 6H), 2.75 (t, J = 7.5 Hz, 2H). 13C NMR (125 MHz, MeOD): δ 140.45, 129.89, 129.67, 127.59, 51.72, 46.09, 35.12. ESI-LRMS: [M+H]+ = m/z 165.2.

(3-Phenylpropyl)hydrazine dihydrochloride (9c): The title compound was synthesized from 3-phenylpropyl bromide (380 µL, 2.51 mmol) according to general procedure A and isolated as a white solid (0.256 g, 68%). 1H NMR (400 MHz, MeOD): δ 7.24 (m, 5H), 3.05 (m, 2H), 2.72 (t, J = 7.6 Hz, 2H), 1.97 (quin, J = 7.7 Hz, 2H). 13C NMR (125 MHz, MeOD): δ 142.00, 129.74, 129.55, 127.46, 52.22, 33.71, 28.04.

1-Methyl-2-(3-phenylpropyl)hydrazine dihydrochloride (9d): The title compound was synthesized from hydrocinnamaldehyde (263 µL, 2 mmol) according to general procedure B and isolated as a white powder (0.056 g, 12%). 1H NMR (400 MHz, MeOD): δ 7.22 (m, 5H), 3.01 (t, J = 7.6 Hz, 2H), 2.76 (s, 3H), 2.71 (t, J = 7.6 Hz, 2H), 1.92 (quin, J = 7.8 Hz, 2H). 13C NMR (101 MHz, MeOD): δ 142.36, 129.67, 129.56, 127.32, 49.11, 35.56, 33.88, 29.20. ESI-HRMS: calcd. for C10H16N2: [M+H]+ = m/z 165.1391, found: [M+H]+ = m/z 165.1386.

[3-(4-Methoxyphenyl)propyl]hydrazine dihydrochloride (9e): The title compound was synthesized from 1-(3-bromopropyl)-4-methoxybenzene (524 µL, 3 mmol) according to general procedure A and isolated as a white powder (0.052 g, 7.2%). 1H NMR (400 MHz,
1H NMR (500 MHz, MeOD): \( \delta \) 7.13 (d, \( J = 8.6 \) Hz, 2H), 6.85 (d, \( J = 8.6 \) Hz, 2H), 3.76 (s, 3H), 2.98 (m, 2H), 2.75 (s, 3H), 2.66 (t, \( J = 7.5 \) Hz, 2H), 1.88 (quin, \( J = 7.5 \) Hz, 2H).

13C NMR (125 MHz, DMSO-\( d_6 \)): \( \delta \) 157.49, 133.00, 129.20, 113.75, 54.97, 46.77, 34.08, 31.32, 27.70. ESI-HRMS: calcd. for C11H18N2O: [M+H]+ = m/z 195.1496, found: [M+H]+ = m/z 195.1492.

N’-[3-(4-Methoxyphenyl)propyl]acetohydrazine (9g): Under nitrogen on ice, acetylhydrazide (593 mg, 8 mmol) was dissolved in MeOH (20 mL) and 3-(4-methoxyphenyl)propionaldehyde (0.317 mL, 2 mmol) was slowly added. The ice bath was removed after 30 min, and the reaction was left to stir for 2 h. Volatiles were removed in vacuo and saturated sodium bicarbonate (10 mL) was added. The product was extracted with EtOAc (3 x 15 mL) and dried in vacuo. The product was then purified via flash chromatography (2% MeOH/DCM) to yield the intermediate (0.107 g, 24%) as a white powder. Under nitrogen, the intermediate (0.107 g, 0.49 mmol) was dissolved in MeOH (10 mL). Sodium cyanoborohydride (220 mg, 3.5 mmol) was slowly added along with acetic acid (300 \( \mu \)L, 1.5% v/v). The reaction was left to stir overnight. MeOH was then removed in vacuo and saturated sodium bicarbonate (10 mL) was added. The
product was extracted with EtOAc (3 x 15 mL) and dried in vacuo. Purification via flash chromatography (SiO2, 2% MeOH/DCM) yielded the desired product as a white powder (0.095 g, 88%). $^1$H NMR (500 MHz, MeOD): δ 7.10 (d, $J = 8.6$ Hz, 2H), 6.81 (d, $J = 8.6$ Hz, 2H), 3.75 (s, 3H), 2.76 (t, $J = 7.2$ Hz, 2H), 2.60 (t, $J = 7.6$ Hz, 2H), 1.89 (s, 3H), 1.75 (quin, $J = 7.4$ Hz, 2H). $^{13}$C NMR (125 MHz, MeOD): δ 171.61, 159.50, 135.35, 130.46, 114.92, 55.78, 52.21, 33.43, 30.95, 20.74. ESI-HRMS: calcd. for C₁₂H₁₈N₂O₂: [M+H]$^+$ = m/z 223.1437, found: [M+H]$^+$ = m/z 223.1441.

(4-Phenylbutyl)hydrazine dihydrochloride (9h): The title compound was synthesized from 4-bromobutyl benzene (1.00 mL, 5.70 mmol) according to general procedure A and isolated as a white solid (0.640 g, 45%). $^1$H NMR (400 MHz, MeOD): δ 7.20 (m, 5H), 3.05 (m, 2H), 2.67 (t, $J = 7.3$ Hz, 2H), 1.69 (m, 4H).

(2-Phenoxyethyl)hydrazine dihydrochloride (10a): The title compound was synthesized from beta-bromophenetole (0.500 g, 2.49 mmol) according to general procedure A and isolated as an off-white solid (0.136 g, 36%). $^1$H NMR (400 MHz, MeOD): δ 7.30 (dd, $J_1 = 8.8$ Hz, $J_2 = 7.4$ Hz, 2H), 6.98 (m, 3H), 4.24 (t, $J = 5.0$ Hz, 2H), 3.43 (t, $J = 4.4$ Hz, 2H). $^{13}$C NMR (125 MHz, CDCl₃/MeOD): δ 157.60, 129.46, 121.64, 114.45, 62.68, 49.74.

(3-Phenoxypropyl)hydrazine dihydrochloride (10b): The title compound was synthesized from beta-bromopropyl phenoxy ether (366 µL, 2.32 mmol) according to general procedure A and isolated as an off-white solid (0.179 g, 46%). $^1$H NMR (400 MHz, MeOD):...
167 MHz, MeOD): 7.27 (t, J = 8.0 Hz, 2H), 6.94 (m, 3H), 4.10 (t, J = 5.8 Hz, 2H), 3.26 (t, J = 7.2 Hz, 2H), 2.14 (quin, J = 7.0 Hz, 2H). \(^{13}\)C NMR (125 MHz, MeOD): \(\delta\) 160.12, 130.66, 122.25, 115.67, 66.28, 50.30, 26.63.

4-(3-Hydrazinylpropyl) morpholine (11): Purchased from ChemBridge Screening Library (Catalog #9195784).

4-(4-Chlorophenyl)butanoic acid: 4-(4-Chlorophenyl)-4-oxobutanoic acid (1.06 g, 5 mmol) and KOH (85% by wt., 0.79 g, 12 mmol) were placed in a round-bottomed flask fitted with a Dean-Stark apparatus and a reflux condenser and suspended in diethylene glycol (10 mL) at RT. Then, hydrazine monohydrate (50% by wt., 1.20 g, 12 mmol) was added slowly to the reaction at RT after which it was heated to 120–130 °C for 2 h. The reaction became homogenous after heating for approximately 45 min. After 2 h, the temperature was increased to 180–200 °C and the reaction stirred for an additional 3 h to remove residual hydrazine and water via the Dean–Stark trap. The reaction was then cooled to RT, diluted with H₂O (10 mL), and poured into a 2.5 N aqueous solution of HCl (20 mL). The suspension was cooled in an ice bath and the resulting precipitate was isolated by filtration. To remove residual diethylene glycol, the solid was dissolved in a saturated aqueous solution of K₂CO₃ (20 mL), diluted with H₂O (20 mL), and poured into a 2.5 N aqueous solution of HCl (20 mL). The suspension was again cooled in an ice bath and the precipitate isolated by filtration, washed with cold H₂O (2 x 15 mL), and dried under vacuum. The title compound was isolated as a white solid (0.89 g, 89%). \(^{1}\)H NMR (500 MHz, DMSO-\(d_6\)): \(\delta\) 12.06 (br, 1H), 7.32 (d, J = 8.5 Hz, 2H), 7.21 (d, J = 8.5 Hz,
2H), 2.57 (t, J = 7.4 Hz, 2H), 2.20 (t, J = 7.3 Hz, 2H), 1.77 (q, J = 7.5 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): δ 174.16, 140.57, 130.41, 130.17, 128.20, 33.63, 32.95, 26.11. ESI-LRMS: [M-H]$^-$ = m/z 284.3. ESI-HRMS: calcd. for C$_{10}$H$_{11}$ClO$_2$: [M-H]$^-$ = m/z 197.0375, found: [M-H]$^-$ = m/z 197.0379.

4-(4-Fluorophenyl)butanoic acid: 4-(4-Fluorophenyl)-4-oxobutanoic acid (0.98 g, 5 mmol) and KOH (85% by wt., 0.79 g, 12 mmol) were placed in a round-bottomed flask fitted with a Dean-Stark apparatus and a reflux condenser and suspended in diethylene glycol (10 mL) at RT. Then, hydrazine monohydrate (50% by wt., 1.20 g, 12 mmol) was added slowly to the reaction at RT after which it was heated to 120–130 °C for 2 h. The reaction became homogenous after heating for approximately 45 min. After 2 h, the temperature was increased to 180–200 °C and the reaction stirred for an additional 3 h to remove residual hydrazine and water via the Dean–Stark trap. The reaction was then cooled to RT, diluted with H$_2$O (10 mL), and poured into a 2.5 N aqueous solution of HCl (20 mL). The organic products were extracted with EtOAc (3 x 15 mL), washed with brine (10 mL), dried with anhydrous Na$_2$SO$_4$, filtered, and concentrated in vacuo. Purification by column chromatography (30–50% EtOAc/hexanes) afforded the desired product as a clear, viscous oil (0.32 g, 35%). $^1$H NMR (500 MHz, CDCl$_3$): δ 11.50 (br, 1H), 7.16 (m, 2H), 7.00 (m, 2H), 2.67 (t, J = 7.6 Hz, 2H), 2.40 (t, J = 7.4 Hz, 2H), 1.97 (quin, J = 7.5 Hz, 2H). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 180.16, 161.34 (d, J = 243.4 Hz), 136.74, 129.76 (d, J = 7.3 Hz), 115.09 (d, J = 20.9 Hz), 34.09, 33.20, 26.24.
**N-[4-(2-Hydroxyethyl)phenyl]-3-phenylpropanamide (16c):** The title compound was synthesized from 3-phenylpropanoic acid (1.50 g, 10 mmol) according to general procedure F and isolated as a white solid (2.36 g, 88%). $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 9.80 (s, 1H), 7.46 (d, $J = 8.5$ Hz, 2H), 7.27 (m, 4H), 7.18 (m, 1H), 7.11 (d, $J = 8.5$ Hz, 2H), 4.59 (t, $J = 5.2$ Hz, 1H), 3.55 (td, $J_1 = 7.1$ Hz, $J_2 = 5.3$ Hz, 2H), 2.90 (t, $J = 7.6$ Hz, 2H), 2.65 (t, $J = 7.2$ Hz, 2H), 2.60 (t, $J = 7.7$ Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 170.08, 141.19, 137.12, 134.08, 128.95, 128.27, 128.21, 125.89, 118.99, 62.24, 38.45, 37.88, 30.85. ESI-HRMS: calcd. for C$_{17}$H$_{19}$NO$_2$: [M+H]$^+$ = m/z 270.1489, found: [M+H]$^+$ = m/z 270.1501.

**N-[4-(2-Hydroxyethyl)phenyl]-5-phenylpentanamide (16e):** The title compound was synthesized from 5-phenylpentanoic acid (0.89 g, 5 mmol) according to general procedure F. Purification by recrystallization from EtOAc facilitated by the dropwise addition of hexanes afforded the desired product as a white, crystalline solid (1.12 g, 75%). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.44 (d, $J = 8.3$ Hz, 2H), 7.36 (s, 1H), 7.30 (m, 2H), 7.19 (m, 4H), 3.84 (t, $J = 6.4$ Hz, 2H), 2.84 (t, $J = 6.5$ Hz, 2H), 2.67 (t, $J = 7.4$ Hz, 2H), 2.37 (t, $J = 7.2$ Hz, 2H), 1.79 (m, 2H), 1.72 (m, 2H), 1.65 (br, 1H). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 171.21, 142.07, 136.28, 134.41, 129.45, 128.36, 128.31, 125.78, 120.19, 63.57, 38.53, 37.48, 35.65, 30.97, 25.22. ESI-HRMS: calcd. for C$_{19}$H$_{23}$NO$_2$: [M+H]$^+$ = m/z 298.1802, found: [M+H]$^+$ = m/z 298.1807.

**4-(4-Chlorophenyl)-N-[4-(2-hydroxyethyl)phenyl]butanamide (16f):** The title compound was synthesized from 4-(4-chlorophenyl)butanoic acid (0.57 g, 3 mmol)
according to general procedure F. Purification by recrystallization from EtOAc facilitated by the dropwise addition of hexanes afforded the desired product as a white solid (0.89 g, 93%). $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 9.78 (s, 1H), 7.47 (d, $J = 8.5$ Hz, 2H), 7.33 (m, 2H), 7.24 (d, $J = 8.3$ Hz, 2H), 7.11 (d, $J = 8.5$ Hz, 2H), 4.59 (t, $J = 5.2$ Hz, 1H), 3.55 (td, $J_1 = 7.1$ Hz, $J_2 = 5.3$ Hz, 2H), 2.65 (t, $J = 7.2$ Hz, 2H), 2.61 (t, $J = 7.6$ Hz, 2H), 2.28 (t, $J = 7.5$ Hz, 2H), 1.87 (q, $J = 7.5$ Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 170.56, 140.68, 137.20, 134.02, 130.37, 130.21, 128.92, 128.18, 119.01, 62.27, 38.46, 35.52, 33.82, 26.57. ESI-HRMS: calcd. for C$_{18}$H$_{20}$ClNO$_2$: [M+H]$^+$ = m/z 318.1255, found: [M+H]$^+$ = m/z 318.1268.

**4-(4-Fluorophenyl)-N-[4-(2-hydroxyethyl)phenyl]butanamide (16g):** The title compound was synthesized from 4-(4-fluorophenyl)butanoic acid (0.32 g, 1.8 mmol) according to general procedure F and isolated as a white solid (0.53 g, 94%). $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 9.77 (s, 1H), 7.47 (d, $J = 8.3$ Hz, 2H), 7.24 (m, 2H), 7.10 (m, 4H), 4.59 (t, $J = 5.2$ Hz, 1H), 3.55 (td, $J_1 = 7.1$ Hz, $J_2 = 5.3$ Hz, 2H), 2.65 (t, $J = 7.2$ Hz, 2H), 2.60 (t, $J = 7.5$ Hz, 2H), 2.28 (t, $J = 7.5$ Hz, 2H), 1.86 (quin, $J = 7.5$ Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 170.61, 160.60 (d, $J = 241.6$ Hz), 137.76 (d, $J = 2.7$ Hz), 137.20, 134.03, 130.02 (d, $J = 8.2$ Hz), 128.92, 119.01, 114.91 (d, $J = 20.9$ Hz), 62.27, 38.46, 35.58, 33.70, 26.83. ESI-HRMS: calcd. for C$_{18}$H$_{20}$FNO$_2$: [M+H]$^+$ = m/z 302.1551, found: [M+H]$^+$ = m/z 302.1559.

**N-[4-(2-Hydroxyethyl)phenyl]-4-(4-methoxyphenyl)butanamide (16h):** The title compound was synthesized from 4-(4-methoxyphenyl)butanoic acid (0.58 g, 3 mmol)
according to general procedure F. Purification by column chromatography (SiO2, 25–75% EtOAc/hexanes) afforded the desired product as a white solid (0.74 g, 79%). $^1$H NMR (500 MHz, CDCl3): δ 7.42 (m, 3H), 7.15 (d, J = 8.3 Hz, 2H), 7.10 (d, J = 8.6, 2H), 6.83 (d, J = 8.5 Hz, 2H), 3.81 (t, J = 6.5 Hz, 2H), 3.78 (s, 3H), 2.81 (t, J = 6.5 Hz, 2H), 2.64 (t, J = 7.4 Hz, 2H), 2.31 (t, J = 7.5 Hz, 2H), 2.02 (quin, J = 7.4 Hz, 2H), 1.76 (s, 1H). $^{13}$C NMR (125 MHz, CDCl3): δ 171.17, 157.84, 136.26, 134.42, 133.34, 129.43, 129.35, 120.18, 113.80, 63.53, 55.22, 38.52, 36.59, 34.10, 27.07. ESI-HRMS: calcd. for C$_{19}$H$_{23}$NO$_3$: [M+H]$^+$ = m/z 314.1751, found: [M+H]$^+$ = m/z 314.1763.

$N$-[4-(2-Hydroxyethyl)phenyl]-4-(4-nitrophenyl)butanamide (16i): The title compound was synthesized from 4-(4-nitrophenyl)butanoic acid (1.05 g, 5 mmol) according to general procedure F and isolated as a white solid (1.49 g, 90%). $^1$H NMR (500 MHz, DMSO-d$_6$): δ 9.79 (s, 1H), 8.16 (d, J = 8.8 Hz, 2H), 7.51 (d, J = 8.8 Hz, 2H), 7.46 (d, J = 8.5 Hz, 2H), 7.11 (d, J = 8.6 Hz, 2H), 4.60 (br, 1H), 3.55 (m, 2H), 2.65 (t, J = 7.2 Hz, 2H), 2.31 (t, J = 7.4 Hz, 2H), 1.93 (quin, J = 7.5 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-d$_6$): δ 170.44, 150.23, 145.87, 137.16, 134.69, 129.67, 128.95, 123.45, 119.04, 62.29, 38.47, 35.48, 34.35, 26.20. ESI-HRMS: calcd. for C$_{18}$H$_{20}$N$_2$O$_4$: [M+H]$^+$ = m/z 329.1496, found: [M+H]$^+$ = m/z 329.1501.

$N$-[4-(2-Hydroxyethyl)phenyl]-3-(2-hydroxyphenyl)propanamide (16j): The title compound was synthesized from 3-(2-hydroxyphenyl)propanoic acid (0.83 g, 5 mmol) according to general procedure F and isolated as a white solid (1.43 g, 82%). $^1$H NMR (500 MHz, DMSO-d$_6$): δ 9.78 (s, 1H), 9.32 (s, 1H), 7.47 (d, J = 8.5 Hz, 2H), 7.11 (d, J =
8.5 Hz, 2H), 7.08 (dd, \( J = 7.4 \) Hz, 1.4 Hz, 1H), 7.00 (td, \( J = 7.7 \) Hz, 1.7 Hz, 1H), 6.78 (dd, \( J_1 = 8.0 \) Hz, \( J_2 = 0.9 \) Hz, 1H), 6.69 (td, \( J_1 = 7.4 \) Hz, \( J_2 = 1.1 \) Hz, 1H), 4.58 (br, 1H), 3.55 (t, \( J = 7.2 \) Hz, 2H), 2.82 (t, \( J = 7.8 \) Hz, 2H), 2.65 (t, \( J = 7.2 \) Hz, 2H), 2.55 (t, \( J = 7.8 \) Hz, 2H). \(^{13}\)C NMR (125 MHz, DMSO-\( \text{d}_6 \)): \( \delta \) 170.57, 155.07, 137.20, 134.00, 129.63, 128.94, 127.24, 126.98, 119.01, 118.84, 114.84, 62.27, 38.46, 36.25, 25.59. ESI-HRMS: calcd. for C\(_{17}\)H\(_{19}\)NO\(_3\): [M+H]\(^+\) = \( m/z \) 286.1438, found: [M+H]\(^+\) = \( m/z \) 286.1445.

\( N \)-[4-(2-Hydroxyethyl)phenyl]-3-(3-hydroxyphenyl)propanamide (16k): The title compound was synthesized from 3-(3-hydroxyphenyl)propanoic acid (0.83 g, 5 mmol) according to general procedure F. Purification by column chromatography (SiO\(_2\), 5% MeOH/DCM) afforded the desired product as a clear, viscous oil that solidified on standing overnight to form a white solid (0.67 g, 47%). \(^1\)H NMR (500 MHz, DMSO-\( \text{d}_6 \)): 9.80 (s, 1H), 9.25 (s, 1H), 7.46 (d, \( J = 8.3 \) Hz, 2H), 7.12 (d, \( J = 8.3 \) Hz, 2H), 7.05 (t, \( J = 7.8 \) Hz, 1H), 6.64 (m, 2H), 6.57 (dt, \( J_1 = 8.0 \) Hz, \( J_2 = 1.2 \) Hz, 1H), 4.59 (t, \( J = 5.3 \) Hz, 1H), 3.55 (td, \( J_1 = 7.1 \) Hz, \( J_2 = 5.3 \) Hz, 2H), 2.80 (t, \( J = 7.7 \) Hz, 2H), 2.65 (t, \( J = 7.1 \) Hz, 2H), 2.55 (t, \( J = 7.7 \) Hz, 2H). \(^{13}\)C NMR (125 MHz, DMSO-\( \text{d}_6 \)): \( \delta \) 170.15, 157.28, 142.61, 137.16, 134.08, 129.19, 128.96, 119.01, 118.80, 115.24, 112.88, 62.27, 38.47, 37.86, 30.86. ESI-HRMS: calcd. for C\(_{17}\)H\(_{19}\)NO\(_3\): [M+H]\(^+\) = \( m/z \) 286.1438, found: [M+H]\(^+\) = \( m/z \) 286.1449.

\( N \)-[4-(2-Hydrazinylethyl)phenyl]-3-phenylpropanamide sulfate (12c): The title compound was synthesized from \( N \)-[4-(2-hydroxyethyl)phenyl]-3-phenylpropanamide 16c (0.28 g, 1 mmol) according to general procedure G and the sulfate salt was prepared

172
according to general procedure H. The desired product was isolated as a white solid (0.25 g, 67%). $^1$H NMR (500 MHz, MeOD): δ 7.47 (d, J = 8.2 Hz, 2H), 7.22 (m, 7H), 3.25 (t, J = 7.8 Hz, 2H), 2.99 (t, J = 7.6 Hz, 2H), 2.91 (t, J = 7.8 Hz, 2H), 2.66 (t, J = 7.6 Hz, 2H).

$^{13}$C NMR (125 MHz, MeOD): δ 173.91, 142.23, 138.76, 134.18, 130.23, 129.62, 129.54, 127.38, 122.03, 53.71, 39.87, 32.93, 32.12. ESI-HRMS: calcd. for C$_{17}$H$_{21}$N$_3$O: [M+H]$^+$ = m/z 284.1757, found: [M+H]$^+$ = m/z 284.1770.

**N-[4-(2-Hydrazinylethyl)phenyl]-5-phenylpentanamide oxalate (12e):** The title compound was synthesized from N-[4-(2-Hydroxyethyl)phenyl]-5-phenylpentanamide 16e (0.30 g, 1 mmol) according to general procedure G and the oxalate salt was prepared according to general procedure I. The desired product was isolated as a white solid (0.32 g, 80%). $^1$H NMR (500 MHz, MeOD): δ 7.51 (d, J = 8.5 Hz, 2H), 7.20 (m, 7H), 3.24 (m, 2H), 2.91 (t, J = 7.7 Hz, 2H), 2.66 (t, J = 7.2 Hz, 2H), 2.39 (t, J = 7.2 Hz, 2H), 1.71 (m, 4H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): δ 171.02, 163.93, 142.04, 137.77, 132.34, 128.74, 128.25, 128.23, 125.63, 119.21, 51.48, 36.19, 34.89, 30.78, 30.63, 24.80. ESI-HRMS: calcd. for C$_{19}$H$_{25}$N$_3$O: [M+H]$^+$ = m/z 312.2070, found: [M+H]$^+$ = m/z 312.2081.

**4-(4-Chlorophenyl)-N-[4-(2-hydrazinylethyl)phenyl]butanamide sulfate (12f):** The title compound was synthesized from 4-(4-chlorophenyl)-N-[4-(2-hydroxyethyl)phenyl]butanamide (16f) (0.32 g, 1 mmol) according to general procedure G and the sulfate salt was prepared according to general procedure H. The desired product was isolated as a white solid (0.31 g, 73%). $^1$H NMR (500 MHz, MeOD): δ 7.50 (d, J = 8.6 Hz, 2H), 7.23 (m, 6H), 3.25 (m, 2H), 2.92 (t, J = 7.8 Hz, 2H), 2.68 (t, J = 7.7
Hz, 2H), 2.38 (t, J = 7.4 Hz, 2H), 1.99 (quin, J = 7.5 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): δ 170.73, 140.67, 137.77, 132.29, 130.39, 130.22, 128.76, 128.21, 119.27, 51.48, 35.54, 33.82, 30.81, 26.57. ESI-HRMS: calcd. for C$_{18}$H$_{22}$ClN$_3$O: [M+H]$^+$ = m/z 332.1524, found: [M+H]$^+$ = m/z 332.1537.

4-(4-Fluorophenyl)-N-[4-(2-hydrazinylethyl)phenyl]butanamide sulfate (12g): The title compound was synthesized from 4-(4-fluorophenyl)-N-[4-(2-hydroxyethyl)phenyl]butanamide 16g (0.30 g, 1 mmol) according to general procedure G and the sulfate salt was prepared according to general procedure H. The desired product was isolated as a white solid (0.24 g, 58%). $^1$H NMR (500 MHz, MeOD): δ 7.51 (d, J = 8.5 Hz, 2H), 7.21 (m, 4H), 6.99 (t, J = 8.8 Hz, 2H), 3.25 (m, 2H), 2.92 (t, J = 7.4 Hz, 2H), 2.37 (t, J = 7.7 Hz, 2H), 2.38 (t, J = 7.4 Hz, 2H), 1.98 (quin, J = 7.5 Hz, 2H). $^{13}$C NMR (125 MHz, MeOD): δ 174.39, 162.94 (d, J = 242.5 Hz), 139.00 (d, J = 3.63 Hz), 138.95, 134.11, 131.23 (d, J = 7.3 Hz), 130.23, 121.90, 116.08 (d, J = 21.8 Hz), 53.72, 37.29, 35.56, 32.20, 28.79. ESI-HRMS: calcd. for C$_{18}$H$_{22}$FN$_3$O: [M+H]$^+$ = m/z 316.1820, found: [M+H]$^+$ = m/z 316.1825.

N-[4-(2-Hydrazinylethyl)phenyl]-4-(4-methoxyphenyl)butanamide sulfate (12h): The title compound was synthesized from N-[4-(2-Hydroxyethyl)phenyl]-4-(4-methoxyphenyl)butanamide 16h (0.63 g, 2 mmol) according to general procedure G and the sulfate salt was prepared according to general procedure H. The desired product was isolated as a white solid (0.57 g, 67%). $^1$H NMR (500 MHz, MeOD): δ 7.51 (d, J = 8.5 Hz, 2H), 7.22 (d, J = 8.5 Hz, 2H), 7.12 (d, J = 8.6 Hz, 2H), 6.83 (d, J = 8.6 Hz, 2H), 3.75
(s, 3H), 3.25 (m, 2H), 2.91 (t, J = 7.8 Hz, 2H), 2.62 (t, J = 7.5 Hz, 2H), 2.36 (t, J = 7.5 Hz, 2H), 1.96 (quin, J = 7.5 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 170.93, 157.44, 137.82, 133.51, 132.30, 129.25, 128.78, 119.29, 113.73, 54.98, 51.53, 35.72, 33.74, 30.82, 27.07. ESI-HRMS: calcd. for C$_{19}$H$_{25}$N$_3$O$_2$: [M+H]$^+$ = m/z 328.2020, found: [M+H]$^+$ = m/z 328.2026.

$N$-[4-(2-Hydrazinylethyl)phenyl]-4-(4-nitrophenyl)butanamide sulfate (12i): The title compound was synthesized from $N$-[4-(2-Hydroxyethyl)phenyl]-4-(4-nitrophenyl)butanamide 16i (0.33 g, 1 mmol) according to general procedure G and the sulfate salt was prepared according to general procedure H. The desired product was isolated as a white solid (0.29 g, 65%). $^1$H NMR (500 MHz, MeOD): $\delta$ 8.15 (d, J = 8.8 Hz, 2H), 7.48 (t, J = 9.0 Hz, 4H), 7.21 (d, J = 8.5 Hz, 2H), 3.25 (m, 2H), 2.92 (t, J = 7.9 Hz, 2H), 2.84 (t, J = 8.6 Hz, 2H), 2.42 (t, J = 7.4 Hz, 2H), 2.06 (quin, J = 7.5 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 170.63, 150.24, 145.90, 137.75, 132.37, 129.71, 128.80, 123.48, 119.31, 51.52, 35.52, 34.36, 30.83, 26.22. ESI-HRMS: calcd. for C$_{18}$H$_{22}$N$_4$O$_3$: [M+H]$^+$ = m/z 343.1765, found: [M+H]$^+$ = m/z 343.1768.

2-(3-[[4-(2-Hydrazinylethyl)phenyl]amino]-3-oxopropyl)phenyl methanesulfonate oxalate (12j): The title compound was synthesized from $N$-[4-(2-Hydroxyethyl)phenyl]-3-(2-hydroxyphenyl)propanamide 16j (0.29 g, 1 mmol) according to general procedure G and the oxalate salt was prepared according to general procedure I. The desired product was isolated as a white solid (0.16 g, 34%). $^1$H NMR (500 MHz, MeOD): $\delta$ 7.49 (d, J = 8.5 Hz, 2H), 7.40 (dd, $J_1$ = 7.0 Hz, $J_2$ = 2.3 Hz, 1H), 7.36 (dd, $J_1$ = 7.6 Hz, $J_2$ = 1.7 Hz,
1H), 7.28 (m, 2H), 7.21 (d, J = 8.6 Hz, 2H), 3.34 (s, 3H), 3.24 (m, 2H), 3.11 (t, J = 7.8 Hz, 2H), 2.91 (t, J = 7.9 Hz, 2H), 2.70 (t, J = 7.6 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): δ 169.97, 163.85, 147.33, 137.64, 134.27, 132.49, 130.55, 128.78, 127.73, 127.22, 122.09, 119.27, 51.46, 38.30, 36.09, 30.80, 25.03. ESI-HRMS: calcd. for C$_{18}$H$_{23}$N$_3$O$_4$S: [M+H]$^+$ = m/z 378.1482, found: [M+H]$^+$ = m/z 378.1499.

3-(3-[[4-(2-Hydrazinylethyl)phenyl]amino]-3-oxopropyl)phenyl methanesulfonate oxalate (12k): The title compound was synthesized from N-[4-(2-Hydroxyethyl)phenyl]-3-(3-hydroxyphenyl)propanamide 16k (0.29 g, 1 mmol) according to general procedure G and the oxalate salt was prepared according to general procedure I. The desired product was isolated as an off-white solid (56 mg, 12%). $^1$H NMR (500 MHz, DMSO-$d_6$/MeOD): δ 7.45 (d, J = 8.5 Hz, 2H), 7.33 (t, J = 7.9 Hz, 1H), 7.21 (d, J = 7.7 Hz, 1H), 7.18 (m, 1H), 7.11 (m, 3H), 3.19 (s, 3H), 3.08 (t, J = 7.7 Hz, 2H), 2.94 (t, J = 7.5 Hz, 2H), 2.77 (t, J = 7.7 Hz, 2H), 2.60 (t, J = 7.7 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): δ 170.00, 164.09, 149.13, 143.76, 137.61, 132.54, 129.92, 128.79, 127.32, 121.93, 119.77, 119.27, 51.48, 37.43, 37.36, 30.79, 30.40. ESI-HRMS: calcd. for C$_{18}$H$_{23}$N$_3$O$_4$S: [M+H]$^+$ = m/z 378.1482, found: [M+H]$^+$ = m/z 378.1499.

N-[4-(2-Hydroxyethyl)phenyl]-3-(1H-indol-3-yl)propanamide (18a): The title compound was synthesized from 3-(1H-indol-3-yl)propanoic acid (0.57 g, 3 mmol) according to general procedure F and isolated as a white solid (0.82 g, 89%). $^1$H NMR (500 MHz, DMSO-$d_6$): δ 10.75 (s, 1H), 9.82 (s, 1H), 7.56 (d, J = 7.9 Hz, 1H), 7.48 (d, J = 8.5 Hz, 2H), 7.32 (d, J = 8.0 Hz, 1H), 7.12 (m, 3H), 7.06 (td, J$_1$ = 7.5 Hz, J$_2$ = 1.0 Hz,
1H, 6.98 (td, J₁ = 7.5 Hz, J₂ = 0.9 Hz, 1H), 4.60 (t, J = 5.3 Hz, 1H), 3.56 (td, J₁ = 7.2 Hz, J₂ = 5.3 Hz, 2H), 3.01 (t, J = 7.5 Hz, 2H), 2.66 (t, J = 7.4 Hz, 4H). ¹³C NMR (125 MHz, DMSO-₆): δ 170.71, 137.23, 136.21, 134.02, 128.95, 127.01, 122.13, 120.90, 119.00, 118.34, 118.14, 113.70, 111.31, 62.27, 38.47, 37.22, 20.83. ESI-HRMS: calcd. for C₁₉H₂₀N₂O₂: [M+H]⁺ = m/z 309.1598, found: [M+H]⁺ = m/z 309.1603.

**N-[4-(2-Hydroxyethyl)phenyl]-4-(1H-indol-3-yl)butanamide (18b):** The title compound was synthesized from 4-(1H-indol-3-yl)butanoic acid (0.61 g, 3 mmol) according to general procedure F and isolated as a white solid (0.41 g, 43%). ¹H NMR (500 MHz, DMSO-₆): δ 10.77 (s, 1H), 9.79 (s, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.49 (d, J = 8.3 Hz, 2H), 7.33 (d, J = 8.2 Hz, 1H), 7.11 (m, 3H), 7.06 (dt, J₁ = 7.1 Hz, J₂ = 0.9 Hz, 1H), 6.97 (dt, J₁ = 7.1 Hz, J₂ = 0.9 Hz, 1H), 4.60 (t, J = 5.2 Hz, 1H), 3.56 (td, J₁ = 7.1 Hz, J₂ = 5.3 Hz, 2H), 2.73 (t, J = 7.4 Hz, 2H), 2.66 (t, J = 7.2 Hz, 2H), 2.35 (t, J = 7.5 Hz, 2H), 1.96 (quin, 7.4 Hz, 2H). ¹³C NMR (125 MHz, DMSO-₆): δ 170.97, 137.29, 136.31, 133.97, 128.93, 127.17, 122.28, 120.81, 119.01, 118.29, 118.10, 114.01, 111.31, 62.29, 38.48, 36.14, 25.95, 24.31. ESI-HRMS: calcd. for C₂₀H₂₂N₂O₂: [M+H]⁺ = m/z 323.1754, found: [M+H]⁺ = m/z 323.1759.

**N-[4-(2-Hydrazinylethyl)phenyl]-3-(1H-indol-3-yl)propanamide sulfate (15a):** The title compound was synthesized from N-[4-(2-Hydroxyethyl)phenyl]-3-(1H-indol-3-yl)propanamide 18a (0.31 g, 1 mmol) according to general procedure G and the sulfate salt was prepared according to general procedure H. The desired product was isolated as a white solid (0.26 g, 62%). ¹H NMR (500 MHz, DMSO-d₆/MeOD): δ 7.53 (d, J = 7.9
Hz, 1H), 7.49 (d, J = 8.5 Hz, 2H), 7.29 (d, J = 8.2 Hz, 1H), 7.12 (d, J = 8.5 Hz, 2H), 7.03 (m, 2H), 6.95 (m, 1H), 3.09 (t, J = 7.4 Hz, 2H), 3.02 (t, J = 7.5 Hz, 2H), 2.78 (t, J = 7.8 Hz, 2H), 2.65 (t, J = 7.6 Hz, 2H). $^{13}$C NMR (125 MHz, MeOD/DMSO-$d_6$): $\delta$ 173.88, 139.09, 138.11, 134.05, 130.30, 128.73, 123.34, 122.56, 121.65, 119.82, 119.68, 115.34, 112.59, 53.55, 39.03, 32.14, 22.48. ESI-HRMS: calcd. for C$_{19}$H$_{22}$N$_4$O: [M+H]$^+$ = m/z 323.1866, found: [M+H]$^+$ = m/z 323.1871.

$N$-[4-(2-Hydrazinylethyl)phenyl]-4-(1H-indol-3-yl)butanamide sulfate (15b): The title compound was synthesized from $N$-[4-(2-hydroxyethyl)phenyl]-4-(1H-indol-3-yl)butanamide 18b (0.32 g, 1 mmol) according to general procedure G and the sulfate salt was prepared according to general procedure H. The desired product was isolated as an off-white solid (84 mg, 19%). $^1$H NMR (500 MHz, MeOD/DMSO-$d_6$): $\delta$ 7.63 (d, J = 8.3 Hz, 3H), 7.43 (d, J = 8.0 Hz, 1H), 7.27 (d, J = 8.5 Hz, 2H), 7.17 (m, 3H), 7.08 (m, 1H), 3.25 (t, J = 7.4 Hz, 2H), 2.94 (t, J = 7.7 Hz, 2H), 2.88 (t, J = 7.5 Hz, 2H), 2.48 (t, J = 7.5 Hz, 2H), 2.12 (quin, J = 7.4 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 171.12, 137.86, 136.31, 132.20, 128.76, 127.15, 122.28, 120.81, 119.27, 118.27, 118.09, 113.98, 111.33, 51.48, 36.14, 30.82, 25.95, 24.29. ESI-HRMS: calcd. for C$_{20}$H$_{24}$N$_4$O: [M+H]$^+$ = m/z 337.2023, found: [M+H]$^+$ = m/z 337.2025.

($2E$)-$N$-[4-(2-Hydroxyethyl)phenyl]-3-phenylprop-2-enamide (19): The title compound was synthesized from ($2E$)-3-phenylprop-2-enoic acid (0.74 g, 5 mmol) according to general procedure F and isolated as a white, crystalline solid (1.34 g, 88%). $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 10.13 (s, 1H), 7.61 (m, 5H), 7.42 (m, 3H), 7.18 (d, J =
8.5 Hz, 2H), 6.85 (d, J = 15.6 Hz, 1H), 4.62 (t, J = 5.3 Hz, 1H), 3.59 (td, J₁ = 7.1 Hz, J₂ = 5.2 Hz, 2H), 2.69 (t, J = 7.1 Hz, 2H). ^1^C NMR (125 MHz, DMSO-d₆): δ 163.30, 139.90, 137.17, 134.76, 134.54, 129.69, 129.13, 128.99, 127.66, 122.37, 119.15, 62.25, 38.51. ESI-HRMS: calcd. for C₁₇H₁₇NO₂: [M+H]^+ = m/z 268.1332, found: [M+H]^+ = m/z 268.1342.

(2E)-N-[4-(2-Hydrazinylethyl)phenyl]-3-phenylprop-2-enamide sulfate (13): The title compound was synthesized from (2E)-N-[4-(2-hydroxyethyl)phenyl]-3-phenylprop-2-enamide 19 (0.27 g, 1 mmol) according to general procedure G and the sulfate salt was prepared according to general procedure H. The desired product was isolated as a white solid (0.24 g, 64%). ^1^H NMR (500 MHz, MeOD): δ 7.63 (m, 5H), 7.41 (m, 3H), 7.27 (d, J = 8.5 Hz, 2H), 6.80 (d, J = 15.6 Hz, 1H), 3.27 (m, 2H), 2.94 (t, J = 7.8 Hz, 2H). ^1^C NMR (125 MHz, DMSO-d₆): δ 163.48, 140.04, 137.80, 134.75, 132.80, 129.75, 129.00, 128.96, 127.76, 122.38, 119.44, 51.49, 30.89. ESI-HRMS: calcd. for C₁₇H₁₉N₃O: [M+H]^+ = m/z 282.1601, found: [M+H]^+ = m/z 282.1608.

N-[4-((t-Butyl(dimethyl)silyl)oxy)ethyl]phenyl]-4-phenylbutanamide (20): N-[4-(2-hydroxyethyl)phenyl]-4-phenylbutanamide 16d (0.99 g, 3.5 mmol) was dissolved in anhydrous DCM (8 mL) and to it was added triethylamine (1.22 mL, 8.75 mmol) and DMAP (43 mg, 0.35 mmol) at RT. Upon dissolution of 16d, tert-butyldimethylsilyl chloride (0.63 g, 4.2 mmol) was dissolved in anhydrous DCM (7 mL) and added to the reaction in one portion. The reaction was then stirred at RT for 2 h after which it was poured into H₂O (15 mL) and the organic layer isolated. The aqueous layer was further
extracted with DCM (2 x 15 mL). The combined organic fractions were washed with brine (15 mL), dried with anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue obtained was dissolved in a 1:1 mixture of EtOAc/hexanes and passed through a 3-inch pad of silica gel (60 Å, 200–400 mesh). The filtrate was concentrated in vacuo which afforded the desired product as a clear, viscous oil (1.29 g, 92%). ¹H NMR (500 MHz, CDCl₃): δ 7.43 (m, 3H), 7.30 (m, 2H), 7.21 (m, 3H), 7.15 (d, J = 8.3 Hz, 2H), 3.79 (t, J = 7.1 Hz, 2H), 2.80 (t, J = 7.1 Hz, 2H), 2.71 (t, J = 7.5 Hz, 2H), 2.34 (t, J = 7.5 Hz, 2H), 2.07 (quin, J = 7.5 Hz, 2H), 0.90 (s, 9H), 0.01 (s, 6H). ¹³C NMR (125 MHz, CDCl₃): δ 170.95, 141.31, 136.01, 135.08, 129.52, 128.44, 128.36, 125.95, 119.75, 64.43, 38.94, 36.65, 35.02, 26.84, 25.88, 18.27, 5.42. ESI-HRMS: calcd. for C₂₄H₃₅NO₂Si: [M+H]+ = m/z 398.2510, found: [M+H]+ = m/z 398.2526.

**N-[4-(2-Hydroxyethyl)phenyl]-N-methyl-4-phenylbutanamide (21a):** Sodium hydride (95% by wt., 33 mg, 1.3 mmol) was placed under argon, suspended in anhydrous THF (2 mL), and cooled to 0 °C in an ice bath. Then, N-[4-((tert-butyldimethyl)silyl)oxy]ethylphenyl]-4-phenylbutanamide 20 (0.40 g, 1 mmol) was dissolved in anhydrous THF (3 mL) and added slowly to the reaction at 0 °C. Stirring was continued for 5 min and then methyl iodide (2 M solution in THF, 1.0 mL, 2 mmol) was added dropwise to the reaction. The reaction was stirred at 0 °C for 30 min after which it was warmed to RT and stirred for an additional 16 h. The reaction was then partitioned between saturated aqueous ammonium chloride (15 mL) and EtOAc (15 mL). The organic layer was isolated and the aqueous layer was further extracted with EtOAc (2 x 15 mL). The combined organic extracts were washed with brine (10 mL), dried with
anhydrous Na$_2$SO$_4$, filtered, and concentrated in vacuo. Purification by column chromatography (SiO$_2$, 25% EtOAc/hexanes) afforded the desired product as a clear viscous oil (0.34 g, 82%). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.24 (m, 4H), 7.15 (m, 1H), 7.11 (d, $J = 7.2$ Hz, 2H), 7.05 (d, $J = 8.0$ Hz, 2H), 3.84 (t, $J = 6.6$ Hz, 2H), 3.25 (s, 3H), 2.84 (t, $J = 6.7$ Hz, 2H), 2.54 (t, $J = 7.7$ Hz, 2H), 2.11 (t, $J = 7.4$ Hz, 2H), 1.91 (quin, $J = 7.5$ Hz, 2H), 0.87 (s, 9H), -0.02 (s, 6H). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 172.84, 142.16, 141.78, 139.12, 130.44, 128.33, 128.17, 126.88, 125.68, 64.09, 38.96, 37.28, 35.22, 33.42, 26.99, 25.85, 18.27, -5.45. ESI-HRMS: calcd. for C$_{25}$H$_{37}$NO$_2$Si: [M+H]$^+$ = m/z 412.2666, found: [M+H]$^+$ = m/z 412.2676.

$N$-[4-(2-{{[tert-butyl(dimethyl)silyl]oxy}ethyl}phenyl)-$N$-methyl-4-phenylbutanamide (0.32 g, 0.8 mmol) was dissolved in anhydrous THF (5 mL) and to it was added tetra-$n$-butylammonium fluoride (1 M solution in THF, 2.4 mL, 2.4 mmol) at RT. Stirring was continued until the reaction was complete as evidenced by TLC (approximately 24 h). Then, the reaction was poured into H$_2$O (10 mL) and the organic products were extracted with DCM (3 x 10 mL). The combined organic fractions were washed with brine (10 mL), dried with anhydrous Na$_2$SO$_4$, filtered, and concentrated in vacuo. Purification by column chromatography (SiO$_2$, 25–50% EtOAc/hexanes) afforded the desired product as a clear, viscous oil that solidified under vacuum (0.22 g, 94%). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.23 (m, 4H), 7.15 (m, 1H), 7.09 (d, $J = 7.7$ Hz, 2H), 7.07 (d, $J = 8.2$ Hz, 2H), 3.90 (t, $J = 6.6$ Hz, 2H), 3.25 (s, 3H), 2.90 (t, $J = 6.6$ Hz, 2H), 2.54 (t, $J = 7.6$ Hz, 2H), 2.10 (t, $J = 7.3$ Hz, 2H), 1.90 (quin, $J = 7.4$ Hz, 2H). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 172.91, 142.30, 141.70, 138.37, 130.20, 128.33, 128.16, 127.19, 125.68, 63.31, 38.62,
37.26, 35.16, 33.35, 26.94. ESI-HRMS: calcd. for C_{19}H_{23}NO_{2}: [M+H]^+ = m/z 298.1802, found: [M+H]^+ = m/z 298.1810.

**N-Benzyl-N-[4-(2-hydroxyethyl)phenyl]-4-phenylbutanamide (21b):** Potassium tert-butoxide (0.14 g, 1.2 mmol) was placed under argon, suspended in 4 mL of a 1:1 mixture of anhydrous DCM/DMF, and cooled to 0 °C in an ice bath. Then, N-[4-(2-tert-butyldimethylsilyloxy)ethyl]phenyl]-4-phenylbutanamide 20 (0.40 g, 1 mmol) dissolved in an additional 4 mL of a 1:1 mixture of anhydrous DCM/DMF was added slowly at 0 °C. The reaction was stirred for 15 min after which benzyl bromide (0.13 mL, 1.1 mmol) dissolved in 2 mL of a 1:1 mixture of anhydrous DCM/DMF was added dropwise to the reaction at 0 °C. The reaction was allowed to warm to RT and then heated to 60 °C for 16 h. The reaction was quenched by the addition of H_{2}O (30 mL), then DCM (15 mL) was added and the organic layer isolated. The aqueous layer was further extracted with DCM (2 x 10 mL) and the combined organic fractions were washed with H_{2}O (3 x 30 mL), brine (10 mL), dried with anhydrous Na_{2}SO_{4}, filtered, and concentrated in vacuo. Purification by column chromatography (SiO_{2}, 20% EtOAc/hexanes) afforded the desired product as a clear, viscous oil (0.40 g, 82%).

^1H NMR (500 MHz, CDCl_{3}): δ 7.29 (m, 7H), 7.19 (m, 5H), 6.89 (d, J = 8.5 Hz, 2H), 4.92 (s, 2H), 3.86 (t, J = 6.6 Hz, 2H), 2.85 (t, J = 6.5 Hz, 2H), 2.61 (t, J = 7.8 Hz, 2H), 2.16 (t, J = 7.4 Hz, 2H), 1.99 (quin, J = 7.5 Hz, 2H), 0.91 (s, 9H), 0.00 (s, 6H). ^13C NMR (125 MHz, CDCl_{3}): δ 172.63, 141.75, 140.36, 139.31, 137.72, 130.24, 128.79, 128.34, 128.24, 128.17, 127.95, 127.18, 125.67, 63.97, 52.93, 38.92, 35.17, 33.63, 26.98, 25.84, 18.24, -5.47.
N-Benzyl-N-[4-\{\text{[}t\text{ert}-\text{butyl}(\text{dimethyl})\text{silyl}]\text{oxy}\}\text{ethyl}\}\text{phenyl}]\text{-4-phenylbutanamide}

(0.35 g, 0.7 mmol) was dissolved in anhydrous THF (5 mL) and to it was added tetra-\text{n-}
butylammonium fluoride (1 M solution in THF, 2.2 mL, 2.2 mmol) at RT. Stirring was
continued until reaction was complete as evidenced by TLC (approximately 24 h). Then,
the reaction was poured into H\text{2}O (10 mL) and the organic products extracted with DCM
(3 x 10 mL). The combined organic fractions were washed with brine (10 mL), dried with
anhydrous Na\text{2}SO\text{4}, filtered, and concentrated in vacuo. Purification by column
chromatography (SiO\text{2}, 25–50\% EtOAc/hexanes) afforded the desired product as a clear,
viscous oil (0.25 g, 92\%). \text{1H NMR} (500 MHz, CDCl\text{3}): \text{δ} 7.26 (m, 7H), 7.17 (m, 3H),
7.11 (d, \text{J} = 7.2 \text{ Hz}, 2H), 6.89 (d, \text{J} = 8.0 \text{ Hz}, 2H), 4.89 (s, 2H), 3.89 (q, \text{J} = 6.1 \text{ Hz}, 2H),
2.88 (t, \text{J} = 6.6 \text{ Hz}, 2H), 2.58 (t, \text{J} = 7.6 \text{ Hz}, 2H), 2.12 (t, \text{J} = 7.4 \text{ Hz}, 2H), 1.96 (quin, \text{J} =
7.5 \text{ Hz}, 2H). \text{13C NMR} (125 MHz, CDCl\text{3}): \text{δ} 172.70, 141.73, 140.72, 138.41, 137.67,
129.99, 128.69, 128.38, 128.31, 128.19, 127.24, 125.71, 63.30, 52.95, 38.64, 35.16,
33.63, 26.95. ESI-HRMS: calcd. for C\text{25}H\text{27}NO\text{2}: [M+H]\text{+} = m/z 374.2115, found: [M+H]\text{+}
= m/z 374.2125.

\text{N-[4-(2-Hyrazinylethyl)phenyl]-N-methyl-4-phenylbutanamide oxalate (12l): The}
title compound was synthesized from \text{N-[4-(2-Hydroxyethyl)phenyl]-N-methyl-4-}
phenylbutanamide 21a (0.20 g, 0.68 mmol) according to general procedure G and the
oxalate salt was prepared according to general procedure I. The desired product was
isolated as a white solid (0.11 g, 40\%). \text{1H NMR} (500 MHz, MeOD): \text{δ} 7.32 (d, \text{J} = 8.0
Hz, 2H), 7.19 (m, 4H), 7.13 (m, 1H), 7.05 (d, \text{J} = 6.9 \text{ Hz}, 2H), 3.27 (t, \text{J} = 7.8 \text{ Hz}, 2H),
3.21 (s, 3H), 2.98 (t, \text{J} = 7.5 \text{ Hz}, 2H), 2.50 (t, \text{J} = 6.9 \text{ Hz}, 2H), 2.08 (t, \text{J} = 6.8 \text{ Hz}, 2H),
1.84 (br, 2H). $^{13}$C NMR (125 MHz, MeOD/DMSO-$d_6$): δ 175.16, 165.52, 144.00, 142.98, 138.91, 131.44, 129.57, 129.50, 128.78, 127.05, 53.20, 37.88, 36.19, 34.35, 32.40, 28.41. ESI-HRMS: calcd. for C$_{19}$H$_{25}$N$_3$O: [M+H]$^+$ = m/z 312.2070, found: [M+H]$^+$ = m/z 312.2079.

$N$-Benzyl-$N$-[4-(2-hydrazinylethyl)phenyl]-4-phenylbutanamide oxalate (12m): The title compound was synthesized from $N$-Benzyl-$N$-[4-(2-hydroxyethyl)phenyl]-4-phenylbutanamide 21b (0.25 g, 0.66 mmol) according to general procedure G and the oxalate salt was prepared according to general procedure I. The desired product was isolated as an off-white solid (0.23 g, 47%). $^1$H NMR (500 MHz, MeOD): δ 7.20 (m, 10H), 7.06 (d, $J = 7.2$ Hz, 2H), 6.95 (d, $J = 8.0$ Hz, 2H), 4.88 (s, 2H), 3.23 (m, 2H), 2.93 (t, $J = 8.2$ Hz, 2H), 2.53 (t, $J = 7.5$ Hz, 2H), 2.10 (t, $J = 7.4$ Hz, 2H), 1.88 (quin, $J = 7.4$ Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$): δ 171.61, 164.24, 141.57, 140.53, 137.76, 137.57, 129.54, 128.27, 128.20, 128.18, 128.06, 127.74, 126.97, 125.69, 51.95, 50.95, 34.39, 32.78, 30.92, 26.64. ESI-HRMS: calcd. for C$_{25}$H$_{29}$N$_3$O: [M+H]$^+$ = m/z 388.2383, found: [M+H]$^+$ = m/z 388.2396.
Appendix II: Compound 9-15 $^1$H NMR and $^{13}$C NMR Spectra
Appendix III: LSD1 and MAO A/B Kinetic Parameter Data Tables;

Compound 9-15 LSD1 Kinetic Plots of $k_{\text{obs}}$ Versus Inhibitor Concentration

Appendix III Table 1. Replicate #2 GST-LSD1 inhibition kinetics of compounds 12a, 12b, 12d (bizine), and 14.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_{\text{i(inact)}}$ ($\mu$M)</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$k_{\text{inact}}$/$K_{\text{i(inact)}}$ ($\mu$M$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenelzine</td>
<td>6.4±1.79</td>
<td>0.19±0.02</td>
<td>0.030±0.0089</td>
</tr>
<tr>
<td>12a</td>
<td>0.34±0.13</td>
<td>0.15±0.01</td>
<td>0.44±0.17</td>
</tr>
<tr>
<td>12b</td>
<td>0.34±0.07</td>
<td>0.18±0.02</td>
<td>0.53±0.12</td>
</tr>
<tr>
<td>12d</td>
<td>0.23±0.094</td>
<td>0.19±0.04</td>
<td>0.83±0.38</td>
</tr>
<tr>
<td>14</td>
<td>1.3±0.68</td>
<td>0.11±0.032</td>
<td>0.085±0.051</td>
</tr>
</tbody>
</table>
Appendix III Table 2. Replicate #1 MAO-A inhibition kinetics (best fit) of compounds 12a, 12b, 12d (bizine), and 14.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_{i\text{inact}}$ (µM)</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$k_{\text{inact}}/K_{i\text{inact}}$ (µM$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenelzine</td>
<td>0.81±0.46</td>
<td>0.24±0.057</td>
<td>0.30±0.18</td>
</tr>
<tr>
<td>12a</td>
<td>2.8±1.3</td>
<td>0.29±0.050</td>
<td>0.10±0.051</td>
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<tr>
<td>12b</td>
<td>3.9±6.0</td>
<td>0.39±0.28</td>
<td>0.10±0.17</td>
</tr>
<tr>
<td>12d</td>
<td>2.6±2.3</td>
<td>0.30±0.11</td>
<td>0.12±0.11</td>
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<tr>
<td>14</td>
<td>2.1±0.38</td>
<td>0.28±0.016</td>
<td>0.13±0.025</td>
</tr>
</tbody>
</table>

Appendix III Table 3. Replicate #2 MAO-A inhibition kinetics (best fit) of compounds 12a, 12b, 12d (bizine), and 14.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_{i\text{inact}}$ (µM)</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$k_{\text{inact}}/K_{i\text{inact}}$ (µM$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenelzine</td>
<td>0.76±0.27</td>
<td>0.31±0.040</td>
<td>0.41±0.15</td>
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<tr>
<td>12a</td>
<td>4.9±2.3</td>
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<td>0.084±0.042</td>
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<tr>
<td>12b</td>
<td>5.0±1.3</td>
<td>0.37±0.035</td>
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<tr>
<td>12d</td>
<td>5.0±3.9</td>
<td>0.57±0.22</td>
<td>0.11±0.099</td>
</tr>
<tr>
<td>14</td>
<td>2.0±0.78</td>
<td>0.25±0.034</td>
<td>0.13±0.052</td>
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</tbody>
</table>
Appendix III Table 4. Replicate #1 MAO-B inhibition kinetics (best fit) of compounds 12a, 12b, and 12d (bizine).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_{i\text{(inact)}}$ (µM)</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$k_{\text{inact}}/K_{i\text{(inact)}}$ (µM$^{-1}$min$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>phenelzine</td>
<td>3.9±1.7</td>
<td>0.20±0.040</td>
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<tr>
<td>12a</td>
<td>2.1±0.59</td>
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<td>0.095±0.031</td>
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<tr>
<td>12b</td>
<td>3.1±0.78</td>
<td>0.20±0.031</td>
<td>0.065±0.019</td>
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<tr>
<td>12d</td>
<td>6.5±4.6</td>
<td>0.26±0.13</td>
<td>0.04±0.035</td>
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</table>

Appendix III Table 5. Replicate #2 MAO-B inhibition kinetics (best fit) of compounds 12a, 12b, and 12d (bizine).

<table>
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<tr>
<th>Inhibitor</th>
<th>$K_{i\text{(inact)}}$ (µM)</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$k_{\text{inact}}/K_{i\text{(inact)}}$ (µM$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenelzine</td>
<td>1.9±0.61</td>
<td>0.48±0.090</td>
<td>0.25±0.094</td>
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<tr>
<td>12a</td>
<td>3.3±0.79</td>
<td>0.44±0.085</td>
<td>0.13±0.041</td>
</tr>
<tr>
<td>12b</td>
<td>12.7±15.9</td>
<td>1.0±1.1</td>
<td>0.079±0.13</td>
</tr>
<tr>
<td>12d</td>
<td>10.1±10.8</td>
<td>0.38±0.33</td>
<td>0.038±0.052</td>
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</table>
Phenelzine (3):

![Graph of 3 - LSD1 - 0-20 min]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>kinact</td>
<td>0.3545</td>
<td>0.0584</td>
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<tr>
<td>Kiinact</td>
<td>89.5833</td>
<td>20.7782</td>
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</tbody>
</table>
9a:
9b:

![Graph showing the effect of different concentrations of a compound on product formation over time. The graph includes data points for [I]=0μM, [I]=100μM, and [I]=100μM.]
9c:
9d:

![Graph showing enzyme kinetics](image)

**9d – LSD1**

\[ y = 0.0725x + 3.3887 \]

\[ R^2 = 0.8746 \]

\[ IC_{50} = 47 \mu M \]
\(9e:\)

![Graph: 9e - LSD1 - 0-20 min](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{\text{max}})</td>
<td>0.1453</td>
<td>0.0225</td>
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<td>(K_m)</td>
<td>24.1930</td>
<td>10.6825</td>
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</table>
9f:

![Graph showing the relationship between [9f] (µM) and 1/V (min µM⁻¹). The line of best fit is given by the equation y = 0.0317x + 3.73 with an R² value of 0.7908. The IC₅₀ value is 117.6 µM.](image)
9g - LSD1 - 0-20 min

- [I] = 0 uM
- [I] = 100 uM
- [I] = 100 uM
- [I] = 50 uM
9h:
10a:
10b:

Graph showing the relationship between $K_{obs}$ (min$^{-1}$) and [10b] (μM) for LSD1 over 0-20 minutes.
11 - LSD1

The graph shows the relationship between $1/V$ (1/min µM$^{-1}$) and $[11]$ (µM) with the equation $y = 0.0197x + 2.4284$ and an $R^2$ value of 0.8155. The IC$_{50}$ value is 123.3 µM.
12a:

**12a - LSD1 - 0-20 min**

<table>
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<tr>
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<th>Value</th>
<th>Std. Error</th>
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<td>Kiinact</td>
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<td>1.8134</td>
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</table>
12b:

**Graph:**

- **Title:** 12b - LSD1 - 0-20 min
- **Y-axis:** $k_{obs}$ (min$^{-1}$)
- **X-axis:** [12b] (µM)

<table>
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<th>Std. Error</th>
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<td>0.0087</td>
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<td>$K_{inact}$</td>
<td>5.8976</td>
<td>0.5085</td>
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</table>
12c - LSD1 - 0-20 min

\[ K_{\text{obs}} \text{ (min}^{-1} \text{)} \]

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**12d - LSD1 - 0-20 min**

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### 12e - LSD1 - 0-20 min

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12f - LSD1 - 0-20 min

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**12g - LSD1 - 0-20 min**

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**12h - LSD1 - 0-20 min**

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12i:

![Graph showing the relationship between [12i] (µM) and $k_{obs}$ (min⁻¹) for 12i-LSD1 over 0-20 min. The graph includes a line of best fit with data points and a table below with parameter values and standard errors.]

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$12j$:

**12j - LSD1 - 0-20 min**

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12k - LSD1 - 0-20 min

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12I - LSD1 - 0-20 min

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**12m - LSD1 - 0-20 min**

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13 - LSD1 - 0-20 min

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14 - LSD1 - 0-20 min

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15a - LSD1 - 0-20 min

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15b:  

15b - LSD1 - 0-20 min  

### Parameters

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References

Histone demethylation catalysed by LSD1 is a flavin-dependent oxidative process. 

LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. 

LSD1 blocks alpha-herpesvirus lytic replication and reactivation from latency. 

LSD1 is a subunit of a new family of histone deacetylase-containing complexes. 

LSD1 and targets the metastasis programs in breast cancer. 

LSD1 histone demethylase activity by its associated factors. 


(125) Schmidt, D. M. Z., and McCafferty, D. G. (2007) trans-2-
Phenylcyclopropylamine is a mechanism-based inactivator of the histone
demethylase by the antidepressant trans-2-phenylcyclopropylamine. Biochemistry 46, 8058–8065.
(136) Abdulla, A., Zhao, X., and Yang, F. (2013) Natural Polyphenols Inhibit Lysine-


142 Yu, Y. (2013) Inhibition of DNA methyltransferases, histone deacetylases and lysine-specific demethylase-1 suppresses the tumorigenicity of the ovarian cancer ascites cell line SKOV3. Int. J. Oncol.


Disclosure of Potential Conflicts of Interest

Polina Prusevich, the author of this dissertation, declares no conflict of interest.
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Polina Prusevich     September 2014

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Research rotation 2010 Lab of Diane Hayward, Hopkins
Research rotation 2010 Lab of Caren L. Freel-Meyers, Hopkins
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Salutatorian 2006 Oyster River High School

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Posters:

Publications:
Patents:
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Student Research Mentor, Johns Hopkins University School of Medicine
Dawn Hayward, Heather Roberson, Kirubel Frew, Sabrina Schatzman, Amer Alkhouja
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Publication: Cell Research

Publisher: Nature Publishing Group

Date: Dec 25, 2012

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