Examination of the Role of Lysine Specific Demethylase 1 (LSD1) and Associated Proteins in Breast Cancer Proliferation using 2-Phenylcyclopropylamine Inhibitors

by

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Michael Fitzgerald

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the Graduate School of Duke University

2011
ABSTRACT

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Abstract

Lysine specific demethylase 1 (LSD1) is a FAD-dependent amine oxidase enzyme responsible for removing methyl groups from the side chain nitrogen of lysine within histones in order to regulate gene transcription. By its interaction with various transcriptional complexes, including those containing estrogen receptor α (ERα), LSD1 mediates expression of many genes important in cancer proliferation and progression. Herein, we report our efforts towards understanding the function of LSD1 in breast cancer. We have developed a straightforward method for the syntheses of 2-arylcyclopropylamines as irreversible mechanism-based inactivators of LSD1. We employed these small molecules as probes of LSD1 activity, and together with experiments involving the knockout of LSD1 by small interfering RNA (siRNA), we have shown that LSD1 activity is essential for both ERα-positive and ERα-negative breast cancer proliferation. LSD1 inhibitors induce a dramatic cell cycle arrest without causing apoptosis.

Furthermore, we observe that LSD1 and ERα work cooperatively to express certain estrogen-target genes through simultaneous recruitment to promoters; LSD1 inhibition diminishes ERα recruitment. Similarly, knockdown of CoREST, a binding partner of LSD1, results in comparable changes in gene expression. Although, we have not observed a direct interaction between LSD1 and ERα, we believe that CoREST may
be facilitating this interaction. We have made efforts to inhibit the interaction between LSD1 and CoREST *in vitro* in hopes of targeting this interface in breast cancer cells in order to disrupt the necessary functional complex and prevent LSD1 activity.
To my parents who have always supported my dreams
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List of Abbreviations

2-PCPA 2-phenylcyclopropylamine
AR Androgen receptor
Boc tert-butylcarbamate
ChIP Chromatin immunoprecipitation
DA Dopamine
DAT Dopamine transporter
DDD Dopamine-deficient dopamine transporter knockout
DMF Dimethylformamide
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
DPPA Diphenylphosphorylazide
E2 Estradiol
ERE Estrogen response element
ERα Estrogen receptor α
FAD Flavin adenine dinucleotide
FPLC Fast protein liquid chromatography
HAT Histone acetyltransferase
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<tr>
<td>HDAC</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>PD</td>
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<td>2,2,6,6-tetramethylheptane-3,5-dione</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsilyl</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank my boss Dewey McCafferty for taking me in as an organic chemist and opening my eyes to the world of biology, for allowing me to follow the science into the unknown and for encouraging me to grow as a teacher and a researcher.

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1. Introduction

1.1 Eukaryotic gene regulation

Genetic material is encoded as DNA, and although DNA is not directly involved in running specific operations of the cells, it programs all of its activities. In normal cells, finely controlled regulation of gene expression allows for cells to replicate, grow and die in a balanced manner. However, in disease states, such as cancer, this balance is disrupted. Mutations within specific genes can lead to a gain in function accelerating cell division or a loss of function of tumor suppressors. Alternatively, there are not always specific variations within the DNA sequences, but instead changes in the regulation of gene expression. In many cases, this disregulation causes the normal process of programmed cell death to no longer operate functionally. In order to identify ways to combat cancer progression, it is essential to fully understand how this misregulation of gene expression arises and what cellular components are responsible.

1.1.1 The nucleosome

Within eukaryotes, DNA is organized into a compact form called chromatin. One of the main organizational features is the nucleosome, the individual repeating unit of chromatin that is subsequently packaged further into more compact states. Nucleosomes are made up of an octamer of histone proteins (H2A, H2B, H3 and H4) around which approximately 146 base pairs of DNA is wound (Figure 1) [1]. The assembly of a stable
nucleosomal core depends on initial heterodimerization of H3 with H4 and subsequent
dimerization of H3 to form the H3-H4 tetramer. Histones H2A and H2B form a stable
heterodimer that binds to both sides of the H3-H4 tetramer to form the core octamer. An
additional histone, H1, acts as a linker that stabilizes adjacent nucleosomes and the DNA
strands between them. Together, this combination of nucleosomes and linker histones
forms an organized structure that appears as “beads on a string”. The positively charged
histone residues such as arginine contact the negatively charged phosphate groups of
the DNA to form weak interactions that collectively form the strong stable interactions
between the histones and DNA. These precise histone-DNA interactions constrain all
DNA sequences, regardless of inherent sequence dependent structure, to adopt a
relatively similar conformation in the nucleosome [2].
Figure 1. Nucleosome structure. DNA (orange) wraps around the histone octamer core (green). Figure was generated using the 1AOI pdb file.
Heterochromatin, the condensed structure of chromatin, inhibits gene transcription, DNA replication and DNA recombination, while euchromatin is a loosely packed structure associated with active transcription (Figure 2). By preventing the access of RNA polymerase and other regulatory proteins to the DNA, the heterochromatin functions to repress these cell processes [3]. A series of chromatin remodeling complexes alter the structure of chromatin, enabling accessibility to specific elements such as promoters and enhancers in an organized and controlled manner [4].
Figure 2. Structural differences between euchromatin and heterochromatin.
1.1.2 Post-translational modifications of histones

The flexible tails of histones that make up approximately 25% of the mass of the histones extend freely beyond the core nucleosomal structure and are readily accessible to other proteins and transcription factors. These N- and C-terminal tails were initially defined by their sensitivity to proteases [5], however, their capacity to be altered post-translationally via histone-modifying enzymes has more completely defined their functions. The post-translational modifications decorate all the histone core proteins and are typically localized to the N- and C-terminal tails with only a few found in the globular domains themselves. These histone modifications along with DNA methylation are known as epigenetics, and together they alter chromatin structure and affect gene transcription, thus amplifying the information stored in DNA without affecting the sequence [6]. Histone modifications, including methylation, phosphorylation, acetylation and biotinylation (Figure 3) as well as ubiquitination and SUMOylation, can correlate with either positive or negative transcriptional states depending on the site of modification. Typically, in response to cytoplasmic signaling, positive-acting marks are established across promoters activating gene transcription through recruitment of relevant DNA-bound activators and RNA polymerase. Alternatively, negative-acting marks are added across genes during repression by DNA-bound repressor proteins or to heterochromatic regions of the genome [6].
Figure 3. Histone H3 N-terminal tail modifications.
For example, the acetylation of histones at specific residues in H3 and H4 has been typically associated with transcriptional activity [7]. Active genes exhibit high levels of acetylation while histones associated with repressed heterochromatin are hypoacetylated. In the case of lysine, the positively charged amino group becomes neutral upon acetylation and therefore weakens the interaction between the histone and the phosphate backbone of DNA. Generally, phosphorylation of threonines and serines is associated with activated gene transcription while SUMOylation of lysine represses transcription [6]. Methylation, on the other hand, has been associated with both activation and repression, depending on the specific residue involved [8]. Similarly, ubiquitination has different functions [6].

These generalized classifications are beginning to be questioned as contradictory observations have appeared. For example, lysine 12 of histone H4 (H4K12) has been found to be acetylated in heterochromatin [9]. Also, the functional outcome of H3K4 methylation, although initially thought to be a positive-acting modification, has been found to recruit numerous proteins whose regulatory functions are not only activating but also repressing. The chromatin remodeler Chd1 binds to H3K4-Me, assisting the recruitment of acetyltransferases and enhancing acetylation [10]. Alternatively, the Sin3-HDAC1 deacetylation complex binds to H3K4-Me3 and reduces acetylation [11]. From these examples and many others, it is clear that the epigenetic landscape is multifaceted. Indeed, examining an individual modification may not reveal the entire story. The
number, variety, and interdependence of histone modifications led to the “histone code” hypothesis predicting that “multiple histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions” [12]. In order to more completely understand this “histone code”, it is essential to understand the proteins that manipulate the histone tails by adding and erasing modifications.

The enzymes responsible for post-translational modifications, in particular acetylation and methylation, play important roles in the regulation of gene expression, and consequently, the proteins affect major cellular processes such as proliferation and differentiation. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) counteract each other to control acetylation levels of lysines. Methylation of lysine and arginine residues is regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs). In most cases, these enzymes are present in multisubunit protein complexes where the partners are believed to regulate enzymatic activity, modulate substrate specificity, recruit cofactors, and carry out other undefined functions. The approximately 20 human HDACs are divided into three classes based on their sequence similarity. Class I and II HDACs are zinc-dependent metallohydrolases found in many protein complexes that function in cell-cycle regulation, maintenance of stem cell pluripotency, and cellular differentiation [13,14]. Treatment of tumor cells with general inhibitors of class I and II HDACs results in growth arrest, differentiation, and apoptosis.
[15-17]. Recent studies have revealed that many of the complexes containing HDACs are associated with HDMs, and therefore much work has focused on understanding the enzymes that erase methyl marks.

1.2 Histone lysine methylation and demethylation

Histones may be methylated on either lysine (K) or arginine (R) residues. A methyl group is relatively small and its addition to either of these residues does not neutralize its charge. Therefore, methylation itself does not affect chromatin structure significantly, but instead, these methyl groups are thought to create binding sites for regulatory proteins [18]. Arginine side chains may be mono-methylated or di-methylated (symmetrically or asymmetrical), whereas lysine side chains can be mono-, di-, or tri-methylated (Figure 4). Because of the considerable number of distinct lysine and arginine residues present in the N-terminal tails of histones as well as the multiple methylation states, there are a substantial number of possibilities.
Lysine methylation states

Arginine methylation states

Figure 4. Potential methylation states of lysine and arginine.
The mechanistic and functional consequences of lysine methylation are limited to the proteins and domain that recognize the modification. For example, heterochromatin protein 1 (HP1) specifically recognizes the repressive methylation mark of H3K9 whereas chromodomain helicase DNA-binding protein (CHD1) binds to the activating methylated H3K4 [19]. Alternatively, the DNA-repair checkpoint protein p53-binding protein 1 (p53BP1) recognizes methylated H3K79 [10], and the vertebrate transcriptional activator WDR5 binds both di- and tri-methylated H3K4 [20]. Almost all characterized methylation marks have been shown to play a role in transcription, and recently the role of methylation has been extended to establishing the checkpoint control following DNA damage [20].

Methylation of lysine residues is executed by histone methyltransferases (HMTs), and until the discovery of the first histone lysine demethylase (KDM), the methylation was thought to be irreversible [21]. However, since then two classes of KDMs have been established. The amine oxidases containing proteins (LSD1 and LSD2) use a FAD-cofactor to generate the demethylated lysine, a reduced FADH₂ and formaldehyde. The JmjC-domain containing proteins (including KDM2A, KDM4C, KDM5A) are metalloenzymes that require iron and α-ketoglutarate as cofactors and produce succinate, carbon dioxide, and formaldehyde in addition to the demethylated lysine. The latter class is capable of erasing all methyl modifications while the former only works on mono- and di-methylated lysines.
Figure 5. Schematic representation of members of the two families of histone lysine demethylases.
Considerable progress has been made towards understanding the dynamic landscape of histone lysine methylation regulation, but the biological function of the newly found KDMs are just beginning to be uncovered. Global levels of lysine methylation have been linked to clinical outcome and progression of multiple types of cancer (Table 1). Furthermore, mutations or overexpression of several KDMs have recently been linked to cancer, raising the possibility of therapeutic potential.

Table 1. Patterns of global lysine methylation cancer.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Methylation site and state</th>
<th>Alternation in cancer (as compared to normal tissue)</th>
<th>Associated cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K4-Me</td>
<td>Decreased initially, increased upon progression</td>
<td>Prostate</td>
</tr>
<tr>
<td>H3K4-Me2</td>
<td>Decreased initially, increased upon progression</td>
<td>Lung, kidney, prostate, non small cell lung carcinoma, hepatocellular carcinoma, breast, pancreatic adenocarcinoma</td>
</tr>
<tr>
<td>H3K4-Me3</td>
<td>Increased upon progression</td>
<td>Prostate</td>
</tr>
<tr>
<td>H3K9-Me2</td>
<td>Decreased</td>
<td>Pancreatic adenocarcinoma, prostate, kidney</td>
</tr>
<tr>
<td>H3K9-Me3</td>
<td>Increased</td>
<td>Gastric adenocarcinoma</td>
</tr>
<tr>
<td>H3K27-Me3</td>
<td>Decreased</td>
<td>Prostate</td>
</tr>
<tr>
<td>H3K27-Me3</td>
<td>Increased</td>
<td>Breast, ovarian, pancreatic Paragangliomas</td>
</tr>
<tr>
<td>H4K20-Me3</td>
<td>Decreased</td>
<td>Lymphomas, colorectal adenocarcinoma, breast carcinoma</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Adapted from Varier, et. al. [18]
Accumulating evidence suggests that HDMs participate in nuclear receptor (NR) signaling. There are approximately 50 mammalian NRs that mediate biological effects of hormones [22]. Transcriptional activation by NR is a multistep process and involves primary and secondary coactivators. Binding of hormone to the receptor induces a structural reorganization that allows the NR to recognize specific DNA elements at promoter regions [23]. The conformational changes result in recruitment of coactivators, many of which process intrinsic enzymatic activities, such as demethylation. The simultaneous action of these proteins usually results in changes in local chromatin structure and subsequent recruitment of RNA polymerase II transcriptional machinery [24]. Recently HDMs have emerged as key players in the regulation of androgen receptor (AR)-mediated transcription. Both LSD1 and KDM4C have been shown to interact with AR and induce demethylation of H3K9 at the prostate-specific antigen (PSA) promoter [25,26]. Similarly, activation of thyroid receptor (TR)-dependent transcription has been attributed to demethylation of H3K9 presumably by a JmjC-domain containing family member [27]. Although studies have linked arginine methylation to estrogen receptor (ER) function, very little has been done in elucidating the role of lysine methylation in regulating ER-dependent transcription. Recently, several H3K9 HMTs have been implicated in repressing ER targets pS2 and GREB1 in the absence of estrogen [28]. However, the role of LSD1 in estrogen signaling has not been completely defined.
1.3 LSD1

1.3.1 LSD1 mechanism and function

As mentioned above, LSD1 is member of the FAD-dependent amine oxidase class of KDMs. LSD1 facilitates demethylation by oxidizing the carbon-nitrogen bond between the methyl group and the epsilon amine of lysine residues, forming an imine intermediate that is nonenzymatically hydrolyzed to produce formaldehyde and the demethylated lysine (Scheme 1). LSD1 can only demethylate mono- and dimethylated lysines, and not trimethylated lysines, due to the necessity of a lone pair of electrons on the amino group to form the imine intermediate.

![Scheme 1. Depiction of LSD1 reaction mechanism.](image)

LSD1 was initially shown to repress transcription of neuronal genes by demethylation of H3K4 [29], suggesting that LSD1 functions generally as a gene repressor. However, studies of LSD1 with the androgen receptor (AR) revealed that LSD1 was also able to demethylate H3K9 and subsequently upregulate expression of AR-target genes [30]. In one study, approximately 80% of the promoters that were
occupied by LSD1 also were bound to RNA polymerase II, revealing LSD1 association with more active than inactive genes [28]. LSD1 has been implicated in the maintenance of diseases such as neuroblastoma [31] and high-risk prostate cancer [32]. The enzyme plays important regulatory roles in cell differentiation [33] and is required for transcriptional repression of the hTERT gene in both normal and cancerous cells [34]. In addition to histone substrates, LSD1 has been shown to demethylate K370 of the tumor suppressor p53, repressing its function by disrupting the interaction with 53BP1, a DNA damage checkpoint [35]. It has been seen that the SNAG domain of Snail1 resembles a H3-like structure and functions as a molecular hook for recruitment of LSD1 to repress gene expression in metastasis [36]. Furthermore, LSD1 is highly expressed in breast cancer cells and has been deemed an essential mediator of the interchromosomal interactions necessary for estrogen-dependent transcription [37,38].

1.3.2 LSD1 structure

Homology and crystallographic studies reveal that LSD1 is composed of three domains (Figure 6) [39,40]. The amine oxidase domain contains the active site consisting of a substrate-binding cavity and a FAD-binding domain; it shares large homology with polyamine oxidase (PAO) and monoamine oxidases (MAOs). However, LSD1 includes a 102 amino acid tower domain insert that separates the N- and C-terminus of the amine oxidase domain and adopts a typical anti-parallel coiled-coil conformation that extends
out from the core of LSD1. The tower domain has been shown to interact with the corepressor protein, CoREST \([41,42]\). Lastly, LSD1 possesses a SWIRM domain of undefined function. SWIRM domains of other histone-modification proteins have been shown to bind DNA; however, the SWIRM domain of LSD1 is not composed of the typical conserved and solvent exposed positively charged patch responsible for interaction with DNA \([43]\). Instead, the SWIRM domain has been implicated in substrate binding, although the function has yet to be elucidated.
Figure 6. LSD1 structure consists of three domains, an FAD-dependent amine oxidase (blue) with a tower insert (green) and N-terminal SWIRM domain (red). Figure was generated using the 2HKO pdb file.
Although LSD1 on its own is capable of demethylating histones and peptide substrates, CoREST is essential for LSD1-mediated demethylation of nucleosomal substrates. The crystal structure of the two proteins in complex reveal a substantial interaction between the tower domain of LSD1 and an α-helix in CoREST (Figure 7). CoREST also contains a SANT2 domain that interacts with DNA and therefore arbitrates the interaction between LSD1 and the nucleosome.
Figure 7. Crystal structure of LSD1 (grey) in complex with CoREST (purple). Figure was generated using the 2UXX pdb file.
1.3.3 LSD1 inhibition

Because of the wide-spread knowledge of the importance of LSD1 function in the progression of multiple diseases, the identification of LSD1 inhibitors has been the focus of many recent reports (Figure 8). Initially, small molecule MAO inhibitors including tranylcypromine and phenelzine were shown to potently inhibit nucleosomal demethylation of H3K4 [44]; although phenelzine has been shown to be more potent against recombinant LSD1, tranylcypromine has been the most promising small molecule inhibitor in cellular environments [44,45]. Global increases in H3K4 methylation were observed in embryonal carcinoma cells after treatment with tranylcypromine [44]. Various derivatives of tranylcypromine have been shown to induce differentiation of promyelocytic leukemia cells and slow the growth of prostate cancer cell lines [45,46]. Pargyline, a MAO B selective inhibitor, has also been reported to inhibit LSD1-catalyzed demethylation of H3K9 in the presence of the androgen receptor at 5 mM [25]. When the dimethyl lysine peptide substrate is replaced with residue conjugated as a propargylamine or an aziridine, significant inhibition of LSD1 activity in observed in vitro [47]. The aziridine peptide acted as reversible competitive inhibitor, but the propargylamine peptide exhibited time-dependent inactivation of the enzyme by modification of the FAD. Lastly, bisguanidine and biguanide polyamine analogs have been shown to be effective noncompetitive LSD1 inhibitors and treatment results in re-expression of silenced tumor suppressor genes in colon cancer [48,49]. Very recently,
these polyamine analogs have been shown to modulate gene expression in breast cancer cells [50].

**Small molecule MAO inhibitors**

- Tranylcypromine: \( K_i \approx 500 \mu M \)
- Phenelzine: \( K_i \approx 50 \mu M \)
- Pargyline: \( K_i \approx 5 \text{ mM} \)

**Peptide-based inhibitors**

- ART N<br>\( \text{QTARKSTGGKAPRKQLA} \): \( K_i \approx 20 \mu M \)
- ART N<br>\( \text{QTARKSTGGKAPRKQLA} \): \( I_{50} \approx 16 \mu M \)

**Polyamine analogs**

- Noncompetitive inhibition at 2.5 \( \mu M \)

Figure 8. Reported classes of LSD1 inhibitors.
1.4 Thesis objectives

Few studies thus far have focused on the role of LSD1 in breast cancer proliferation. Efforts towards understanding the function of LSD1 in breast cancer using small molecule probes will be presented in this thesis. In Chapter 2, we discuss a straightforward and effective method for synthesis of two potential classes of small molecule inactivators of LSD1, 2-arylcyclopropylamines and propargylamines. In Chapter 3, we show that 2-arylcyclopropylamines are potent irreversible mechanism-based inhibitors of LSD1, while propargylamines are only inhibitory at high concentrations. Chapter 4 presents data supporting the essential role of LSD1 in cellular proliferation of ERα-positive and ERα-negative breast cancer using both 2-arylcyclopropylamine inhibitors and small interfering RNA (siRNA) to knockdown LSD1 protein levels. We also determined that LSD1’s demethylation activity is required for expression of some, but not all, estrogen-dependent genes. In Chapter 5, we present initial experiments to elucidate the protein-protein interactions and transcriptional partner requirement for LSD1-mediated processes in breast cancer cells. We have found that CoREST may be an important facilitator between LSD1 and ERα and that potential peptides or peptidomimetics that disrupt the interaction between LSD1 and CoREST may allow for selective targeting of LSD1 in breast cancer.
2. Synthesis of small molecule compounds: 2-arylcylopropylamines and propargylamines

2.1 Background information

The amine oxidase domain of LSD1 is homologous to equivalent domains found in polyamine oxidase (PAO, 22.4 % sequence identity), monoamine oxidase A (MAO A, 17.6 % sequence identity), and monoamine oxidase B (MAO B, 17.6 % sequence identity). All four enzymes require a FAD cofactor to facilitate enzymatic activity. Monoamine oxidase inhibitors (MAOIs) are well-known drugs that have been used clinically for the treatment of depression, anxiety and Parkinson’s disease [51-53]. Many classes of MAOIs are effective because they inactivate the enzyme by covalent modification of the active site FAD [54-56]. Two of MAOIs of particular interest are 2-phenylcylopropylamine (2.1, 2-PCPA, tranylcypromine, brand name Parnate) and pargyline (2.2) (Figure 9).

![Figure 9. Structures of MAOIs: 2-PCPA (2.1) and pargyline (2.2).](image)

Because of the similarities between the catalytic mechanisms and architecture of the enzyme active sites of MAO A and B and LSD1, we hypothesized that flavin-
targeted irreversible inhibitors of MAOs might also inhibit the function of LSD1, perhaps leading to the beneficial pleiotropic effects of these therapeutics. Indeed, upon evaluation of this hypothesis, it was discovered that clinically relevant concentration of 2-PCPA proved effective at inhibiting LSD1-mediated demethylation of histones in vivo and in vitro [57]. Schmidt and McCafferty subsequently published the first mechanistic studies of LSD1 inactivation by 2-PCPA [58]. Later that year, a crystal structure of LSD1 after inhibition by 2-PCPA was obtained [59], and a mechanism of inhibition was proposed involving the formation of a five-membered ring adduct (Scheme 2). Alternatively, in a following crystal structure, Mimasu et. al. observed not only the five-membered ring model but also a N(5) model (Figure 10) [60]. Regardless, both of these adducts are different than the one observed upon inhibition of MAO B (Figure 10).
Scheme 2. The proposed mechanism of inhibition of LSD1 by 2-PCPA via flavin adduct formation.
Figure 10. Adducts of 2-PCPA covalently bound to FAD. 5-membered ring and N(5) are observed in LSD1 structures while C(4) adduct is observed with MAO B.
Because of the difference between the complexes observed for LSD1 vs MAO B and the difference in the size of their active sites, we set out to synthesize derivatives of 2-PCPA that may be selective for LSD1. We believed that sterically large compounds may inhibit LSD1 while no longer inhibiting MAOs. We recognized that it may not only be sterics that allow for selective inhibition, but the oxidation potential of the inhibitor since it must first donate an electron before covalently linking to the FAD (Scheme 2). Lastly, we modeled a para-methoxy derivative of 2.1 and identified a potential hydrogen bond between the para-oxygen and a neighboring Thr335 of LSD1 (Figure 11) and therefore rationally designed molecules that would test this hypothesis.
Figure 11. Predicted hydrogen bond between para-oxygen of a 2.1 derivative and Thr335 in the crystal structure of LSD1 using the pdb 2UXX.
Pargyline (2.2) and other small molecule propargylamines such as deprenyl and clorgyline were devoid of any inhibitory activity [57]. However, a peptide containing the propargylamine moiety was found to sufficient at inhibiting LSD1 function in vitro [47]. Therefore, we wanted to develop routes to not only substituted trans-2-arylcyclopropylamines but also propargylamines to pave the way for LSD1-selective inhibitor discovery.

2.2 Synthesis of 2-arylcyclopropylamine derivatives

2.2.1. Design of synthetic plan

In order to synthesize an array of substituted 2-arylcyclopropylamine derivatives, we envisioned a parallel synthesis that would incorporate a number of starting materials. The general synthetic plan began with the cyclopropanation of trans-2-arylpropenoate esters followed by hydrolysis and subsequent Curtius rearrangement [61] to yield the cyclopropylamines (Scheme 3). Initially, we synthesized these compounds as racemic mixtures of the trans-cyclopropylamines. We envisioned that if a good inhibitor of LSD1 was identified, we could generate it enantioselectively or resolve the enantiomers by chiral chromatography to determine the effects of the individual stereoisomers. The absolute stereochemistry of the individual enantiomers could be determined by x-ray crystallography.
Some \textit{trans}-2-arylpropenoic acids are commercially available and therefore can easily be subjected to standard esterification conditions to generate the corresponding esters. To expand beyond the commercially available carboxylic acids, we planned to utilize a Horner-Emmons-Wadsworth olefination of substituted benzaldehydes to generate additional \textit{trans}-2-arylpropenoic esters \cite{62,63}. To make additional non-commercially available substituted benzaldehydes for olefination, we utilized a cross-coupling between \textit{para}-halobenzaldehydes and a variety of phenols and thiophenols (Scheme 4).

\textbf{2.2.2 Results}

We utilized a copper-catalyzed Ullmann coupling to generate biaryl ethers and thioethers from the corresponding phenols and thiophenols and halobenzaldehydes.
These reactions proceeded with moderate yields (Table 2), with yields decreased in the two cases of phenols containing electron-withdrawing substituents (entries 8 and 10). In particular, the para-nitrophenol (entry 8) did not generate any desired product. Typically, Ullmann condensations are more successful with aryl iodides than bromides [66]. However, in our hands, the iodide (entries 1-3) did not improve yields significantly (entries 4-6). Therefore, the para-bromobenzaldehyde was used for the remaining reactions because of its lower cost.
Table 2. Ullmann coupling between halobenzaldehydes and phenol/thiophenols.

![Reaction Scheme]

where \( Y = \text{O or S} \)

where \( X = \text{I or Br} \)

<table>
<thead>
<tr>
<th>Entry</th>
<th>(thio)phenol</th>
<th>X</th>
<th>Product</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="OH phenol" /></td>
<td>I</td>
<td>2.3</td>
<td>54</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="SH phenol" /></td>
<td>I</td>
<td>2.4</td>
<td>62</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="SH phenol" /></td>
<td>I</td>
<td>2.5</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="OH phenol" /></td>
<td>Br</td>
<td>2.3</td>
<td>52</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="SH phenol" /></td>
<td>Br</td>
<td>2.4</td>
<td>54</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="SH phenol" /></td>
<td>Br</td>
<td>2.5</td>
<td>46</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="H3CO phenol" /></td>
<td>Br</td>
<td>2.6</td>
<td>44</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="O2N phenol" /></td>
<td>Br</td>
<td>2.7</td>
<td>N.R.</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9" alt="OH phenol" /></td>
<td>Br</td>
<td>2.8</td>
<td>38</td>
</tr>
<tr>
<td>10</td>
<td><img src="image10" alt="NC phenol" /></td>
<td>Br</td>
<td>2.9</td>
<td>18</td>
</tr>
</tbody>
</table>
Two additional benzaldehyde derivatives (2.10 and 2.11) were produced from \textit{para}-hydroxybenzaldehyde. The former (2.10) was made in excellent yield after reaction with propargyl bromide in the presence of potassium carbonate (Scheme 5), while the latter (2.11) was made in good yield after reaction with methyl chloromethyl ether in the presence of diisopropylethylamine (Scheme 6).

\begin{center}
\begin{align*}
&\begin{array}{c}
\text{HO} \\
\text{H} \\
\text{O} \\
\text{Br} \\
\text{K}_2\text{CO}_3 \\
\text{DMF} \\
\text{rt, 5 h, 92%}
\end{array} \\
&\begin{array}{c}
\text{HO} \\
\text{O} \\
\text{2.10}
\end{array}
\end{align*}
\end{center}

\textbf{Scheme 5. Synthesis of propargyl phenol derivative to yield 2.10.}

\begin{center}
\begin{align*}
&\begin{array}{c}
\text{HO} \\
\text{H} \\
\text{O} \\
\text{MOMCl} \\
\text{DIPEA} \\
\text{CH}_2\text{Cl}_2 \\
\text{rt, 1.5 h, 83%}
\end{array} \\
&\begin{array}{c}
\text{O} \\
\text{2.11}
\end{array}
\end{align*}
\end{center}

\textbf{Scheme 6. Protection of phenol as methoxymethyl ether (MOM) to yield 2.11.}

Three methods were used to generate \textit{trans}-3-arylpropenoate esters (2.12-2.32). The first used acidic methanol to esterify commercially available \textit{trans}-2-arylpropenoic acids in good to excellent yields (Table 3). Alternatively, commercially available \textit{trans}-2-arylpropenoic acids were esterified using TMS-diazomethane with near quantitative yields in all cases (Table 4). A Horner-Emmons-Wadsworth olefination [62,63] was
chosen to generate 2.20-2.32 from the corresponding benzaldehydes (commercially available or 2.3-2.11). The use of the diethyl methyl phosphonate carbanion allowed for reaction with all of the aldehydes under mild conditions and resulted in good to excellent yields with complete (E)-selectivity (Table 5). The trans-stereochemistry was confirmed by the vicinal ¹H-¹H coupling constants (J_{ab} = 14-16 Hz versus J_{ab} = 9-11 for cis-alkenes).

Table 3. Esterification of commercially available trans-3-arylpropenoic acids using acidic methanol.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Product</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="image" /></td>
<td>2.12</td>
<td>73</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="image" /></td>
<td>2.13</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="image" /></td>
<td>2.14</td>
<td>77</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="image" /></td>
<td>2.15</td>
<td>89</td>
</tr>
</tbody>
</table>
Table 4. Esterification of commercially available trans-3-arylpropenoic acids with TMSCHN₂.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Rxn time (h)</th>
<th>Product</th>
<th>% yield</th>
</tr>
</thead>
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</tr>
<tr>
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<td><img src="image" alt="Structure 7" /></td>
<td>0.5</td>
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<td>2.19</td>
</tr>
</tbody>
</table>
Table 5. Horner-Emmons-Wadsworth reaction to produce $E$-alkenes.

\[
\begin{align*}
\text{EtO-P(O)(OEt)_2-OMe} & \xrightarrow{\text{a. NaH, THF}} \text{R=EtO, H} \quad 0 \degree C \text{ to rt, 45 min} \\
& \xrightarrow{\text{b. toluene, -78 \degree C to rt, 7 h}} \text{R=EtO, H} \\
\end{align*}
\]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Aldehyde</th>
<th>Product</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
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<td>95</td>
</tr>
<tr>
<td>2</td>
<td>(\text{i-PrO-\text{Ph}})</td>
<td>2.21</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>(\text{Me_2N-\text{Ph}})</td>
<td>2.22</td>
<td>86</td>
</tr>
<tr>
<td>4</td>
<td>(\text{O-O-\text{Ph}})</td>
<td>2.23</td>
<td>87</td>
</tr>
<tr>
<td>5</td>
<td>(\text{O-O-\text{Ph}})</td>
<td>2.24</td>
<td>98</td>
</tr>
<tr>
<td>6</td>
<td>(\text{BnO-\text{Ph}})</td>
<td>2.25</td>
<td>83</td>
</tr>
<tr>
<td>7</td>
<td>(\text{PhO-\text{Ph}})</td>
<td>2.26</td>
<td>67</td>
</tr>
<tr>
<td>8</td>
<td>(\text{O-O-\text{Ph}})</td>
<td>2.27</td>
<td>89</td>
</tr>
<tr>
<td>9</td>
<td>(\text{S-O-\text{Ph}})</td>
<td>2.28</td>
<td>76</td>
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<tr>
<td>Entry</td>
<td>Aldehyde</td>
<td>Product</td>
<td>% Yield</td>
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<td>---------</td>
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</tr>
<tr>
<td>13</td>
<td><img src="image4.png" alt="Image" /></td>
<td>2.6</td>
<td>82</td>
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</tbody>
</table>

Reaction of the α,β-unsaturated esters (2.16-2.32) with diazomethane and palladium (II) acetate afforded the corresponding cyclopropanated products (2.33-2.49) in excellent yield and complete stereoselectivity (Table 6) [67]. The trans-stereochemistry of the 2-arylcyclopropylcarboxylate esters was confirmed by the vicinal 1H-1H coupling constants of the cyclopropyl moiety ($J_{ab} = 4.2-4.5$ Hz) [68].
Table 6. Cyclopropanation using diazomethane.

![Reaction scheme](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Product</th>
<th>% Yield</th>
</tr>
</thead>
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<tr>
<td>Entry</td>
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<td>Product</td>
<td>% Yield</td>
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<td>-------------------</td>
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<tr>
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<td><img src="#" alt="Image" /> 2.30</td>
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<tr>
<td>17</td>
<td><img src="#" alt="Image" /> 2.31</td>
<td>2.49</td>
<td>95</td>
</tr>
</tbody>
</table>
Only 2.45 was not produced using this method; presumably this is because the alkyne disrupts desired palladium-mediated reaction by interacting with the metal. Therefore, in order to generate the 2.45, we removed the MOM protecting group from 2.39 using traditional methods of trifluoroacetic acid in dichloromethane to generate 2.50 followed by installation of the propargyl through an S$_2$2 reaction between the phenoxide of 2.50 and propargyl bromide (Scheme 7) [69]. Both of these reactions occurred in good yields.

Scheme 7. Removal of MOM protecting group and installation of propargyl group.

The next step of the synthesis was to hydrolyze the methyl esters (2.33-2.49) to give the carboxylic acids (2.51-2.67). This was accomplished under basic conditions in good to excellent yields (Table 7) with the exception of entry 15. The desired product was not isolated from this reaction presumably because of loss of the ammonium salt under aqueous workup.
Table 7. Alkaline hydrolysis of esters.

\[
\begin{align*}
\text{Ar} & \quad \text{OMe} & \quad 2.33-2.49 & \quad \text{i. } 2 \text{ M NaOH, MeOH} & \quad \text{ii. } 12 \text{ M HCl} & \quad \text{Ar} & \quad \text{OH} & \quad 2.51-2.67
\end{align*}
\]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Product</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<td>73</td>
</tr>
<tr>
<td>2</td>
<td>EtO</td>
<td>2.52</td>
<td>78</td>
</tr>
<tr>
<td>3</td>
<td>i-PrO</td>
<td>2.53</td>
<td>78</td>
</tr>
<tr>
<td>4</td>
<td>F$_3$C</td>
<td>2.54</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>Br</td>
<td>2.55</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>PhO</td>
<td>2.56</td>
<td>58</td>
</tr>
<tr>
<td>7</td>
<td>BnO</td>
<td>2.57</td>
<td>76</td>
</tr>
<tr>
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<td>MOMO</td>
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<td>67</td>
</tr>
<tr>
<td>Entry</td>
<td>Starting material</td>
<td>Product</td>
<td>% Yield</td>
</tr>
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<td>---------</td>
</tr>
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<tr>
<td>14</td>
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<td>2.64</td>
<td>74</td>
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<td>0(^a)</td>
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<tr>
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<td>72</td>
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<tr>
<td>17</td>
<td><img src="2.49" alt="image" /></td>
<td>2.67</td>
<td>41</td>
</tr>
</tbody>
</table>

\(\text{a}^a\) Desired product not isolated; presumably lost during workup procedure.
The subsequent step was the Curtius rearrangement [61]. For the majority of the compounds, we used diphenylphosphorylazide and triethylamine to generate the intermediate isocyanate that was trapped with tert-butyl alcohol allowing the carbamates (2.68-2.78) to be isolated and purified [67]. These reactions proceeded in low to good yields (Table 8). Potentially acid-sensitive substrates were trapped as the 2-(trimethylsilyl)ether carbamates (6) following Curtius rearrangement (Table 9) [70].

Table 8. Curtius rearrangement using diphenylphosphorylazide to yield tert-butyl carbamate protected amines.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting Material</th>
<th>Time (h)</th>
<th>Product</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
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<td>2.68</td>
<td>67</td>
</tr>
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<td>45</td>
<td>2.69</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
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<td>57</td>
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<tr>
<td>Entry</td>
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<td>Product</td>
<td>% Yield</td>
</tr>
<tr>
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<td>------------------</td>
<td>----------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>5</td>
<td><img src="image1" alt="Image" /></td>
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<td>2.72</td>
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</tr>
<tr>
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<td><img src="image2" alt="Image" /></td>
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<td>2.74</td>
<td>27</td>
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<td><img src="image7" alt="Image" /></td>
<td>45</td>
<td>2.78</td>
<td>43</td>
</tr>
</tbody>
</table>
Table 9. Curtius rearrangement using sodium azide and TMS-ethanol to yield 2-(trimethylsilyl)ethyl carbamate protected amines.

\[
\text{Ar} \begin{array}{c}
\text{2.59-2.60} \\
\text{OH}
\end{array} \xrightarrow[1. \text{TEA, NaN}_3, \text{ethylchloroformate}]{\text{2.90 °C, 2 h}} \text{Ar} \begin{array}{c}
\text{HN}
\end{array} \begin{array}{c}
\text{O}
\end{array} \begin{array}{c}
\text{O}
\end{array} \begin{array}{c}
\text{Si}
\end{array} \begin{array}{c}
\text{2.79-2.80}
\end{array} \\
\text{2.59-2.77} \xrightarrow[2. \text{TMSethanol}]{\text{60 °C, 18 h}} \text{2.60-2.80}
\]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Product</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>2</td>
<td>2.60</td>
<td>2.80</td>
<td>26</td>
</tr>
</tbody>
</table>

The t-butylicarbamates (2.68-2.77) were hydrolyzed to yield the cyclopropylamine hydrochloride salts (2.81-2.91) using hydrochloric acid in tetrahydrofuran (Table 10). Reaction times were determined using thin layer chromatography and upon complete consumption of starting material, the reaction was stopped. This reaction proceeded with good to excellent yield. The methoxymethyl ether starting material (entry 8) did not generate the corresponding amine because MOM protecting group is acid labile resulting in release of the free phenol. There was not enough material for 2.80 to be taken on to the next step, but 2.79 was hydrolyzed using tetra-N-butylammonium fluoride in tetrahydrofun to generate the hydrochloride salt 2.92 in low yield (Scheme 8).
Table 10. Hydrolysis of \( t \)-butylcarbamates to yield cyclopropylamine hydrochloride salts.

\[
\begin{array}{c}
\text{R} \quad \text{NHBoc} \quad \xrightarrow{6 \text{ M HCl}} \quad \text{R} \quad \text{NH}_2\text{HCl}
\end{array}
\]

\( 2.81-2.91 \)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material ( \text{H}_3\text{CO} )</th>
<th>Time (h)</th>
<th>Product ( \text{NH}_2\text{HCl} )</th>
<th>% Yield</th>
</tr>
</thead>
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<td>8</td>
<td>( 2.81 )</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>( \text{EtO} ) ( 2.69 )</td>
<td>24</td>
<td>( 2.82 )</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>( \text{PrO} ) ( 2.70 )</td>
<td>13</td>
<td>( 2.83 )</td>
<td>84</td>
</tr>
<tr>
<td>4</td>
<td>( \text{F}_3\text{C} ) ( 2.71 )</td>
<td>5</td>
<td>( 2.84 )</td>
<td>99</td>
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<tr>
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<td>( \text{Br} ) ( 2.72 )</td>
<td>5</td>
<td>( 2.85 )</td>
<td>99</td>
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<tr>
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<td>( 2.86 )</td>
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<td>( 2.87 )</td>
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<td>( 2.88 )</td>
<td>0 ( a )</td>
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<td>Entry</td>
<td>Starting material</td>
<td>Time (h)</td>
<td>Product</td>
<td>% Yield</td>
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<td>---------</td>
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<td><img src="2.78" alt="Image" /></td>
<td>6</td>
<td>2.91</td>
<td>70</td>
</tr>
</tbody>
</table>

*Desired product not isolated; reaction resulted in removal of the MOM protecting group to yield unprotected phenol.

Scheme 8. Removal of 2-(trimethylsilyl)ethyl protecting group to yield free amine 2.92.

Overall, twelve 2-arylcyclopropylamines were synthesized. The Curtius rearrangement was the lowest yielding reaction, and as it is the second to last step, the overall yields are decreased. More derivatives could be synthesized, but none of these compounds are selective inhibitors of LSD1 (see Chapter 3) and, therefore, we suspended continued synthetic efforts.
2.3 Synthesis of propargylamine derivatives

Propargylamines are an alternate class of compounds that have been shown to inactivate FAD-containing proteins [54,56]. Therefore, we set out to synthesize propargylamine derivatives to investigate their inhibitory potential against LSD1. These propargylamine are less synthetically challenging than the cyclopropylamines, only requiring a reductive amination from the corresponding benzaldehyde and propargyl amine. The reaction proceeded in low to moderate yields for the three substrates tested (Table 11).

Table 11. Reductive amination to generate propargylamines.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Product</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>H</td>
<td>2.93</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
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<td>2.94</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>OPh</td>
<td>2.95</td>
<td>34</td>
</tr>
</tbody>
</table>

Ideally, an entire library of propargylamine inhibitors could be synthesized and their inactivation parameters tested in vitro. We initially hypothesized that this library would allow us to rationally direct a plan for other 2-arylcyclopropylamines because their ease of synthesis would allow for larger library generation in shorter times. If we
were able to find a propargylamine with increased potency against LSD1, the substitution pattern would be repeated on the more effective cyclopropylamine warhead. However, the propargylamines did not inhibit LSD1 \textit{in vitro} at concentrations lower than 5 mM (see Chapter 3); therefore, efforts were discontinued.

\section*{2.4 Methylation of 2-PCPA}

We hypothesized that an $N$-methyl-2-phenylcyclopropylamine may also be effective at inhibiting LSD1. Regardless, it would reveal information about the importance of oxidation potential in inhibiting LSD1. Primary and secondary aliphatic amines have different rates of oxidation and the secondary radical cation produced upon oxidation is significantly more stable than the primary radical cation\cite{71}. We also predicted that a methylated amine would more resemble the methylated histone substrate of LSD1 and therefore may increase binding.

The conversion of primary amines to secondary amines is not a trivial process. Typically, alkylation of the primary amines with alkyl halides, especially using methyl iodide, leads to the formation of the undesired tertiary amines and/or quaternary ammonium salts. To avoid these multi-alkylated products, reductive $N$-alkylation of amides is typically employed \cite{72}. We desired a direct route from the commercially available 2-PCPA (2.1). In order to avoid the use of formaldehyde in the reductive amination reaction, a method developed by Fukuyama was employed. The nitrogen of
the amine is protected and activated, followed by methylation and subsequent deprotection [73,74]. The primary amine of 2-PCPA was transformed into the corresponding o-nitrobenzensulfoamide (nosylamide) 2.96 in good yield. Then it was methylated using MeI (Scheme 9). Crude 2.97 was deprotected initially using thiophenol. However, there was difficulty in separating the desired product 2.98 from the disulfide byproduct using flash column chromatography with silica gel. As a result, we used a polystyrene linked thiophenol resin [75]; this permitted isolation of desired product isolated from the unwanted disulfide that remained on the resin. The overall yield of the second two steps was 50% (Table 12).

![Scheme 9. Synthetic scheme for methylated 2-PCPA.](image)

Table 12. Methods for the removal of nosyl protecting group.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Thiol</th>
<th>Base</th>
<th>Solvent</th>
<th>Time (h)</th>
<th>Temp</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PhSH</td>
<td>K2CO3</td>
<td>ACN</td>
<td>24.5</td>
<td>50 °C</td>
<td>impure</td>
</tr>
<tr>
<td>2</td>
<td>PS-thiophenol</td>
<td>Cs2CO3</td>
<td>THF</td>
<td>67</td>
<td>rt</td>
<td>50% yield</td>
</tr>
</tbody>
</table>
2.5 Mechanistic evaluation of Corey-Chaykovsky cyclopropanation

Currently, the generation of diazomethane, an explosive reagent, is utilized for the cyclopropanation of the 2-arylcyclopropylamines as described above; this requires the use of specialized glassware and a maximum scale of 2 mmol. Ideally, the reactions to produce the LSD1 inhibitors should be safe, general, and applicable on a large scale. In order to avoid the use of diazomethane, a mechanistic study was performed utilizing sulfoxonium ylides as developed by Corey and Chaykovsky [76,77]. These ylides are capable of adding at the β-position of Michael acceptors and upon collapse of the intermediate, the cyclopropane is formed by intramolecular ring closure, expelling DMSO as a leaving group. Typical drawbacks of this reaction include poor yields and competition between 1,2- or 1,4-addition. We hypothesized that a Lewis acid would coordinate the carbonyl and as a result activate the substrate for Michael addition. Ultimately, it was thought that chiral Lewis acids could be used to induce enantioselectivity in the reaction. A wide array of reactions was performed with various Lewis acids and \textit{trans}-methylcinnamic ester as the substrate (Table 13).
Table 13. Results of mechanistic studies of Corey-Chaykovsky cyclopropanation.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Base</th>
<th>Lewis Acid</th>
<th>LA (%) mol</th>
<th>Solvent</th>
<th>Temp (°C)</th>
<th>Time (h)</th>
<th>% conv.</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KOt-Bu</td>
<td>none</td>
<td>N/A</td>
<td>DMSO</td>
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<td>1</td>
<td>100</td>
<td>14</td>
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<tr>
<td>2</td>
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<td>DMSO</td>
<td>23</td>
<td>1</td>
<td>95</td>
<td>32</td>
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<tr>
<td>3</td>
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<td>N/A</td>
<td>DMSO</td>
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<td>100</td>
<td>18</td>
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<td>100</td>
<td>64</td>
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<tr>
<td>5</td>
<td>KOt-Bu</td>
<td>none</td>
<td>N/A</td>
<td>THF</td>
<td>23</td>
<td>18</td>
<td>0</td>
<td>0</td>
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<tr>
<td>6</td>
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<td>N/A</td>
<td>Et₂O</td>
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<td>50</td>
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<tr>
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<td>0</td>
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<tr>
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</tr>
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<td>TiCl₄</td>
<td>100</td>
<td>DMSO</td>
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<td>48</td>
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<td>0</td>
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<td>TiCl₄</td>
<td>10</td>
<td>DMSO</td>
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<td>51</td>
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<tr>
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<tr>
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<td>10</td>
<td>DMSO</td>
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<td>0.25</td>
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<td>KOt-Bu</td>
<td>BF₃(OEt)₂</td>
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<td>DMSO</td>
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<td>1</td>
<td>100</td>
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<tr>
<td>16</td>
<td>NaH</td>
<td>BF₃(OEt)₂</td>
<td>10</td>
<td>DMSO</td>
<td>23</td>
<td>1</td>
<td>88</td>
<td>51</td>
</tr>
<tr>
<td>17</td>
<td>KOt-Bu</td>
<td>ZnI₂</td>
<td>10</td>
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<td>1</td>
<td>94</td>
<td>26</td>
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<td>18</td>
<td>NaH</td>
<td>ZnI₂</td>
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<td>19</td>
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<td>ZnCl₂</td>
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<td>DMSO</td>
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<td>1</td>
<td>95</td>
<td>19</td>
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<tr>
<td>20</td>
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<td>DMSO</td>
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<td>1</td>
<td>95</td>
<td>36</td>
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<tr>
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<td>KOt-Bu</td>
<td>MgCl₂</td>
<td>10</td>
<td>DMSO</td>
<td>23</td>
<td>1</td>
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<td>52</td>
</tr>
<tr>
<td>23</td>
<td>KOt-Bu</td>
<td>Ti(Oi-Pr)₄</td>
<td>10</td>
<td>DMSO</td>
<td>R.T.</td>
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<td>100</td>
<td>35</td>
</tr>
<tr>
<td>24</td>
<td>NaH</td>
<td>Ti(Oi-Pr)₄</td>
<td>10</td>
<td>DMSO</td>
<td>R.T.</td>
<td>1</td>
<td>100</td>
<td>46</td>
</tr>
</tbody>
</table>

As seen above, the Lewis acids did not help to improve the cyclopropanation. In fact, in the case of methyl cinnamic ester, the Lewis acids hindered the ability of the
ylide to add to the substrate. Using one equivalent of titanium tetrachloride inhibited the desired reaction from proceeding (entries 9-10, Table 13). This may be due to an interaction between the Lewis acid and the base or ylide, and thus, impeding their ability to participate in the reaction scheme. Another problem with this reaction is the poor to moderate yield of the desired cyclopropane without recovery of starting material. This is most likely due to the reaction medium of DMSO and the requirement of vigorous washing with water in order to isolate the product. Potentially, both the starting material and product are partially soluble in water and are partitioning between both the aqueous and organic layers during extraction. In order to improve yields, the solvent for performing the reaction was changed (entries 5-8), and the workup was adjusted. However, these changes did not increase yields. Because the nonchiral Lewis acids did not help and may have not been compatible with the reaction, we did not attempt to use any chiral catalysts. We ended the methodology project here because if we could not use this reaction to induce enantioselectivity, we decided the higher yielding diazomethane reaction was sufficient for our synthesis of the 2-arylcyclopropylamines.

2.6 Conclusions and future directions

In this chapter, we have described the synthesis of two classes of small molecule inhibitors of FAD-dependent amine oxidases, the 2-arylcyclopropylamines and the
propargylamines. The synthesis of the propargylamines was a one-step reductive amination that proceeded in good yields. For the 2-arylcyclopropylamines, the cyclopropyl group was installed using diazomethane in excellent yields for all derivatives and the Curtius rearrangement, although low yielding, installed the amine. These general routes can be applied to a multitude of starting materials in order to fully examine structure-activity relationships for any FAD-containing amine oxidase inhibition.

In the next chapter, we will investigate the inhibitory potential of these compounds against LSD1. If a selective inhibitor is identified from the racemic mixtures we have made, it will be prudent to evaluate the impact of the stereochemistry on the molecule. In order to obtain enantiopure 2-arylcyclopropylamines, we will employ the use of a chiral handle such as enoyl sultams. By incorporating either L-\((+)-camphorsultam\) or D-\((-)-camphorsultam\), the cyclopropanation step using diazomethane would yield the corresponding cyclopropanoyl derivatives in high diasteromeric purity (Scheme 10) [78,79].

Scheme 10. Proposed preparation of enantiopure cyclopanes by diastereoselective cyclopropanation of \((+)-enoyl sultams\). (i) L-\((+)-camphorsultam; (ii) CH$_2$N$_2$, Pd(OAc)$_2$; (iii) Ti(i-PrO)$_4$, BzOH followed by 2 M NaOH, MeOH.
2.7 Experimental section

**General Considerations:** Unless stated to the contrary, where applicable, the following conditions apply. Air sensitive reactions were carried out using dried solvents (see below) and under a slight static pressure of Ar (pre-purified quality) that had been passed through a column of Drierite. Glassware was dried in an oven at 120 °C for at least 12 h prior to use and then assembled quickly while hot, sealed with rubber septa, and allowed to cool under a stream of Ar. Reactions were stirred magnetically using Teflon-coated magnetic stirring bars. Teflon-coated magnetic stirring bars and syringe needles were dried in an oven at 120 °C for at least 12 h prior to use. Commercially available Norm-Ject disposable syringes were used. All $^1$H and $^{13}$C NMR spectra were recorded on 300 MHz or 400 MHz Varian Mercury spectrometers as noted. $^1$H spectra were referenced to CHCl$_3$ at 7.26 ppm and $^{13}$C spectra were referenced to CDCl$_3$ at 77.23 ppm. All spectra were taken in CDCl$_3$ unless otherwise noted. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F$_{254}$ aluminum backed plates and visualized using 254 nm UV light. Flash chromatographic purifications were performed using silica gel (40-60 μm) purchased from Agela Technologies (Newark, DE). Compounds and solvents were obtained from Fisher, Sigma-Aldrich, and VWR and used without further purification unless noted below.
The following example is representative for the formation of all diaryl ethers and diaryl ethers from their respective phenols or thiophenols and para-bromobenzaldehyde.

\[
\begin{align*}
\text{Ph-SH} + \text{Ph-Br} \xrightarrow{\text{CuBr} \quad \text{Cs}_2\text{CO}_3 \quad \text{THMD} \quad \text{NMP}} \text{Ph-S-Ph}
\end{align*}
\]

4-\textit{(4-tert-butylphenylthio)benzaldehyde} (2.4): Under argon, an oven dried round bottom flask was charged with the 4-\textit{tert}-butylthiophenol (0.69 mL, 4 mmol, 2 eq) and anhydrous N-methyl-2-pyrrolidone (5 mL). Cesium carbonate (1.3 g, 4 mmol, 2 eq) was added to the stirring solution and it immediately turned cloudy. Para-bromobenzaldehyde (370 mg, 2 mmol, 1 eq) was added, followed by copper (I) bromide (143 mg, 1 mmol, 0.5 eq) and 2,2,6,6-tetramethyl-3,5-heptanediione (41 μL, 0.2 mmol, 0.1 eq). The flask was equipped with a reflux condenser and heated to 70-80 °C for 15.5 h while stirring. After cooling to rt, the reaction mixture was diluted with methyl \textit{tert}-butyl ether (100 mL) and vacuum filtered. The residue was washed with MTBE (100 mL) and the combined filtrates were washed with 2 N HCl (100 mL), 0.6 N HCl (100 mL), 2 M NaOH (100 mL), and saturated NaCl (100 mL). The organic layer was dried over MgSO₄, filtered, and concentrated \textit{in vacuo}. The desired 4-\textit{(4-tert-butylphenylthio)benzaldehyde} was isolated by flash chromatography over silica gel with 10:1 hexanes:ethyl acetate to afford a gold oil in 62% yield (0.065 g). \textit{1H NMR} (400 MHz, CDCl₃): δ 9.88 (1H, s), 7.69 (2H, d, J=8.4 Hz), 7.44 (4H, m), 7.20 (2H, d, J=8.4 Hz),
1.34 (9H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$191.2, 152.7, 148.0, 134.4, 133.5, 130.1, 127.3, 126.9, 126.7, 34.8, 31.2.

![4-(m-tolyloxy)benzaldehyde](image)

4-(m-tolyloxy)benzaldehyde (2.3): 0.049 g, 54%, white solid. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$9.92 (1H, s), 7.83 (2H, m), 7.27 (1H, m), 7.03 (3H, m), 6.90 (2H, m), 2.36 (3H, s). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$191.0, 132.2, 130.1, 126.0, 121.3, 117.8, 117.6, 21.6.

![4-(naphthalen-2-ylthio)benzaldehyde](image)

4-(naphthalen-2-ylthio)benzaldehyde (2.5): 0.071 g, 60%, off-white solid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$9.90 (1H, s), 8.07 (1H, s), 7.82 (3H, m), 7.70 (2H, m), 7.54 (3H, m), 7.27 (2H, m). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$191.2, 147.1, 134.0, 133.8, 133.7, 133.1, 130.7, 130.2, 129.6, 127.9, 127.8, 127.3, 127.2, 126.9.

![4-(3-methoxyphenoxy)benzaldehyde](image)

4-(3-methoxyphenoxy)benzaldehyde (2.6): 0.204 g, 44%, yellow oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$9.89 (1H, s), 7.83 (2H, m), 7.28 (1H, m), 7.07 (2H, m), 6.77 (1H, m), 6.66 (2H, m),
3.77 (3H, s). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$190.6, 162.8, 161.0, 156.0, 131.8, 131.2, 130.4, 117.5, 112.2, 110.4, 106.2, 55.2.

$\text{O} \quad \text{O} \quad \text{H}$

$4$‐(3,5‐dimethylphenoxy)benzaldehyde (2.8): 0.176 g, 38%, gold oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$9.91 (1H, s), 7.82 (2H, m), 7.03 (2H, m), 6.85 (1H, s), 6.70 (2H, s), 2.31 (6H, s). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$191.0, 163.7, 155.2, 140.3, 132.2, 131.3, 126.9, 118.3, 117.7, 21.5.

$\text{O} \quad \text{O} \quad \text{N} \quad \text{C}$

$4$‐(4‐formylphenoxy)benzonitrile (2.9): 0.082 g, 18%, gold solid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$9.98 (1H, s), 7.92 (2H, m), 7.68 (2H, m), 7.15 (4H, m). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$190.9, 159.8, 134.7, 133.0, 132.4, 128.8, 119.9, 119.7, 118.6, 107.9.

$\text{O} \quad \text{H}$

$4$‐(prop‐2‐ynyloxy)benzaldehyde (2.10): An oven‐dried round bottom flask was charged with anhydrous dimethylformamide (35 mL), 4‐hydroxybenzaldehyde (2.08 g, 16.4
mmol, 1 eq) and anhydrous potassium carbonate (6.80 g, 49.2 mmol, 3 eq) and was stirred at 55 °C for 30 min. The reaction mixture was cooled to rt and propargyl bromide (1.75 mL, 19.7 mmol, 1.2 eq) was added. The reaction was stirred for an additional 5 h at rt. The crude reaction mixture was poured on ice water (100 mL) and stirred for 10 min. The desired 4-(prop-2-ynyloxybenzaldehyde) was isolated by vacuum filtration and dried in vacuo over CaSO₄ to afford a brown solid in 92% yield (2.520 g). ¹H NMR (300 MHz, CDCl₃): δ 9.90 (1H, s), 7.86 (2H, m), 7.10 (2H, m), 4.79 (2H, d, J=2.7 Hz), 2.57 (1H, J=2.4 Hz). ¹³C NMR (75 MHz, CDCl₃): δ 191.0, 132.1, 115.4, 76.6, 56.2.

4-(methoxymethoxy)benzaldehyde (2.11): Diisopropylethylamine (7.1 mL, 41 mmol, 2.5 eq) was added slowly dropwise to a stirring solution of 4-hydroxybenzaldehyde (2.0 g, 16.4 mmol, 1 eq) in dichloromethane (50 mL). Chloromethyl methyl ether (1.9 mL, 24.6 mmol, 1.5 eq) was added slowly producing a gas. The reaction was stirred at rt for 1.25 h. The reaction was quenched by adding water (50 mL). The organic products were extracted with ethyl acetate (3 X 25 mL), dried with anhydrous Na₂SO₄ and concentrated to a golden oil under reduced pressure. The desired 4-(methoxymethoxy)benzaldehyde was isolated by flash chromatography over silica gel with 4:1 hexanes:ethyl acetate to afford a clear colorless oil in 83% yield (2.227 g). ¹H NMR (400 MHz, CDCl₃): δ 9.87 (1H,
The following example is representative for the formation of all methyl esters from their respective cinnamic acids with acidic methanol.

(E)-methyl 3-(4-methoxyphenyl)acrylate (2.13): In a round bottom flask, 4-methoxycinnamic acid (3.14 g, 17 mmol, 1 eq) was suspended in methanol (20 mL). Sulfuric acid (650 μL) was added dropwise. The reaction was brought to 70 °C and allowed to reflux for 3.5 hours, until starting material was consumed as observed by TLC. The crude reaction mixture was poured on ice water (30 mL). The organic products were extracted with ether (1 X 60 mL, 2 X 30 mL), washed with brine (30 mL) and dried using anhydrous MgSO₄. The crude product was concentrated in vacuo. The desired (E)-methyl 3-(4-methoxyphenyl)acrylate was isolated by flash chromatography using 5:1 hexanes:ethyl acetate to afford a white solid in 96% yield (3.241 g). ¹H NMR (300 MHz, CDCl₃): δ 7.61 (1H, d, J=15.9 Hz), 7.41 (2H, d, J=8.7 Hz), 6.84 (2H, d, J=8.7 Hz), 6.27 (1H, d, J=15.9 Hz), 3.83 (3H, s), 3.79 (3H, s). ¹³C NMR (75 MHz, CDCl₃): δ 167.8, 161.6, 144.6, 129.9, 127.2, 115.3, 114.4, 55.4, 51.6.
methyl cinnamate (2.12): 7.947 g, 73%, white solid. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.70 (1H, d, J=16 Hz), 7.51 (2H, m), 7.37 (2H, m), 6.46 (1H, d, J=1.8 Hz), 6.42 (1H, d, J=1.8 Hz), 3.81 (3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 145.1, 134.6, 130.5, 129.1, 128.3, 118.1, 51.9.

(E)-methyl 3-(2-bromophenyl)acrylate (2.14): 0.094 g, 89%, light gold oil. $^1$H NMR (300 MHz, CDCl$_3$): δ 8.05 (1H, d, J=15.9 Hz), 7.58 (2H, m), 7.29 (2H, m), 6.39 (1H, d, J=15.9 Hz), 3.82 (3H, s).

(E)-methyl 3-(3-bromophenyl)acrylate (2.15): 1.622 g, 77%, white solid. $^1$H NMR (300 MHz, CDCl$_3$): δ 7.50 (1H, d, J=16.2 Hz), 7.44 (1H, s), 7.36 (1H, d, J=8.1 Hz), 7.28 (1H, d, J=8.1 Hz), 7.10 (1H, t, J=7.8 Hz), 6.30 (1H, J=16.2 Hz), 3.70 (3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 167.0, 143.2, 136.6, 133.2, 130.9, 130.5, 126.8, 123.2, 119.4.
The following example is representative for the formation of all methyl esters from their respective cinnamic acids with TMSCHN₂.

\[
\begin{align*}
\text{F}_3\text{C} & \quad \text{OH} & \quad \text{F}_3\text{C} & \quad \text{O} & \quad \text{Me} \\
\text{O} & \quad \text{OH} & \quad \text{OMe} & & &
\end{align*}
\]

(\(E\))-methyl 3-(4-trifluoromethyl)phenylacrylate (2.16): A solution of TMSCHN₂ (2.0 M in hexanes, 1.6 eq) was added dropwise with stirring to a 0 °C solution of the (\(E\))-3-(4-(trifluoromethyl)phenyl)acrylic acid (0.25 M) in benzene:methanol (2:1). The reaction was allowed to warm to rt over the course of 0.5 h. Concentration of the reaction mixture afforded the desired (\(E\))-methyl 3-(4-trifluoromethyl)phenylacrylate as a white solid in 99% yield (0.898 g). \(^1\)H NMR (300 MHz, CDCl₃): \(\delta\) 7.69 (1H, d, \(J=15.9\) Hz), 7.62 (4H, m), 6.50 (1H, d, \(J=15.9\) Hz), 3.87 (3H, s). \(^{13}\)C NMR (75 MHz, CDCl₃): \(\delta\) 167.0, 143.2, 138.0, 128.4, 126.1, 126.0, 120.6, 52.1.

\[
\begin{align*}
\text{Br} & \quad \text{O} & \quad \text{OMe} \\
\text{O} & \quad \text{H} & \quad \text{OMe} \\
\end{align*}
\]

(\(E\))-methyl 3-(4-bromophenyl)acrylate (2.17): 1.47 g, 99%, off-white solid. \(^1\)H NMR (300 MHz, CDCl₃): \(\delta\) 7.62 (1H, d, \(J=15.9\) Hz), 7.51 (2H, d, \(J=8.1\) Hz), 7.37 (2H, d, \(J=8.1\) Hz), 6.42 (1H, d, \(J=15.9\) Hz), 3.80 (3H, s). \(^{13}\)C NMR (75 MHz, CDCl₃): \(\delta\) 167.4, 143.7, 133.5, 132.4, 129.7, 124.8, 118.7, 52.0.
(E)-methyl 3-(thiophen-3-yl)acrylate (2.18): 1.22 g, 99%, light brown solid. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.67 (1H, d, J=15.6 Hz), 7.49 (1H, m), 7.29 (1H, m), 7.33 (1H, m), 6.26 (1H, d, J=15.6 Hz), 3.79 (3H, s). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 167.9, 138.6, 137.8, 128.3, 127.2, 125.4, 117.7, 51.9.

(E)-methyl 3-(furan-3-yl)acrylate (2.19): 1.11 g, 98%, off-white solid. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.63 (1H, s), 7.56 (1H, d, J=15.6 Hz), 7.41 (1H, s), 6.57 (1H, s), 6.14 (1H, d, J=15.6 Hz), 3.76 (3H, s). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 167.6, 144.7, 144.6, 135.0, 122.8, 117.8, 107.6, 51.8.

The following example is representative for the formation of all trans-alkenes from the corresponding benzaldehyde and methyl diethylphosphonoacetate through a Horner-Emmons-Wadsworth olefination.
(E)-methyl 3-(4-ethoxyphenyl)acrylate (2.20): Under argon, sodium hydride (252 mg, 10 mmol, 1.7 eq) was dissolved in anhydrous THF (40 mL) in an oven dried round bottom flask and cooled to 0 °C. Methyl diethyl phosphonoacetate (1.7 mL, 9.6 mmol, 1.6 eq) was added dropwise and stirred for 45 min while allowing to warm to rt. In round bottom flask under argon, para-ethoxybenzaldehyde (830 μL, 6 mmol, 1.0 eq) was dissolved in anhydrous toluene (60 mL) and cooled to -78 °C. The phosphonate anion solution was transferred to the aldehyde via cannula and the reaction was allowed to warm to rt over the course of 6.75 h. Saturated Rochelle’s salt (20 mL) was added and stirred for 10 min. CH₂Cl₂ (20 mL) and deionized water (20 mL) was added and the layers separated. The organic products were extracted with CH₂Cl₂ (3 x 30 mL), dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure. The desired (E)-methyl 3-(4-ethoxyphenyl)acrylate was isolated by flash chromatography using 100% CH₂Cl₂ to yield a white solid in 95% yield (1.164 g). ¹H NMR (300 MHz, CDCl₃): δ7.64 (1H, d, J=15.9 Hz), 7.46 (2H, d, J=8.7 Hz), 6.88 (2H, d, J=8.7 Hz), 6.30 (1H, d, J=15.9 Hz), 4.05 (2H, q, J=6.9 Hz), 4.79 (3H, s), 1.42 (3H, t, J=6.9 Hz). ¹³C NMR (75 MHz, CDCl₃): δ168.0, 161.0, 144.8, 129.9, 127.2, 115.3, 115.1, 63.8, 51.8, 14.9.
(E)-methyl 3-(4-isoproxyphenyl)acrylate (2.21): 0.651 g, 95%, clear, colorless oil. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta 7.62\) (1H, d, J=15.9 Hz), 7.37 (2H, m), 6.91 (2H, m), 6.20 (1H, d, J=15.9 Hz), 3.74 (3H, s), 2.96 (6H, s). \(^1\)C NMR (75 MHz, CDCl\(_3\)): \(\delta 168.5, 152.0, 145.6, 130.0, 122.3, 112.2, 112.0, 51.6, 40.3\).

(E)-methyl 3-(4-(dimethylamino)phenyl)acrylate (2.22): 0.456 g, 86%; light yellow solid.

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta 7.62\) (1H, d, J=15.9 Hz), 7.37 (2H, m), 6.91 (2H, m), 6.20 (1H, d, J=15.9 Hz), 3.74 (3H, s), 2.96 (6H, s). \(^1\)C NMR (75 MHz, CDCl\(_3\)): \(\delta 168.5, 152.0, 145.6, 130.0, 122.3, 112.2, 112.0, 51.6, 40.3\).
(E)-methyl 3-(4-(prop-2-ynyloxy)phenyl)acrylate (2.23): 1.359 g, 87%, white solid. 1H NMR (400 MHz, CDCl3): δ7.48 (1H, d, J=16 Hz), 7.32 (2H, d, J=8.8 Hz), 6.82 (2H, d, 8.8 Hz), 6.17 (1H, d, J=16 Hz), 4.56 (2H, d, J=2.4 Hz), 3.62 (3H, s), 2.57 (1H, t, J=2.4 Hz).

(O2Me)2O
\[\overset{\text{O}}{\text{O}}\]  \(\overset{\text{O}}{\text{O}}\)
\(\overset{\text{O}}{\text{O}}\)

(E)-methyl 3-(4-(methoxymethoxy)phenyl)acrylate (2.24): 1.362 g, 98%, white solid. 1H NMR (400 MHz, CDCl3): δ7.64 (1H, d, J=16 Hz), 7.44 (2H, m), 7.02 (2H, m), 6.32 (1H, d, J=16 Hz), 5.19 (2H, s), 3.75 (3H, s), 3.47 (3H, s). 13C NMR (100 MHz, CDCl3): δ167.8, 159.1, 144.6, 129.8, 128.3, 116.6, 116.0, 94.3, 56.3, 51.7.

(O2Me)2O
\[\overset{\text{O}}{\text{O}}\]  \(\overset{\text{O}}{\text{O}}\)
\(\overset{\text{O}}{\text{O}}\)

(E)-methyl 3-(4-(benzyloxy)phenyl)acrylate (2.25): 1.120 g, 83%, white solid. 1H NMR (300 MHz, CDCl3): δ7.64 (1H, d, J=16 Hz), 7.41 (7H, m), 6.96 (2H, d, J=8.7 Hz), 6.30 (1H, d, J=16 Hz), 5.08 (2H, s), 3.78 (3H, s). 13C NMR (75 MHz, CDCl3): δ144.4, 129.7, 128.6, 128.1, 127.4, 115.3, 115.1, 70.0, 51.5.
(E)-methyl 3-(4-phenoxyphenyl)acrylate (2.26): 0.395 g, 67%, white solid. ¹H NMR (300 MHz, CDCl₃): δ7.65 (1H, d, J=15.9 Hz), 7.45 (2H, m), 7.32 (2H, m), 7.14 (1H, m), 7.00 (4H, m), 6.33 (1H, d, J=15.9 Hz), 3.77 (3H, s). ¹³C NMR (75 MHz, CDCl₃): δ167.7, 159.7, 156.3, 144.3, 130.2, 130.0, 129.4, 124.4, 119.9, 118.6, 116.7, 51.9.

(E)-methyl 3-(benzo[d][1,3]dioxol-5-yl)acrylate (2.27): 1.097 g, 89%, white solid. ¹H NMR (300 MHz, CDCl₃): δ7.28 (1H, d, J=15.9 Hz), 7.00 (2H, m), 6.79 (2H, m), 6.25 (1H, d, J=15.9 Hz), 3.78 (3H, s). ¹³C NMR (75 MHz, CDCl₃): δ167.6, 149.6, 148.3, 144.5, 128.8, 124.4, 115.7, 108.5, 106.5, 101.5, 51.6.

(E)-methyl 3-(4-(4-tert-butylphenylthio)phenyl)acrylate (2.28): 0.298 g, 76%, white solid. ¹H NMR (300 MHz, CDCl₃): δ7.63 (1H, d, J=15.9), 7.38 (6H, m), 7.19 (2H, m), 6.37
(1H, d, J=15.9 Hz), 3.79 (3H, s), 1.33 (9H, s). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 167.3, 151.6, 144.0, 141.1, 133.1, 131.8, 129.2, 128.4, 126.5, 117.0, 51.6, 34.6, 31.1.

\(\text{(E)-methyl 3-}(4\text{-}(\text{naphthalen-2-ylthio})\text{phenyl})\text{acrylate (2.29):} 0.314 \text{ g, 100\%, white solid.} \)

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.97 (1H, s), 7.78 (3H, m), 7.63 (1H, d, J=16.2 Hz), 7.48 (5H, m), 7.25 (2H, m), 6.39 (1H, d, J=16.2 Hz). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 144.3, 132.3, 130.0, 129.5, 128.9, 128.0, 127.9, 127.0, 117.7, 52.0.

\(\text{(E)-methyl 3-}(4\text{-}(\text{m-tolyloxy})\text{phenyl})\text{acrylate (2.30):} 67\%, \text{ white solid.} \)

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.60 (1H, d, J=12 Hz), 7.39 (2H, m), 7.26 (1H, m), 7.16 (1H, m), 6.89 (2H, m), 6.78 (2H, m), 6.30 (1H, m), 3.72 (3H, s).
(E)-methyl 3-(4-(3,5-dimethylphenoxy)phenyl)acrylate (2.31): 0.131 g, 95%, clear gold oil. $^1$H NMR (300 MHz, CDCl$_3$): δ7.66 (1H, d, J=15.9 Hz), 7.46 (2H, m), 6.96 (2H, m), 6.80 (1H, s), 6.66 (2H, s), 6.34 (1H, d, J=15.9 Hz), 3.79 (3H, s), 2.29 (6H, s). $^{13}$C NMR (75 MHz, CDCl$_3$): δ167.8, 160.0, 156.2, 144.5, 140.1, 130.0, 129.1, 126.1, 118.5, 117.6, 116.5, 51.9, 21.5.

(E)-methyl 3-(4-(3-methoxyphenoxy)phenyl)acrylate (2.32): 0.213 g, 82%, clear gold oil. $^1$H NMR (300 MHz, CDCl$_3$): δ7.65 (1H, d, J=15.9 Hz), 7.45 (2H, m), 7.23 (1H, m), 6.97 (2H, m), 6.69 (1H, m), 6.60 (2H, m), 6.34 (1H, d, J=15.9 Hz), 3.77 (3H, s), 3.76 (3H, s). $^{13}$C NMR (75 MHz, CDCl$_3$): δ167.8, 161.3, 159.5, 157.5, 144.3, 130.6, 130.0, 129.5, 118.8, 116.7, 111.9, 110.0, 105.9, 55.6, 51.9.

The following example is representative for the cyclopropanation of the acrylates using diazomethane.

Methyl trans-2-(4-ethoxyphenyl)cyclopropanecarboxylate (2.34): The diazomethane generator was used. The (E)-methyl 3-(4-ethoxyphenyl)acrylate (1.8 mmol, 1 eq) and
palladium (II) acetate catalyst (8.0 mg, 1.6 mol%) were dissolved in diethyl ether (26 mL) in the round bottom flask. 85% Potassium hydroxide pellets (2.81 g, 42 mmol, 23 eq) was dissolved in water (10 mL) and diethylene glycol monoethyl ether (15 mL) in the distillation chamber and brought to 60-70 °C using an oil bath. The cold finger was brought to -72 °C using isopropyl alcohol/dry ice and the round bottom brought to < -25 °C using ethylene glycol/dry ice. Diazald (3.90 g, 18 mmol, 10 eq) dissolved in diethyl ether (30 mL) was added dropwise from the addition funnel to the distillation chamber. The produced diazomethane was distilled into the round bottom collecting the clear golden yellow liquid. The round bottom capped loosely and stirred overnight (12-20 h) allowing to warm to rt. The reaction mixture was run over a plug of celite to remove the catalyst and the solvent removed by in vacuo. The reaction was monitored by ¹H NMR and if alkene was still present (1H d ~ 6.3 ppm, 1H d ~ 7.6 ppm) additional equivalents of reagents were added to drive reaction to completion. No other purification was necessary to yield the white solid in 99% yield (0.286 g). ¹H NMR (400 MHz, CDCl₃): δ6.99 (2H, m), 6.79 (2H, m), 3.97 (2H, q, J=7.6 Hz), 3.69 (3H, s), 2.48 (1H, ddd, J= 4.2, 4.4, 11.3 Hz), 1.81 (2H, quintet, J=4.2), 1.54 (1H, ), 1.38 (3H, t, J=4.2), 1.25 (1H, ). ¹³C NMR (100 MHz, CDCl₃): δ174.2, 157.9, 132.0, 127.5, 114.7, 63.6, 52.0, 26.0, 23.8, 16.9, 15.0.
Methyl \textit{trans}-2-(4-methoxyphenyl)cyclopropanecarboxylate (2.33): 0.343 g, 91%, light yellow solid. \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 7.00 (2H, m), 6.78 (2H, m), 3.72 (3H, s), 3.70 (3H, s), 2.49 (1H, ddd, \(J=4.5, 6.6, 8.4\) Hz), 1.83 (2H, quintet, \(J=4.5\) Hz), 1.56 (1H, m), 1.27 (1H, m). \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}): \(\delta\) 174.0, 158.7, 132.1, 127.6, 114.2, 55.3, 51.9, 25.8, 23.8, 16.8.

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\text{Me} \quad \text{O} \quad \text{Me}
\]

Methyl \textit{trans}-2-(4-isopropoxyphenyl)cyclopropanecarboxylate (2.35): 0.408 g, 100%, clear yellow oil. \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 6.98 (2H, m), 6.78 (2H, m), 4.47 (1H, sep, \(J=6\) Hz), 2.47 (1H, ddd, \(J=4.3, 4.5, 11.3\)), 1.82 (1H, q, \(J=4.2\) Hz), 1.54 (1H, q, \(J=4.2\) Hz). \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}): \(\delta\) 174.1, 156.9, 131.9, 127.6, 116.2, 70.1, 51.9, 26.0, 23.9, 22.2, 16.9.

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\text{Me} \quad \text{O} \quad \text{Me}
\]

Methyl \textit{trans}-2-(4-(trifluoromethyl)phenyl)cyclopropanecarboxylate (2.36): 0.423 g, 96%, pale yellow solid. \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 7.51 (2H, d, \(J=8.1\) Hz), 7.17 (2H, d, \(J=8.1\) Hz), 3.71 (3H, s), 2.56 (1H, ddd, \(J=4.2, 6.3, 9.0\) Hz), 1.94 (1H, ddd, \(J=4.2, 5.4, 8.7\) Hz), 73
1.65 (1H, m), 1.33 (1H, ddd, J=4.8, 6.6, 8.4 Hz). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$173.6, 144.5, 125.6, 52.3, 26.0, 24.5, 17.5.

Methyl trans-2-(4-bromophenyl)cyclopropanecarboxylate (2.37): 0.435 g, 94%, pale yellow oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$7.39 (2H, d, J=8.4 Hz), 6.97(2H, d, J=8.4 Hz), 3.72 (3H, s), 2.48 (1H, ddd, J=4.5, 6.6, 8.4 Hz), 1.87 (1H, ddd, J=4.2, 5.1, 8.4 Hz), 1.60 (1H, ddd, J=4.5, 5.4, 9.3 Hz), 1.28 (1H, ddd, J=4.8, 6.6, 8.4 Hz). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$173.8, 139.3, 131.8, 128.2, 120.4, 52.2, 25.2, 24.1, 17.2.

Methyl trans-2-(4-phenoxyphenyl)cyclopropanecarboxylate (2.38): 0.279 g, 100%, light gold oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$7.28 (2H, m), 7.02 (3H, m), 6.94 (4H, m), 3.69 (3H, s), 2.51 (1H, ddd), 1.86 (1H, ddd), 1.58 (1H, ddd), 1.27 (1H, ddd). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$174.1, 157.6, 156.1, 135.1, 130.0, 127.9, 123.4, 119.3, 118.9, 52.1, 30.0, 26.0, 24.1, 17.1.
Methyl trans-2-(3-(benzyloxy)phenyl)cyclopropanecarboxylate (2.39): 0.298 g, 100%, off-white solid. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.37 (4H, m), 7.05 (2H, m), 6.92 (2H, m), 5.04 (2H, s), 3.73 (3H, s), 2.53 (1H, ddd), 1.86 (1H, ddd), 1.59 (1H, ddd), 1.29 (1H, ddd).

$^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$147.2, 157.8, 137.3, 132.5, 128.9, 128.2, 127.7, 115.2, 70.3, 52.1, 26.0, 24.0, 17.0.

Methyl trans-2-(4-(methoxymethoxy)phenyl)cyclopropanecarboxylate (2.40): 0.420 g, 100%, white solid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$7.00 (2H, m), 6.94 (2H, m), 5.11 (2H, s), 3.68 (3H, s), 3.43 (3H, s), 2.47 (1H, ), 1.82 (1H, ), 1.54 (1H, ), 1.25 (1H, ). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$174.0, 156.1, 133.4, 127.5, 116.5, 94.6, 56.0, 51.9, 25.8, 23.8, 16.8.

Methyl trans-2-(thiophen-3-yl)cyclopropanecarboxylate (2.41): 0.163 g, 99%, amber oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$7.23 (1H, m), 6.95 (1H, m), 6.83 (1H, m), 3.71 (3H, s), 2.56
(1H, ddd, J=4.2, 6.6, 9.0 Hz), 1.87 (1H, ddd, J=4.2, 5.1, 8.4 Hz), 1.56 (1H, ddd, J=4.5, 5.1, 9.0 Hz), 1.26 (1H, ddd, J=4.5, 6.3, 8.4 Hz). $^{13}$C NMR (75 MHz, CDCl₃): δ173.9, 141.3, 126.2, 126.1, 120.0, 52.1, 23.6, 22.2, 17.1.

Methyl trans-2-(furan-3-yl)cyclopropanecarboxylate (2.42): 0.052 g, 97%, clear yellow oil. $^1$H NMR (300 MHz, CDCl₃): δ7.32 (1H, m), 7.28 (1H, m), 6.15 (1H, m), 3.71 (3H, s), 2.33 (1H, ddd, J=4.2, 6.6, 9.0 Hz), 1.76 (1H, ddd, J=3.9, 5.1, 8.4 Hz), 1.50 (1H, m), 1.13 (1H, ddd, J=4.5, 6.6, 8.1 Hz). $^{13}$C NMR (75 MHz, CDCl₃): δ174.1, 143.4, 139.4, 124.9, 109.2, 52.1, 22.7, 17.5, 16.3.

Methyl trans-2-(4-(4-tert-butylphenylthio)phenyl)cyclopropanecarboxylate (2.43):
0.246 g, 94%, gold oil. $^1$H NMR (300 MHz, CDCl₃): δ7.25 (5H, m), 6.99 (2H, m), 3.69 (3H, s), 2.41 (1H, ddd), 1.87 (1H, ddd), 1.59 (1H, ddd), 1.29 (10H, m). $^{13}$C NMR (100 MHz, CDCl₃): δ173.9, 150.6, 139.1, 134.5, 132.4, 131.2, 127.2, 126.5, 52.2, 34.8, 31.5, 26.2, 24.3, 17.3.
Methyl trans-2-(4-(naphthalen-2-ylthio)phenyl)cyclopropanecarboxylate (2.44): 0.254 g, 95%, gold oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.70 (4H, m), 7.42 (5H, m), 6.99 (2H, m), 3.69 (3H, s), 2.49 (1H, ddd), 1.88 (1H, ddd), 1.60 (1H, ddd), 1.26 (1H, ddd). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 173.9, 139.7, 134.0, 133.7, 132.4, 131.9, 129.5, 129.1, 128.6, 128.0, 127.6, 127.4, 126.9, 126.4, 52.2, 26.2, 24.4, 17.4.

Methyl 2-(benzo[d][1,3]dioxol-5-yl)cyclopropanecarboxylate (2.46): 0.405 g, 100%, white solid. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 6.68 (2H, m), 6.54 (2H, m), 5.88 (2H, s), 3.68 (3H, s), 2.44 (1H, ddd, $J$=4.2, 6.6, 8.4 Hz), 1.80 (1H, ddd, $J$=4.2, 5.0, 8.4 Hz), 1.52 (1H, ddd, $J$=5.0, 6.6, 7.8 Hz), 1.22 (1H, ddd, $J$=4.2, 6.6, 8.4 Hz). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 173.8, 147.8, 146.3, 133.8, 119.7, 108.1, 106.6, 101.0, 51.8, 26.8, 26.1, 23.7, 16.7.
Methyl trans-2-(4-(dimethylamino)phenyl)cyclopropanecarboxylate (2.47): 0.359 g, 81%, dark gold solid. $^1$H NMR (300 MHz, CDCl₃): $\delta$6.95 (2H, m), 6.64 (2H, m), 3.66 (3H, s), 2.80 (6H, s), 2.45 (1H, ddd, J=4.2, 4.5, 11.4 Hz), 1.79 (1H, ddd, J=4.2, 4.7, 8.4 Hz), 1.52 (1H, dd, J=4.8, 14.1 Hz), 1.24 (1H, ddd, J=4.5, 6.6, 8.3). $^{13}$C NMR (75 MHz, CDCl₃): $\delta$174.4, 149.8, 127.9, 127.4, 113.1, 52.0, 41.0, 26.2, 23.8, 16.8.

![Methyl trans-2-(4-(dimethylamino)phenyl)cyclopropanecarboxylate](image)

Methyl trans-2-(3-(m-tolyloxy)phenyl)cyclopropanecarboxylate (2.48): 0.298 g, 100%, dark gold oil. $^1$H NMR (300 MHz, CDCl₃): $\delta$6.99 (8H, m), 3.71 (3H, s), 2.48 (1H, ddd), 2.33 (3H, s), 1.88 (1H, ddd), 1.57 (1H, ddd), 1.29 (1H, ddd). $^{13}$C NMR (75 MHz, CDCl₃): $\delta$130.2, 129.9, 129.7, 129.5, 125.3, 124.4, 121.1, 199.2, 117.1, 116.8, 116.1, 52.2, 26.3, 24.3, 24.2, 17.4, 17.2.

![Methyl trans-2-(3-(m-tolyloxy)phenyl)cyclopropanecarboxylate](image)

Methyl trans-2-(4-(3,5-dimethylphenoxy)phenyl)cyclopropanecarboxylate (2.49): 0.125 g, 95%, gold oil. $^1$H NMR (300 MHz, CDCl₃): $\delta$7.03 (2H, m), 6.91 (2H, m), 6.72 (1H, s), 6.59 (2H, s), 3.71 (3H, s), 2.51 (1H, ddd), 2.26 (6H, s), 1.85 (1H, ddd), 1.60 (1H, ddd), 1.29
(1H, ddd). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 174.1, 157.6, 156.3, 139.8, 134.8, 127.8, 125.2, 119.3, 116.6, 52.2, 26.0, 24.1, 21.6, 17.1.

methyl 2-(4-hydroxyphenyl)cyclopropanecarboxylate (2.50): A round bottom flask charged with the methyl ester (0.851 g, 3.6 mmol, 1 eq) in CH$_2$Cl$_2$ (20 mL) was brought to 0 °C. Trifluoroacetic acid (2 mL, 26 mmol, 7.2 eq) was added slowly. The reaction was stirred for 24 h allowing to warm to rt. The crude reaction mixture was diluted with CH$_2$Cl$_2$ (50 mL) and then washed with saturated NaHCO$_3$ (50 mL) and saturated NaCl (50 mL), dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure. The desired methyl 2-(4-hydroxyphenyl)cyclopropanecarboxylate was isolated by flash chromatography over silica gel using 3:1 hexanes:ethyl acetate as a light gold oil in 62% yield (0.239 g). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 6.95 (2H, d, $J$=8.4 Hz), 6.75 (2H, d, $J$=8.4 Hz), 3.71 (3H, s), 2.48 (1H, m), 1.82 (1H, m), 1.55 (1H, m), 1.26 (1H, m). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 174.8, 154.9, 131.8, 127.8, 115.6, 52.2, 36.1, 25.6, 24.0, 17.0.
methyl 2-(4-(prop-2-ynyloxy)phenyl)cyclopropanecarboxylate (2.45): An oven-dried round bottom flask charged with anhydrous dimethylformamide (2 mL), methyl 2-(4-hydroxyphenyl)cyclopropanecarboxylate (0.180 g, 0.9 mmol, 1 eq) and anhydrous potassium carbonate (0.418 g, 2.8 mmol, 3 eq) was stirred at 55 °C for 30 min. The reaction mixture was cooled to rt and propargyl bromide (98 µL, 1.1 mmol, 1.2 eq) was added. The reaction was stirred for 5 h at rt. The crude reaction mixture was poured on ice water (25 mL) and no precipitate formed. The organic products were extracted with ethyl acetate (3 x 30 mL), washed with saturated NH₄Cl (25 mL) and saturated NaCl (25 mL), dried over Na₂SO₄ and concentrated in vacuo affording the desired methyl 2-(4-(prop-2-ynyloxy)phenyl)cyclopropanecarboxylate as a dark yellow oil in 79% yield (0.170 g). ¹H NMR (300 MHz, CDCl₃): δ 7.04 (2H, d, J=8.7 Hz), 6.89 (2H, d, J=8.7 Hz), 4.66 (2H, d, J=2.4 Hz), 3.71 (3H, s), 2.50 (2H, m), 1.83 (1H, m), 1.56 (1H, m), 1.27 (1H, m). ¹³C NMR (75 MHz, CDCl₃): δ 174.1, 156.5, 133.2, 127.6, 115.2, 78.8, 75.8, 56.1, 52.1, 25.9, 23.9, 16.9.

The following example is representative for saponification of the methyl cyclopropanecarboxylates to yield the corresponding carboxylic acids.
trans-2-(4-ethoxyphenyl)cyclopropanecarboxylic acid (2.52): To a solution of methyl trans-2-(4-ethoxyphenyl)cyclopropanecarboxylate (0.2862 g, 1.3 mmol, 1 eq) in methanol (3.4 mL) was added 2 M sodium hydroxide (3.4 mL) while stirring. The reaction was monitored by TLC and upon consumption of the ester, the mixture was poured onto ice (~60 mL) and 12 N HCl (1.4 mL) was added dropwise while stirring. The resulting precipitate was isolated by vacuum filtration. The filter cake was washed with portions of ice water until the filtrate was pH neutral and was dried in vacuo to give trans-2-(4-ethoxyphenyl)cyclopropanecarboxylic acid as an off-white solid in 78% yield (0.210 g).  

\[ ^1H \text{NMR (400 MHz, CDCl}_3): \delta 7.02 (2H, m), 6.82 (2H, m), 4.00 (2H, q, J=6.8 Hz), 2.56 (1H, ddd, J=4.0, 6.5, 8.3 Hz), 1.82 (1H, ddd, J=4.0, 5.2, 8.3 Hz), 1.61 (1H, quintet, J=5.2 Hz), 1.40 (3H, t, J=6.8 Hz), 1.35 (ddt, J= 4.0, 6.5, 8.3). \]  

\[ ^{13}C \text{NMR (100 MHz, CDCl}_3): \delta 180.1, 158.0, 131.5, 127.7, 114.8, 63.7, 26.9, 23.9, 17.4, 15.0. \]

trans-2-(4-methoxyphenyl)cyclopropanecarboxylic acid (2.51): 0.237 g, 73%, white solid.  

\[ ^1H \text{NMR (300 MHz, CD}_3\text{OD): } \delta 7.04 (2H, m), 6.82 (2H, m), 4.92 (1H, bs), 3.74 (3H, s), 2.41 (1H, ddd, J=4.2, 6.2, 9.5 Hz), 1.74 (1H, m), 1.47 (1H, quintet, J=4.8 Hz), 1.29 (ddt, J= 4.8, 6.2, 8.1). \]  

\[ ^{13}C \text{NMR (75 MHz, CD}_3\text{OD): } \delta 176.1, 158.7, 132.1, 127.1, 113.8, 54.5, 25.5, 23.5, 15.9. \]
trans-2-(4-isopropoxyphenyl)cyclopropanecarboxylic acid (2.53): 0.384 g, 78%; white solid. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.02 (2H, m), 6.81 (2H, m), 4.50 (1H, sep, J=6.0 Hz), 2.56 (1H, ddd, J=4.0, 6.8, 8.4 Hz), 1.82 (1H, ddd, J=4.0, 5.2, 8.4 Hz), 1.61 (1H, q, J=5.2 Hz), 1.35 (1H, ddd, J=4.0, 6.8, 8.4 Hz), 1.32 (6H, d, J=6.0 Hz). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 180.3, 157.0, 131.5, 127.7, 116.2, 70.2, 26.9, 24.0, 22.2, 17.4.

trans-2-(4-(trifluoromethyl)phenyl)cyclopropanecarboxylic acid (2.54): 0.395 g, 98%, pale yellow solid. $^1$H NMR (300 MHz, CD$_3$OD): δ 7.56 (2H, d, J=8.1 Hz), 7.31 (2H, d, J=8.1 Hz), 2.54 (1H, ddd, J=4.2, 6.0, 9.0 Hz), 1.93 (1H, ddd, J=4.2, 5.4, 8.4 Hz), 1.59 (1H, m) 1.41 (1H, ddd, J=4.8, 6.3, 8.4 Hz). $^{13}$C NMR (75 MHz, CD$_3$OD): δ 176.5, 146.5, 127.7, 126.4, 26.6, 25.5, 17.8.
*trans*-2-(4-bromophenyl)cyclopropanecarboxylic acid (2.55): 0.412 g, 100%, white solid.

$^1$H NMR (300 MHz, CD$_3$OD): $\delta$7.40 (2H, d, J=8.1 Hz), 7.04 (2H, d, J=8.1 Hz), 2.43 (1H, ddd, J=4.2, 6.6, 9.0 Hz), 1.83 (1H, m), 1.52 (1H, m), 1.33 (1H, ddd, J=4.5, 6.3, 8.6 Hz). $^{13}$C NMR (75 MHz, CD$_3$OD): $\delta$176.7, 141.0, 132.5, 129.0, 121.0, 26.5, 25.1, 17.4.

![Chemical structure](image)

*trans*-2-(4-phenoxyphenyl)cyclopropanecarboxylic acid (2.56): 0.148 g, 58%, white solid. $^1$H NMR (300 MHz, CD$_3$OD): $\delta$7.27 (2H, m), 7.04 (3H, m), 6.89 (4H, m), 5.05 (1H, bs), 2.44 (1H, ddd), 1.78 (1H, ddd), 1.50 (1H, ddd), 1.28 (1H, ddd). $^{13}$C NMR (75 MHz, CD$_3$OD): $\delta$175.9, 157.6, 156.1, 135.3, 129.7, 127.5, 123.1, 118.9, 118.5, 25.5, 23.8, 16.2.

![Chemical structure](image)

*trans*-2-(3-(benzyloxy)phenyl)cyclopropanecarboxylic acid (2.57): 0.217 g, 76%, white solid. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$7.30 (5H, m), 7.02 (2H, m), 6.90 (2H, m), 5.03 (2H, s), 2.40 (1H, ddd), 1.74 (1H, ddd), 1.47 (1H, ddd), 1.28 (1H, ddd).
2-(4-(methoxymethoxy)phenyl)cyclopropanecarboxylic acid (2.58): 0.172 g, 67%, white solid. \textsuperscript{1}H NMR (400 MHz, CD$_3$OD): $\delta$7.01 (2H, d, J=8.6 Hz), 6.90 (2H, d, J=8.6 Hz), 5.09 (2H, s), 4.91 (1H, s), 3.39 (2H, s), 2.38 (1H, ddd, J=4.0, 6.4, 9.2 Hz), 1.72 (1H, ddd, J=4.0, 5.2, 8.1 Hz), 1.45 (1H, ddd, J=4.4, 5.2, 9.2 Hz), 1.26 (1H, ddd, J=4.4, 6.4, 8.1 Hz). \textsuperscript{13}C NMR (100 MHz, CD$_3$OD): $\delta$176.0, 156.2, 133.2, 133.5, 127.1, 116.3, 94.4, 54.9, 25.5, 23.6, 16.0.

\[ \text{trans-2-(thiophen-3-yl)cyclopropanecarboxylic acid (2.59): 0.123 g, 90%, yellow solid.} \]

\textsuperscript{1}H NMR (300 MHz, CD$_3$OD): $\delta$7.31 (1H, m), 7.08 (1H, m), 6.89 (1H, m), 2.54 (1H, ddd, J=4.2, 6.6, 9.0 Hz), 1.79 (1H, ddd, J=3.9, 5.1, 8.4 Hz), 1.48 (1H, m), 1.30 (1H, ddd, J=4.2, 6.6, 8.4 Hz). \textsuperscript{13}C NMR (75 MHz, CD$_3$OD): $\delta$177.0, 142.6, 127.0, 126.9, 120.7, 24.5, 22.9, 17.3.

\[ \text{trans-2-(furan-3-yl)cyclopropanecarboxylic acid (2.60): 0.185 g, 65%, yellow solid.} \]

\textsuperscript{1}H NMR (300 MHz, CD$_3$OD): $\delta$7.37 (2H, m), 6.22 (1H, m), 2.28 (1H, ddd, J=3.9, 6.3, 9.3 Hz),
1.70 (1H, ddd, J=4.2, 5.1, 8.4 Hz), 1.42 (1H, m), 1.16 (1H, ddd, J=4.2, 6.3, 8.4 Hz). $^1$C NMR (75 MHz, CD$_3$OD): δ 177.1, 144.4, 140.4, 126.1, 109.8, 23.5, 18.2, 16.5.

trans-2-(4-(4-tert-butylphenylthio)phenyl)cyclopropanecarboxylic acid (2.61): 0.166 g, 73%, yellow oil. $^1$H NMR (300 MHz, CD$_3$OD): δ 7.14 (8H, m), 4.99 (1H, bs), 2.41 (1H, ddd), 1.79 (1H, ddd), 1.49 (1H, ddd), 1.24 (10H, m).

trans-2-(4-(naphthalen-2-ylthio)phenyl)cyclopropanecarboxylic acid (2.62): 0.203 g, 87%, yellow solid. $^1$H NMR (300 MHz, CD$_3$OD): δ 7.72 (4H, m), 7.44 (2H, m), 7.29 (3H, m), 7.13 (2H, m), 2.46 (1H, ddd), 1.84 (1H, ddd), 1.55 (1H, ddd), 1.36 (1H, ddd).

2-(4-(prop-2-ynyloxy)phenyl)cyclopropanecarboxylic acid (2.63): 0.179 g, 70%, white solid. $^1$H NMR (300 MHz, CD$_3$OD): δ 7.07 (2H, d, J=8.6 Hz), 6.90 (2H, d, J=8.6 Hz), 4.86
(1H, s), 4.68 (2H, d, J=2.1 Hz)), 2.90 (1H, t, J=2.1 Hz), 2.42 (1H, m), 1.75 (1H, m), 1.48 (1H, m), 1.30 (1H, m).

2-(benzo[d][1,3]dioxol-5-yl)cyclopropanecarboxylic acid (2.64): 0.281 g, 74%, white solid.

\[
\text{1H NMR (300 MHz, CD}_{3}\text{OD): } \delta 6.62 (3H, m), 5.89 (2H, s), 4.95 (1H, bs), 2.39 (1H, \text{ddd, } J=3.9, 5.1, 9.0 \text{ Hz}), 1.73 (1H, \text{ddd, } J=5.1, 6.5, 6.9), 1.45 (1H, \text{ddd, } J=3.9, 6.5, 9.0 \text{ Hz}).
\]

\[
\text{13C NMR (75 MHz, CD}_{3}\text{OD): } \delta 175.7, 147.9, 146.3, 133.9, 119.3, 107.7, 106.1, 100.9, 25.7, 23.4, 15.8.
\]

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trans-2-(4-(m-tolyloxy)phenyl)cyclopropanecarboxylic acid (2.66): 0.199 g, 72%, dark brown oil.

\[
\text{1H NMR (300 MHz, CD}_{3}\text{OD): } \delta 10.89 (1H, bs), 7.00 (8H, m), 2.54 (1H, \text{ddd}), 2.30 (3H, s), 1.84 (1H, \text{ddd}), 1.60 (1H, \text{ddd}), 1.34 (1H, \text{ddd}).
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trans-2-(4-(3,5-dimethylphenoxy)phenyl)cyclopropanecarboxylic acid (2.67): 0.048 g, 41%, off-white solid. \( ^1\text{H NMR} \) (300 MHz, CD3OD): \( \delta \text{7.09 (2H, m), 6.85 (2H, m), 6.72 (1H, s), 6.54 (2H, s), 4.89 (1H, bs), 2.44 (1H, m), 2.23 (6H, s), 1.77 (1H, m), 1.49 (1H, m), 1.30 (1H, ddd).}

The following example is representative for Curtius rearrangement of carboxylic acids to general the corresponding t-butylcarbamate protected amines using diphenylphosphorylazide, triethylamine and t-butanol. In some cases, the carbamate could not be purified completely so impure material was taken on to the subsequent hydrolysis step.

![Chemical Reaction](image)

tert-Butyl trans-[2-(4-ethoxyphenyl)cyclopropyl]carbamate (2.69):

Diphenylphosphorazidate (125 μL, 0.58 mmol, 1.2 eq) and anhydrous triethylamine (94 μL, 0.67 mmol, 1.4 eq) were added sequentially to a room temperature solution of trans-2-(4-ethoxyphenyl)cyclopropanecarboxylic acid (0.100 g, 0.48 mmol, 1 eq) in anhydrous tert-butanol (1 mL). The reaction was heated to 90 °C with an oil bath for 41 h, cooled to rt and concentrated to dryness under reduced pressure. The resulting residue was partitioned between ethyl acetate (10 mL) and 10% aqueous K₂CO₃ (10 mL). The organic products were extracted with ethyl acetate (2 x 10 mL), dried over Na₂SO₄, filtered, and concentrated in vacuo. The tert-butyl trans-[2-(4-ethoxyphenyl)cyclopropyl]carbamate
was isolated by flash chromatography using 5:1 hexanes:ethyl acetate affording a yellow solid in 30% yield (0.040 g). ¹H NMR (400 MHz, CDCl₃): δ7.06 (2H, m), 6.79 (2H, m), 4.85 (1H, bs), 3.99 (2H, q, J=6.8 Hz) 2.64 (1H, bs), 1.98 (1H, m), 1.45 (9H, s), 1.38 (3H, d, J=6.8 Hz), 1.08 (2H, m). ¹³C NMR (100 MHz, CDCl₃): δ157.5, 132.8, 127.9, 114.6, 63.6, 28.6, 24.5, 16.0, 15.0.

**tert-butyl trans-2-(4-methoxyphenyl)cyclopropylcarbamate (2.68):** 0.186 g, 67%, white solid. ¹H NMR (300 MHz, CDCl₃): δ7.07 (2H, d, J=8.4 Hz), 6.80 (2H, d, J=8.4 Hz), 4.96 (1H, bs), 3.75 (3H, s), 2.64 (1H, m), 1.95 (1H, m), 1.45 (9H, s), 1.07 (2H, m). ¹³C NMR (75 MHz, CDCl₃): δ158.2, 133.2, 128.0, 120.4, 114.0, 79.7, 55.5, 32.4, 28.7, 24.5, 16.0.

**tert-butyl trans-[2-(4-isopropoxyphenyl)cyclopropyl]carbamate (2.70):** 0.077 g, 57%, white solid. ¹H NMR (400 MHz, CDCl₃): δ7.05 (2H, m), 6.79 (2H, m), 4.89 (1H, bs), 4.48 (1H, sep, J=6.0 Hz), 2.65 (1H, bs), 1.98 (1H, ddd, J=3.2, 6.4, 9.3 Hz), 1.45 (9H, s), 1.30 (6H, d, J=6.0 Hz), 1.08 (2H, m). ¹³C NMR (100 MHz, CDCl₃): δ156.4, 132.8, 127.9, 116.1, 70.2, 32.2, 28.6, 24.4, 22.3, 16.0.
tert-butyl trans-[2-(4-(trifluoromethyl)phenyl)cyclopropyl]carbamate (2.71): 0.056 g, 42%, white solid. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$7.50 (2H, d, $J$=8.1 Hz), 7.22 (2H, d, $J$=8.1 Hz), 4.85 (1H, bs), 2.74 (1H, m), 2.09 (1H, m), 1.45 (9H, s), 1.21 (2H, m). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$156.1, 145.4, 128.8, 126.8, 126.0, 79.3, 32.0, 28.6, 23.1, 16.1.

tert-butyl trans-[2-(4-bromophenyl)cyclopropyl]carbamate (2.72): 0.065 g, 50%, white solid. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$7.36 (2H, d, $J$=8.1 Hz), 7.01 (2H, d, $J$=8.1 Hz), 4.84 (1H, bs), 2.66 (1H, m), 1.98 (1H, m), 1.45 (9H, s), 1.13 (2H, m). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$156.5, 140.0, 131.5, 128.6, 119.9, 80.0, 32.8, 28.6, 25.0, 16.4.

tert-butyl trans-[2-(4-phenoxyphenyl)cyclopropyl]carbamate (2.73): 0.035 g, 29%, yellow oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$7.33 (2H, m), 7.10 (3H, m), 6.93 (4H, m), 4.85 (1H, bs), 2.69 (1H, m), 2.03 (1H, m), 1.46 (9H, s), 1.15 (2H, m). $^{13}$C NMR (75 MHz,
CDCl3): δ 157.9, 156.6, 155.5, 136.0, 129.9, 128.1, 123.2, 119.4, 118.7, 79.9, 32.6, 28.6, 24.8, 16.3.

tert-butyl trans-[2-(4-(benzyloxy)phenyl)cyclopropyl]carbamate (2.74): 0.039 g, 27%, yellow solid. ¹H NMR (300 MHz, CDCl3): δ 7.39 (5H, m), 7.07 (2H, d, J=8.7 Hz), 6.87 (2H, d, J=8.7 Hz), 5.03 (2H, s), 4.82 (1H, bs), 2.65 (1H, m), 1.99 (1H, ddd), 1.45 (9H, s), 1.09 (2H, m). ¹³C NMR (75 MHz, CDCl3): δ 157.5, 137.4, 133.3, 128.8, 128.1, 128.0, 127.7, 115.0, 70.3, 32.4, 29.9, 28.7, 24.6, 16.1.

tert-butyl 2-(4-(methoxymethoxy)phenyl)cyclopropylcarbamate (2.75): 0.144 g, 50% yield, white solid. ¹H NMR (300 MHz, CDCl3): δ 7.04 (2H, m), 6.92 (2H, m), 5.11 (2H, s), 5.05 (1H, bs), 3.43 (3H, s), 2.63 (1H, m), 1.97 (1H, m), 1.44 (9H, s), 1.05 (2H, m). ¹³C NMR (75 MHz, CDCl3): δ 156.6, 155.8, 134.4, 127.9, 116.5, 94.8, 79.7, 56.1, 32.5, 28.6, 24.5, 16.1.
**tert-butyl trans-[2-(4-(4-tert-butylphenylthio)phenyl)carbamate (2.76):** 0.034 g, 29%, yellow oil. \( ^1\)H NMR (300 MHz, CDCl\(_3\)): \( \delta \) 7.29 (6H, m), 7.06 (2H, d, J=8.4 Hz), 4.85 (1H, bs), 2.70 (1H, m), 2.01 (1H, m), 1.24 (18H, m), 0.95 (2H, m). \( ^{13}\)C NMR (75 MHz, CDCl\(_3\)): \( \delta \) 150.3, 140.1, 133.4, 131.5, 130.7, 130.3, 127.5, 127.1, 126.4, 34.7, 32.9, 31.5, 29.9, 28.6, 25.1, 16.6.

![tert-butyl trans-[2-(4-(4-tert-butylphenylthio)phenyl)carbamate](image)

**tert-butyl trans-2-(4-(prop-2-ynyloxy)phenyl)cyclopropylcarbamate (2.77):** 0.083 g, 37%, white solid. \( ^1\)H NMR (300 MHz, CDCl\(_3\)): \( \delta \) 7.05 (2H, m), 6.77 (2H, m), 4.90 (1H, bs), 4.64 (2H, d, J=2.4 Hz), 2.64 (1H, m), 2.50 (1H, t, J=2.4 Hz), 2.00 (1H, m), 1.43 (9H, s), 1.09 (2H, m).

![tert-butyl trans-2-(4-(prop-2-ynyloxy)phenyl)cyclopropylcarbamate](image)

**tert-butyl trans-2-(benzo[d][1,3]dioxol-5-yl)cyclopropylcarbamate (2.78):** 0.135 g, 43%, off-white solid. \( ^1\)H NMR (300 MHz, CDCl\(_3\)): \( \delta \) 6.65 (3H, m), 5.87 (2H,s), 4.97 (1H, bs), 2.98 (1H, s), 2.60 (1H, m), 1.95 (1H, m), 1.44 (9H, s), 1.05 (2H, m). \( ^{13}\)C NMR (75 MHz, CDCl\(_3\)): \( \delta \) 156.4, 147.6, 145.8, 134.6, 120.1, 108.0, 100.8, 79.5, 32.2, 28.4, 25.0, 15.8.
The following example is representative for the Curtius rearrangement conditions to form the 2-(trimethylsilyl)ethyl carbamates.

2-(trimethylsilyl)ethyl 2-(thien-3-yl)cyclopropylcarbamate (2.79): Ethylchloroformate (80.5 μL, 1.4 eq) and anhydrous triethylamine (103 μL, 1.2 eq) were added sequentially at -10 to -15 °C to a solution of the carboxylic acid (0.101 g, 1 eq) in anhydrous acetone (3.5 mL). The reaction mixture was stirred for 2 h. A solution of NaN₃ (0.065 g, 1.53 eq) in water (190 μL) was added, and the reaction stirred for 2 h. The reaction was quenched with ice cold water (3.5 mL). The acyl azide was extracted with ethyl ether (4 X 3 mL), dried over anhydrous MgSO₄, and concentrated under reduced pressure. The acyl azide was resuspended in toluene (3.6 mL) and heated to 90 °C while stirred for 2 h to promote the Curtius rearrangement. The reaction mixture was cooled to rt and concentrated under reduced pressure. TMS-ethanol (175 μL) was added and the reaction stirred at 60 °C for 18 h. The excess TMS-ethanol was removed under reduced pressure to afford the desired protected carbamate as a dark amber oil in 93% yield (0.158 g). ¹H NMR (300 MHz, CDCl₃): δ7.21 (m, 1H), 6.90 (m, 2H), 5.10 (bs, 1H), 4.17 (t, J = 8.2 Hz, 2H), 2.68 (bs, 1H), 2.05 (m, 1H), 1.11 (m, 2H), 0.98 (m, 2H), 0.02 (s, 9H). ¹³C NMR (300 MHz, CDCl₃): δ141.9, 127.1, 126.5, 125.8, 119.5, 63.4, 60.3, 32.4, 21.1, 18.0, 16.4, -1.2.
2-(trimethylsilyl)ethyl 2-(furan-3-yl)cyclopropylcarbamate (2.80): 0.007 g, 26%, yellow solid. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$7.68 (m, 1H), 7.50 (m, 1H), 7.28 (m, 1H), 6.19 (bs, 1H), 4.20 (m, 2H), 2.58 (bs, 1H), 1.81 (m, 1H), 1.66 (m, 1H), 1.30 (m, 1H), 0.90 (m, 1H), 0.01 (s, 9H). $^{13}$C NMR (300 MHz, CDCl$_3$): $\delta$157.4, 144.7, 127.0, 123.9, 123.3, 63.5, 33.5, 20.7, 18.0, 17.4, -1.3.

The following example is representative for hydrolysis of the t-butyl carbamates to yield the cyclopropylamines.

trans-2-(4-ethoxyphenyl)cyclopropylamine hydrochloride (2.82): The N-protected carbamate (0.0398 g, 0.14 mmol) was dissolved in THF (0.5 mL) and 6M HCl (0.3 mL). The reaction for stirred at rt for 25 h until TLC indicated complete consumption of starting material. The reaction mixture was concentrated to dryness and the resulting solid residue was dried in vacuo for 24 h over CaSO$_4$, resulting in a yellow solid in 85% yield (0.031 g). $^1$H NMR (300 MHz, CD$_3$OD): $\delta$7.08 (2H, d, J=8.6 Hz), 6.84 (2H, d J=8.6 Hz), 4.86 (3H, bs), 3.99 (2H, q, J=6.9 Hz), 2.75 (1H, m), 2.24 (1H, m), 2.33 (3H, t, J=6.9 Hz),
1.25 (2H, m). \(^{13}\text{C NMR}\) (75 MHz, CD\(_{3}\)OD): \(\delta\) 158.2, 130.3, 127.5, 114.5, 63.3, 30.6, 20.7, 14.0, 12.2.

\[
\text{trans-2-(4-methoxyphenyl)cyclopropylamine hydrochloride (2.81):} 0.131 \text{ g, 94\%, yellow solid.} \quad 1\text{H NMR (300 MHz, CD\(_{3}\)OD):} \delta 7.06 (2H, d, J=8.4 \text{ Hz}), 6.83 (2H, d, J=8.4 \text{ Hz}), 3.75 (3H, s), 2.71 (1H, m), 2.35 (1H, m), 1.36 (1H, m), 1.22 (1H, m). \quad 13\text{C NMR (75 MHz, CD\(_{3}\)OD):} \delta 160.0, 132.2, 128.5, 115.0, 55.7, 32.3, 22.5, 14.1.
\]

\[
\text{trans-2-(4-isopropoxyphenyl)cyclopropylamine hydrochloride (2.83):} 0.840 \text{ g, 84\%, yellow solid.} \quad 1\text{H NMR (400 MHz, CD\(_{3}\)OD):} \delta 7.04 (2H, d, J=8.6 \text{ Hz}), 6.79 (2H, d, J=8.6 \text{ Hz}), 4.50 (1H, quintet, J=6.0 \text{ Hz}), 3.27 (1H, m), 2.71 (1H, m), 2.31 (1H, m), 1.34 (1H, m), 1.23 (6H, d, J=6.0 \text{ Hz}) 1.19 (1H, m). \quad 13\text{C NMR (100 MHz, CD\(_{3}\)OD):} \delta 157.0, 130.4, 127.5, 116.0, 69.8, 30.6, 21.1, 20.7, 12.2.
\]
trans-2-(4-(trifluoromethyl)phenyl)cyclopropylamine hydrochloride (2.84): 0.044 g, 99%, white solid. $^1$H NMR (300 MHz, CD$_3$OD): $\delta$7.60 (2H, d, J=8.7 Hz), 7.37 (2H, d, J=8.7 Hz), 2.95 (1H, m), 2.50 (1H, m), 1.53 (1H, m), 1.41 (1H, m). $^{13}$C NMR (75 MHz, CD$_3$OD): $\delta$144.7, 128.1, 127.5, 126.5, 32.3, 22.3, 14.4.

\[
\text{trans-2-(4-bromophenyl)cyclopropylamine hydrochloride (2.85): 0.052 g, 99%, white solid. $^1$H NMR (300 MHz, CD$_3$OD): $\delta$7.44 (2H, d, J=8.1 Hz), 7.10 (2H, d, J=8.1 Hz), 2.84 (1H, m), 2.39 (1H, m), 1.46 (1H, m), 1.31 (1H, m). $^{13}$C NMR (75 MHz, CD$_3$OD): $\delta$139.2, 132.7, 129.4, 121.3, 32.0, 22.0, 13.9.}

\[
\text{trans-2-(4-phenoxyphenyl)cyclopropylamine hydrochloride (2.86): 0.025 g, 88%, yellow solid. $^1$H NMR (300 MHz, CD$_3$OD): $\delta$7.32 (2H, m), 7.17 (2H, m), 7.09 (1H, m), 6.93 (4H, m), 4.87 (3H, s), 2.82 (1H, m), 2.39 (1H, m), 1.42 (1H, m), 1.29 (1H, m). $^{13}$C NMR (75 MHz, CD$_3$OD): $\delta$157.5, 156.5, 133.5, 129.7, 127.8, 123.3, 118.8, 118.6, 30.7, 20.8, 12.5.}

\[
\text{BnO NH}_2\cdot\text{HCl}
\]
trans-2-(4-(benzyloxy)phenyl)cyclopropylamine hydrochloride (2.87): 0.028 g, 87%, yellow solid. \textsuperscript{1}H NMR (300 MHz, CD\textsubscript{3}OD): \(\delta\)7.36 (5H, m), 7.09 (2H, d, J=8.7 Hz), 6.93 (2H, d, J=8.7 Hz), 5.05 (2H, s), 4.87 (3H, s), 2.75 (1H, m), 2.32 (1H, m), 1.31 (2H, m). \textsuperscript{13}C NMR (75 MHz, CD\textsubscript{3}OD): \(\delta\)158.0, 137.5, 130.7, 128.3, 127.7, 127.5, 127.3, 115.0, 69.8, 30.6, 20.7, 12.2.

\[
\begin{align*}
&\text{trans-2-(4-(4-tert-butyphenylthio)phenyl)cyclopropylamine hydrochloride (2.89):} \\
&0.019 g, 66\%, \text{ yellow solid.} \textsuperscript{1}H NMR (300 MHz, CD\textsubscript{3}OD): \delta7.21 (8H, m), 4.86 (3H, s), 2.83 (1H, ddd), 2.35 (1H, ddd), 1.31 (11H, m). \textsuperscript{13}C NMR (75 MHz, CD\textsubscript{3}OD): \delta137.4, 125.4, 134.2, 131.5, 130.4, 126.9, 126.6, 126.3, 34.3, 30.5, 29.6, 21.0, 12.7.
\end{align*}
\]

\[
\begin{align*}
&\text{2-(4-(prop-2-ynyloxy)phenyl)cyclopropanamine hydrochloride salt (2.90):} 0.055 g, 93\%, \text{ light yellow solid.} \textsuperscript{1}H NMR (300 MHz, CD\textsubscript{3}OD): \delta7.12 (2H, d, J=6.9 Hz), 4.87 (3H, s), 4.69 (2H, d, J=2.1 Hz), 2.92 (1H, t, J=2.1 Hz), 2.77 (1H, m), 2.36 (1H, m), 1.39 (1H, m), 1.26 (1H, m). \textsuperscript{13}C NMR (75 MHz, CD\textsubscript{3}OD): \delta156.7, 131.1, 127.2, 114.8, 78.4, 75.4, 55.3, 30.4, 20.5, 12.1.
\end{align*}
\]
2-(benzo[d][1,3]dioxol-5-yl)cyclopropanamine hydrochloride salt (2.91): 0.089 g, 70%, yellow solid. $^1$H NMR (300 MHz, CD$_3$OD): δ 6.70 (3H, m), 5.90 (2H, s), 4.86 (3H, s), 2.75 (1H, m), 2.32 (1H, m), 1.37 (1H, m), 1.24 (1H, m). $^{13}$C NMR (75 MHz, CD$_3$OD): δ 148.0, 146.6, 132.1, 119.5, 107.8, 106.5, 101.0, 30.4, 21.0, 12.1.

2-(thiophen-3-yl)cyclopropanamine (2.92): The 2-(trimethylsilyl)ethylcarbamate was resuspended in a solution of tetra-N-butylammonium fluoride (180 mg, 0.7 mmol, 1.25 eq) in THF (0.7 mL). The reaction was brought to 50 °C and stirred for 19 h. The reaction was quenched by dropwise addition of water (1.8 mL) and stirring for 30 min. The mixture was acidified with 1 M HCl (2 mL), washed with dichloromethane (4 X 2 mL), alkanilized with aqueous Na$_2$CO$_3$, extracted with EtOAC (3 X 3 mL), dried over K$_2$CO$_3$ and concentrated in vacuo. The desired free amine was isolated by flash chromatography using 100:1 chloroform:triethylamine as a yellow oil in 26% yield (0.021
g). \textbf{\textit{1H NMR}} (300 MHz, CDCl$_3$): $\delta$ 7.20 (m, 1H), 6.79 (m, 2H), 2.50 (m, 1H), 1.90 (m, 1H), 0.97 (m, 1H), 0.89 (m, 1H).

The following is a general protocol for reductive amination of the benzaldehydes to yield the benzylpropargylamines.

![Diagram]

\textbf{N-benzylprop-$2$-yn-$1$-amine (2.93)}: Propargyl amine (2.2 mmol, 1 eq) was dissolved in methanol (6 mL) and the pH adjusted to 5 using 10% HCl. The reaction flask was cooled to 0°C and benzaldehyde (2.5 mmol, 1.1 eq) was added while stirring. The reaction was allowed to warm to rt over 3 h. Then, sodium cyanoborohydride (3.3 mmol, 1.5 eq) was added and the reaction was stirred at rt for 16 h. The reaction mixture was diluted with water (10 mL) and neutralized with 1 M NaOH (3 mL). Then organic products were extracted with CH$_2$Cl$_2$ (3 X 10 mL), washed with brine (10 mL) and dried over anhydrous MgSO$_4$. The products were concentrated under reduced pressure. The desired N-benzylprop2-yn-1-amine was isolated by flash chromatography using 2:1 hexanes:ethyl acetate as a gold oil in 50% yield (0.341 g). \textbf{\textit{1H NMR}} (300 MHz, CDCl$_3$): $\delta$7.32 (5H, m), 3.66 (2H, s), 3.39 (2H, d, J=2.4 Hz), 2.24 (1H, t, J=2.4 Hz). \textbf{\textit{13C NMR}} (75 MHz, CDCl$_3$): $\delta$138.1, 129.5, 129.3, 128.7, 127.7, 29.2, 73.7, 57.4, 42.1.
**N-(4-isopropoxybenzyl)prop-2-yn-1-amine (2.94):** 0.099 g, 50%, yellow solid. \(^1\)H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta 7.24\) (2H, m), 6.84 (2H, m), 4.52 (1H, sep., \(J=6.0\) Hz), 3.80 (2H, s), 3.41 (2H, d, \(J=2.1\) Hz), 2.50 (1H, t, \(J=2.1\) Hz), 1.32 (6H, d, \(J=6.0\) Hz). \(^{13}\)C NMR (75 MHz, CDCl\textsubscript{3}): \(\delta 157.0, 131.2, 129.6, 115.8, 82.0, 71.4, 69.8, 51.6, 37.1, 22.0\).

**N-(4-phenoxybenzyl)prop-2-yn-1-amine (2.95):** 0.178 g, 34%, yellow solid. \(^1\)H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta 7.30\) (4H, m), 7.09 (1H, m), 6.98 (4H, m), 3.83 (2H, s), 3.41 (2H, d, \(J=2.4\) Hz), 2.50 (1H, t, \(J=2.4\) Hz). \(^{13}\)C NMR (75 MHz, CDCl\textsubscript{3}): \(\delta 157.2, 156.2, 134.2, 129.7, 129.6, 123.0, 118.8, 118.6, 81.9, 51.5, 37.1\).

**2-nitro-N-(2-phenylcyclopropyl)benzenesulfonamide (2.96):** Under argon, tranylcypromine hydrochloride salt (0.104 g, 0.59 mmol, 1 eq) was stirred with triethylamine (0.25 mL, 1.77 mmol, 3 eq) and 2-nitrobenzenesulfonyl chloride (0.137 g, 0.59 mmol, 1 eq) in 3 mL of dry dichloromethane at rt for 23 h. The reaction was
monitored using TLC (98:2:1 CH₂Cl₂:MeOH:NH₄Cl). After completion, the mixture was filtered and the solvent removed under reduced pressure. The crude solid was taken up in EtOAc and washed with 10 % NaHCO₃, 10 % citric acid, and saturated NaCl. The organic products were dried over anhydrous Na₂SO₄, filtered, and evaporated under reduced pressure. The desired 2-nitro-N-(2-phenylcyclopropyl)benzenesulfonamide was isolated by flash chromatography using 100:1 CH₂Cl₂:NH₄Cl as a yellow solid in 88% yield (0.172 g). ¹H NMR (300 MHz, CDCl₃): δ 8.08 (1H, m), 7.83 (1H, m), 7.73 (1H, m), 7.65 (1H, m), 7.21 (3H, m), 7.00 (2H, m), 5.86 (1H, bs), 2.50 (1H, m), 2.26 (1H, m), 1.38 (1H, m), 1.23 (1H, m). ¹³C NMR (75 MHz, CDCl₃): δ 148.4, 139.7, 134.3, 133.0, 132.0, 128.7, 126.7, 126.5, 125.6, 34.5, 24.6, 15.6.

\[ \text{N-methyl-2-nitro-N-(2-phenylcyclopropyl)benzenesulfonamide (2.97)} \]: Under argon, 2-nitro-N-(2-phenylcyclopropyl)benzenesulfonamide (0.1718 g, 0.52 mmol) was stirred with K₂CO₃ (0.071 g, mmol) in 5 mL of anhydrous dimethylformamide. Methyl iodide (0.065 mL, mmol) was added dropwise. The reaction was stirred at rt for 16 h. The solid was filtered and the solvent removed under reduced pressure. The bright golden oil was taken into EtOAc (mL) and washed with NaHCO₃ (3X mL), dried with Na₂SO₄, filtered and the solvent removed. The yield for the dark golden oil was not determined. ¹H
**NMR** (300 MHz, CDCl₃): δ 7.91 (1H, m), 7.69 (1H, m), 7.57 (2H, m), 7.25 (3H, m), 7.05 (2H, m), 3.00 (3H, s), 2.41 (1H, m), 2.33 (1H, m), 1.48 (1H, m), 1.28 (1H, m). ¹³C **NMR** (75 MHz, CDCl₃): δ 148.7, 139.6, 134.2, 131.8, 131.6, 130.6, 128.7, 126.7, 126.4, 124.1, 41.3, 37.4, 25.7, 17.0.

\[ \text{N-thiophenol} \quad \text{K}_2\text{CO}_3 \quad \text{ACN} \quad \text{50°C, 24.5 h} \]

**N-methyl-2-phenylcyclopropanamine (2.98):** N-methyl-2-nitro-N-(2-phenylcyclopropylbenzenesulfonylamide was stirred with K₂CO₃ in 10 mL dry acetonitrile under argon. Thiophenol (0.160 mL, mmol) was added dropwise. The reaction was stirred at 50-55 °C for 24.5 hours. The solid was filtered and the solvent removed under reduced pressure. The efforts to isolated the desired N-methyl-2-phenylcyclopropyramine by flash chromatography were unsuccessful.

\[ \text{N-thiophenol} \quad \text{K}_2\text{CO}_3 \quad \text{THF} \]

**N-methyl-2-phenylcyclopropanamine (2.98):** The PS-thiophenol resin was activated by shaking it (0.34 g) with a 0.5 M solution of dithiothreitol in 95:5 THF:H₂O (1.04 g DTT, 12.8 mL THF, 0.7 mL H₂O) for 1 h. The resin was rinsed with anhydrous THF (2X). N-
methyl-2-nitro-\(N\)-(2-phenylcyclopropylbenzenesulfonamide was dissolved in anhydrous THF (5 mL) and Cs\(_2\)CO\(_3\) (0.27 g, 0.88 mmol) was added. This solution was added to the reaction vessel containing the PS-thiophenol resin and was shaken at medium speed for 18 h. The reaction was filtered and washed twice with THF. The solvent was removed under reduced pressure. The \(^1H\) revealed unreacted starting material. Additional activated PS-thiophenol resin (0.42 g) and Cs\(_2\)CO\(_3\) (0.31 g) was added to the crude product in THF (5 mL), and it was shaken for 49 h at rt. After filtration and rinsing with THF (2X), the solvent was removed under reduced pressure. The desired \(N\)-methyl-2-phenylcyclopropyranamine was purified by flash chromatography using 40:60 hexane:EtOAc as a golden oil in 50% yield over two steps (28 mg). \(^1H\) NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.15 (5H, m), 2.51 (3H, s), 2.31 (1H, m), 1.90 (1H, m), 1.68 (1H, bs), 1.06 (1H, m), 0.96 (1H, m). \(^{13}C\) NMR (75 MHz, CDCl\(_3\)): \(\delta\) 140.2, 128.5, 126.1, 125.7, 43.4, 35.9, 25.0, 17.1.

The following is a general protocol for the Corey-Chaykovsky cyclopropanation mechanistic studies.

The ylide/base mixtures were prepared in batch method with the following conditions: KOT-Bu powder (2.99 g) and Me\(_3\)S(O)I powder (5.89 g) were mixed
thoroughly using a mortar and pestle; the mixture was transferred to an amber vial and stored in a desiccator until use as 3.0 mmol methylide/g. NaH powder (0.545 g) and Me₃S(O)I powder (5.01 g) were mixed thoroughly using a mortar and pestle; the mixture was transferred to an amber vial and stored in a desiccator until use as 4.1 mmol methylide/g.

Under argon, an oven dried round bottom flask was charged with methylide/base reagent (1.2 mmol, 2 eq). In a 20 mL scintillation vial, the α,β-unsaturated ester (0.6 mmol, 1 eq) was dissolved in anhydrous DMSO (6 mL). Where applicable, the Lewis acid (X mmol %) was added to the vial and stirred gently to dissolve. The solution from the vial was added at once using a disposable glass pipet to the round bottom flask. The reaction was stirred for the indicated time at the indicated temperature. The reaction was quenched by addition of saturated NaCl (50 mL) and the organic products extracted with diethyl ether (2 X 30 mL). The combined organic layer was washed with water (2 X 50 mL) and saturated NaCl (50 mL), dried over anhydrous MgSO₄, filtered and concentrated in vacuo. Percent conversion was calculated by crude ¹H NMR using a 300 MHz Varian Mercury spectrometer with CDCl₃ as the solvent, referencing to TMS at 0.0 ppm.
3. Inhibition of FAD-dependent amine oxidase enzymes

3.1 Background information

Since its discovery in 2004, the identification of inhibitors the histone demethylase, LSD1, has been the focus of many research groups [80]. LSD1 activity is essential for mammalian development and cellular processes such as cell proliferation and cancer progression [33]. Small molecule inhibitors of LSD1 may be useful biological probes as well as potential cancer therapeutics. A FAD cofactor is enclosed in the active site of LSD1 within the amine oxidase domain. Polyamine oxidase (PAO) and monoamine oxidases A and B (MAO A and MAO B) are also FAD-dependent amine oxidase proteins and the overall folding topology of LSD1 is very similar to those proteins (Figure 12). Despite the similarity of the overall structure of LSD1, PAO, MAO A and MAO B, there is significant variability in the size and shape of their substrate-binding sites. PAO has a long tunnel suited to the aliphatic chain of polyamines (Figure 13D) [81], while MAOs contain a small hydrophobic cavity that accommodates the aromatic substrates (Figure 13B-C) [82]. On the other hand, the substrate-binding site of LSD1 resembles a large funnel that begins at the FAD and opens wide toward the outside of the enzyme to allow for binding of the large histone substrate (Figure 13A) [40,42].
Figure 12. Comparative structural analysis of amine oxidases revealing similar topology. A. LSD1 amine oxidase domain (PDB 2DW4) B. MAO A (PDB 2BXS). C. MAO B (PDB 2XFU). D. PAO (PDB 1H82).
Figure 13. Active sites (boxed) of FAD (green)-containing amine oxidases. A. LSD1 domain (PDB 2DW4) B. MAO A (PDB 2BXS). C. MAO B (PDB 2XFU). D. PAO (PDB 1H82).
Nonselective MAO inhibitors are capable of inhibiting the demethylation by LSD1 of bulk histones and nucleosomes [57]. In particular, 2-phenylcyclopropylamine (2-PCPA, tranylcypropmine, brand name Parnate®) irreversibly inhibits LSD1 *in vitro* and *in vivo* by forming a covalent adduct with the FAD [58,59]. By analyzing structural and stereoelectronic characteristics of 2-PCPA derivatives, we hoped to identify LSD1 selective inhibitors. As described in Chapter 2, we synthesized new small molecules containing the cyclopropylamine scaffold as potential mechanism-based inhibitors. Here, we describe our expression and purification of LSD1, the enzymatic assay and the kinetics parameters of LSD1, MAO A, and MAO B inhibition by 2-PCPA derivatives. Additionally, we show that small molecule propargylamines based on pargyline, a MAO B selective inhibitor, are only able to inhibit LSD1 at concentrations greater than 5 mM.

### 3.2 Optimization of the overexpression and purification of LSD1

A truncated form of human LSD1, lacking the first 150 amino acids in a pET-151b vector was originally expressed and purified, but only yielded less than 1 mg of protein per liter of *E. coli* culture, similar to yields reported by other groups [40,80]. In order to increase the yield and purity of LSD1, a number of test experiments were attempted.
3.2.1 Expression tests of NΔ122

Forneris and coworkers reported a crystal structure of LSD1 using a truncated form of human LSD1 lacking the first 122 amino acids [41]. We performed a series of expression tests of LSD1_{122-852} in pET-151b vector containing a 6xHis tag under various conditions (Table 14). Several E. coli strains were tested due to specific characteristics; BL21(DE3) Star E coli improve protein expression by stabilizing mRNA transcripts while Rosetta 2, RIL BL21(DE3), RP BL21(DE3), and RIPL BL21(DE3) strains all encode for additional tRNAs. Non-bacterial proteins often contain codons not naturally abundant in bacteria, so these tRNAs can aid in E. coli expression.
Table 14. Expression tests of 6xHis LSD1122-852.

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Although most conditions examined were able to overexpress the protein, the highest expression was achieved with the BL21(DE3) Star cells at 23 °C inducing with 0.5 mM IPTG. However, in all cases, the majority of desired protein was insoluble.

### 3.2.2 Efforts towards refolding insoluble LSD1 from inclusion bodies

Since the majority of LSD1 is insoluble, the denaturation and refolding of the protein was attempted. A typical procedure for isolation of proteins from inclusion bodies entails: (1) isolation of the insoluble protein, (2) resuspension in denaturant and
Denaturants, such as guanidine or urea, are capable of hydrogen bonding to the side chain residues and backbone peptide bonds of the protein; as they interact with the protein surface, the protein unfolds creating more surface area for binding. After refolding, it is hoped that the protein will adopt a tertiary structure that is soluble.

Initially, we attempted to solubilize and unfold the protein present in the cell pellet by using 8 M guanidine. In order to refold, we first immobilized the 6xHis-tagged LSD1 on a chelating sepharose resin charged with Ni$^{2+}$. The resin was washed with decreasing concentrations on guanidine, 10 mM imidazole and subsequently eluted with 500 mM imidazole (Figure 14B). Because LSD1 contains a noncovalently bound FAD cofactor, we performed the refolding in the presence of 0.5 mM FAD. After concentration of the eluted protein, excess FAD was removed using dialysis. Activity assays confirmed that the enzyme was inactive after refolding. Despite succeeding attempts to refold the protein using rapid dilution methods, no success was achieved in refolding active LSD1.
Figure 14. Refolding of insoluble LSD1 protein. A. Schematic for refolding process. B. 10% SDS-PAGE gel visualized with Coomassie stain of refolding process using Ni\textsuperscript{2+}-chelating chromatography.
3.2.3 Final overexpression and purification using a codon-optimized clone

Ultimately, a clone optimized for codon usage in *E. coli* increased LSD1 yield to over 4.5 mg/L. The purity of LSD1 was improved from previous methods [58] by performing Ni²⁺-affinity chromatography, gel filtration chromatography and anion-exchange chromatography using Q-sepharose (Figure 15).
Figure 15. LSD1 optimized purification Akta-FPLC traces (left) and 10% SDS/PAGE gels (right). A. Ni²⁺-affinity chromatography. B. S-200 gel filtration chromatography. C. Q-Sepharose chromatography.
3.3 Inhibition of LSD1

3.3.1 LSD1 activity assay

LSD1 catalyzes oxidation of the C-N bond between the carbon of a methyl group and the epsilon nitrogen of a lysine residue, forming an imine intermediate that is nonenzymatically hydrolyzed to produce formaldehyde and the demethylated lysine (Scheme 11). During cleavage of the C-N bond, electrons are transferred from the substrate to the FAD in the active site LSD1, reducing it to FADH₂, which is then converted to its original form by oxygen, forming H₂O₂ in the process [83]. To detect LSD1 activity, and inhibition, we utilized a horseradish peroxidase (HRP) coupled assay. The HRP converts the produced H₂O₂ to O₂ while reducing amplex red into resorufin, a fluorescent molecule, which can be monitored using a fluorimeter.

Scheme 11. Enzymatic scheme for coupled assay to detect LSD1 activity. Horseradish peroxidase (HRP) converts H₂O₂, a byproduct of LSD1 enzymatic activity, and amplex red into fluorescent resorufin.
3.3.2 Reversibility of inhibition of LSD1 by 2-phenylcyclopropylamines

2-PCPA has long been known to act as an irreversible inhibitor of MAO A and MAO B [55,84]. Schmidt and McCafferty published that 2-PCPA results in similar irreversible inhibition of LSD1 [58]. To confirm that the derivatives of 2-PCPA work in the same manner, we incubated 30 μM LSD1 with 250 μM of compounds 3.1-3.6 and 3.12-3.14 overnight at rt, then diluted each sample 100-fold into a solution of coupling enzyme and substrate to measure activity. For freely reversible inhibitors, this 100-fold dilution will reduce the inhibitor concentration in the assay and will allow for recovery of enzymatic activity [85]. However, no residual LSD1 activity was observed with any of the cyclopropylamines (3.1-3.6) indicating irreversible inhibition. On the other hand, the propargylamines (3.12-3.14) retained similar activity to the DMSO control demonstrating that they are not irreversible inhibitors of LSD1.

3.3.3 Time-dependent inhibition of LSD1

In the presence of a time-dependent irreversible inhibitor, progress curves of enzyme reactions initiated by addition of enzyme to assay solutions containing the substrate and inhibitor will be nonlinear relative to assays without the inhibitor. The resulting progress curves can be fit to eq 1 as described in the experimental section to obtain values for $k_{obs}$, the rate of inactivation at each inhibitor concentration, which are then plotted as a function of inhibitor concentration as described in eq 2 resulting in $k_{inact}$.
and $K_i$ values. Plots of $k_{obs}$ versus inhibitor concentration were nonlinear, as expected for a mechanism-based inhibitor. A representative example of the progress curve and $k_{obs}$ versus concentration of inhibitor for compound 3.6 can be seen in Figure 16. Table 15 and Table 16 summarize the resulting values for $k_{inact}$, $K_i$, and $k_{inact}/K_i$. 
Figure 16. A. Representative progress curve for LSD1 activity in the presence of varying concentrations of compound 3.6. B. Rate of inactivation ($k_{obs}$) of LSD1 by compound 3.6 as a function of inhibitor concentration.
Table 15. Kinetic inactivation parameters for inhibition of LSD1 by 2-arylcyclopropylamines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>$k_{\text{inact}}$ (s$^{-1}$)</th>
<th>$K_i$ (μM)</th>
<th>$k_{\text{inact}}/K_i$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-PCPA</td>
<td><img src="image" alt="Structure" /></td>
<td>0.029 ± 0.004</td>
<td>550 ± 150</td>
<td>53</td>
</tr>
<tr>
<td>3.1</td>
<td><img src="image" alt="Structure" /></td>
<td>0.013 ± 0.001</td>
<td>173 ± 37</td>
<td>75</td>
</tr>
<tr>
<td>3.2</td>
<td><img src="image" alt="Structure" /></td>
<td>0.021 ± 0.007</td>
<td>566 ± 73</td>
<td>37</td>
</tr>
<tr>
<td>3.3</td>
<td><img src="image" alt="Structure" /></td>
<td>0.015 ± 0.008</td>
<td>352 ± 95</td>
<td>43</td>
</tr>
<tr>
<td>3.4</td>
<td><img src="image" alt="Structure" /></td>
<td>N.D.</td>
<td>N.D.</td>
<td>&lt; 10$^a$</td>
</tr>
<tr>
<td>3.5</td>
<td><img src="image" alt="Structure" /></td>
<td>0.020 ± 0.002</td>
<td>456 ± 86</td>
<td>45</td>
</tr>
<tr>
<td>3.6</td>
<td><img src="image" alt="Structure" /></td>
<td>0.019 ± 0.002</td>
<td>296 ± 76</td>
<td>54</td>
</tr>
<tr>
<td>3.7</td>
<td><img src="image" alt="Structure" /></td>
<td>0.018 ± 0.002</td>
<td>296 ± 77</td>
<td>59</td>
</tr>
<tr>
<td>3.8</td>
<td><img src="image" alt="Structure" /></td>
<td>0.025 ± 0.005</td>
<td>760 ± 280</td>
<td>33</td>
</tr>
<tr>
<td>3.9</td>
<td><img src="image" alt="Structure" /></td>
<td>0.022 ± 0.003</td>
<td>540 ± 160</td>
<td>40</td>
</tr>
<tr>
<td>3.10</td>
<td><img src="image" alt="Structure" /></td>
<td>0.033 ± 0.003</td>
<td>550 ± 110</td>
<td>60</td>
</tr>
<tr>
<td>3.11</td>
<td><img src="image" alt="Structure" /></td>
<td>0.011 ± 0.003</td>
<td>421 ± 76</td>
<td>26</td>
</tr>
</tbody>
</table>

$^a$Upper limit for $k_{\text{inact}}/K_i$ estimated from average $k_{\text{obs}}$ for lowest concentration showing any inhibitory activity.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>( k_{\text{inact}} ) (s(^{-1}))</th>
<th>( K_i ) (( \mu )M)</th>
<th>( k_{\text{inact}}/K_i ) (M(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>pargyline</td>
<td><img src="image" alt="Structure" /></td>
<td>N.D.</td>
<td>N.D.</td>
<td>&lt; 0.2(^a)</td>
</tr>
<tr>
<td>3.12</td>
<td><img src="image" alt="Structure" /></td>
<td>N.D.</td>
<td>N.D.</td>
<td>&lt; 0.6(^a)</td>
</tr>
<tr>
<td>3.13</td>
<td><img src="image" alt="Structure" /></td>
<td>N.D.</td>
<td>N.D.</td>
<td>&lt; 0.8(^a)</td>
</tr>
<tr>
<td>3.14</td>
<td><img src="image" alt="Structure" /></td>
<td>N.D.</td>
<td>N.D.</td>
<td>&lt; 0.3(^a)</td>
</tr>
</tbody>
</table>

\(^a\)Upper limit for \( k_{\text{inact}}/K_i \) estimated from average \( k_{\text{obs}} \) for lowest concentration showing any inhibitory activity.

Initially, we examined 2-PCPA and compounds 3.1-3.4. From these, we determined that the \( \text{para} \)-methoxy-derivative (3.1) was the most potent inhibitor. This increased activity against LSD1 may result from either the electron-donating capability of the oxygen or to a potential hydrogen bond in the active site of LSD1. In order to take advantage of this feature, we designed inhibitors that contained a similar \( \text{para} \)-substituted oxygen or sulfur moiety (3.5-3.10). Surprisingly, none of these compounds had increased inhibition of LSD1 as compared to 3.1. Generally, the as the bulk increased in the \( \text{para} \)-position, the \( K_i \) also increased; this may be due to steric hindrance in active site only tolerating smaller groups in the \( \text{para} \)-position. The \( N \)-methylated 2-PCPA (3.11)
resulted in a higher $K_i$ than the unmethylated 2-PCPA. However, it afforded a lower $k_{inact}$ which could be contributed to the change in oxidation potential.

On the other hand, none of the propargylamines (pargyline or compounds 3.12-3.14) were effective inhibitors of LSD1 *in vitro*. We observed some inhibition at concentrations higher than 5 mM. Thus, we conclude that small molecule suicide inhibitors containing arylcyclopropylamines are generally better inhibitors than their propargylamine counterparts.

### 3.4 Inhibition of MAO A and MAO B

In order to examine the selectivity of these compounds, we tested their potency against MAO A and MAO B. The time-dependent inhibition assay described above was used with tyramine as the substrate for both enzymes. Once again, a representative example of the progress curve and $k_{obs}$ versus concentration of inhibitor for compound 3.6 can be seen in Figure 17. The resulting values for $k_{inact}$, $K_i$, and $k_{inact}/K_i$ are given in Table 17. With the exception of compound 3.4, all of the compounds tested showed increased inhibitory potential towards MAO A relative to 2-PCPA. Additionally, compounds 3.2 and 3.3 increased potency against MAO B. This increased activity against MAO A could be because the larger active site cavity as compared with MAO B.
Figure 17. A. Representative progress curve for MAO B activity in the presence of varying concentrations of compound 3.6. B. Rate of inactivation ($k_{obs}$) of MAO B by compound 3.6 as a function of inhibitor concentration. C. Representative progress curve for MAO A activity in the presence of varying concentrations of compound 3.6. B. Rate of inactivation ($k_{obs}$) of MAO A by compound 3.6 as a function of inhibitor concentration.
Table 17. Kinetic inactivation parameters for inhibition of MAOA and MAO B.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th></th>
<th>MAO A</th>
<th></th>
<th>MAO B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$k_{\text{inact}}$ (s$^{-1}$)</td>
<td>$K_i$ (μM)</td>
<td>$k_{\text{inact}}/K_i$ (M$^{-1}$ s$^{-1}$)</td>
<td>$k_{\text{inact}}$ (s$^{-1}$)</td>
<td>$K_i$ (μM)</td>
</tr>
<tr>
<td>2-PCPA</td>
<td><img src="image" alt="Structure" /></td>
<td>0.029 ± 0.004</td>
<td>37.3 ± 8.4</td>
<td>469</td>
<td>0.024 ± 0.002</td>
<td>13.6 ± 3.0</td>
</tr>
<tr>
<td>3.1</td>
<td><img src="image" alt="Structure" /></td>
<td>0.023 ± 0.001</td>
<td>20.5 ± 3.0</td>
<td>1112</td>
<td>0.070 ± 0.002</td>
<td>86.6 ± 7.8</td>
</tr>
<tr>
<td>3.2</td>
<td><img src="image" alt="Structure" /></td>
<td>0.070 ± 0.005</td>
<td>36.5 ± 6.2</td>
<td>1918</td>
<td>0.078 ± 0.005</td>
<td>32.9 ± 9.1</td>
</tr>
<tr>
<td>3.3</td>
<td><img src="image" alt="Structure" /></td>
<td>0.085 ± 0.004</td>
<td>29.8 ± 3.3</td>
<td>2859</td>
<td>0.058 ± 0.002</td>
<td>7.7 ± 1.4</td>
</tr>
<tr>
<td>3.4</td>
<td><img src="image" alt="Structure" /></td>
<td>ND</td>
<td>ND</td>
<td>&lt;18$^a$</td>
<td>0.035 ± 0.005</td>
<td>1548 ± 312</td>
</tr>
<tr>
<td>3.5</td>
<td><img src="image" alt="Structure" /></td>
<td>0.014 ± 0.001</td>
<td>4.3 ± 0.8</td>
<td>3140</td>
<td>0.048 ± 0.004</td>
<td>63 ± 10</td>
</tr>
<tr>
<td>3.6</td>
<td><img src="image" alt="Structure" /></td>
<td>0.026 ± 0.002</td>
<td>19.7 ± 4.6</td>
<td>1319</td>
<td>0.046 ± 0.006</td>
<td>106 ± 20</td>
</tr>
<tr>
<td>3.7</td>
<td><img src="image" alt="Structure" /></td>
<td>0.071 ± 0.007</td>
<td>12.4 ± 4.3</td>
<td>5725</td>
<td>0.032 ± 0.002</td>
<td>29.5 ± 4.4</td>
</tr>
</tbody>
</table>

$^a$Upper limit for $k_{\text{inact}}/K_i$ estimated from average $k_{\text{obs}}$ for lowest concentration showing any inhibitory activity.
3.5 Conclusions and future directions

3.5.1. Selectivity issues and other inhibitors

We have shown that derivatives of 2-PCPA are mechanism-based inactivators of LSD1 in vitro. However, we have not identified a selective inhibitor of LSD1, as all of these compounds are also capable of inhibiting MAOs. We believe that developing larger molecules may allow them to inhibit LSD1 but not fit into the active sites of the MAOs. These results indicate that the active sites of the latter enzymes are more flexible than we initially presumed. It may be prudent to mimic the peptide tail of a histone by using a flexible linker or amide bond on the phenyl ring. Additionally, different substitutions on the phenyl ring (ortho or meta) may lead to better inhibitors. Within the last year, two other groups have seen some success with different 2-PCPA derivatives (Figure 18) [86,87]. The first compound increased potency against LSD1 but was not tested against MAOs; the second compound increased selectivity for LSD1 over MAO A and MAO B.

![Figure 18. Other derivatives of 2-PCPA that inhibit LSD1.](image-url)
3.5.2 Lysine specific demethylase 2 (LSD2)

During the course of our research, Zhou et al. reported an evolutionary homologous protein to LSD1 [88]. LSD2, also known as AOF1 and KDM1B, has high identity to the C-terminal amine oxidase and SWIRM domains of LSD1 (Figure 19). Recent enzymatic studies of LSD2 reported substrate recognition almost identical to LSD1 when tested against derivatized peptides [89]. However, LSD2 has not yet been found associated with LSD1 and is presumed to participate in distinct chromatin-remodeling complexes. 2-PCPA is capable of inhibiting LSD2 by modification of the FAD absorption spectrum consistent with the covalent adduct with LSD1 [89]. As a result, LSD1-specific inhibitors must not only be inactive against MAOs but also LSD2; therefore, we have begun efforts to express and purify LSD2 to include in any future selectivity studies.
Figure 19. Domain structures of LSD1 (top) and LSD2 (bottom). Both contain amine oxidase domains (blue) and SWIRM domains (green). LSD1 has a tower domain (purple) while LSD2 has a Zn-CW domain.
Initially, we used SWISS-MODEL [90-92] to predict the structure of LSD2.

Expectedly, the structure prediction model shows the absence of a tower domain and shows similarity within the SWIRM and amine oxidase domains (Figure 20). The first 250 amino acids of LSD2 align well to the solution structure of a zinc finger CW motif [93]. Zinc fingers are present in a number of transcription factors, including histone modification enzymes and are most commonly known for their interactions with DNA and RNA [93].
Figure 20. SWISS-MODEL prediction of LSD2 structure. Alignment with LSD1 (left) and Zn-CW domain (right). Prediction is green while templates are grey.
Karytinos et al. reported initial enzymatic characterization of LSD2 using a construct derived from the mouse AOF1 gene; they saw increased solubility and stability of expressed protein when using an N-terminal truncated (Δ25) construct [89]. We obtained the full length human LSD2 E.coli codon optimized clone in a pET15b vector from Genscript, and we set out to generate two truncation clones (Δ25 and Δ257). The N-terminal truncated (Δ257) construct would allow us to determine if the Zn-CW domain is important for demethylation. First, we amplified the sequences using PCR. We used a Zero-blunt TOPO® vector as a shuttle prior to ligation with T7 ligase (Figure 21). Amplification was confirmed by restriction digestion and sequencing through the Duke Sequencing Facility. We confirmed successful cloning of the NΔ257 clone, however the desired NΔ25 was not produced after ligation.
Figure 21. Cloning of LSD2 truncation products. A. NΔ25 PCR products, TOPO clone and restriction digest by NdeI and XhoI. B. NΔ257 PCR products, TOPO clone and restriction digest by NdeI and XhoI.
The expression of LSD2 and LSD2 NΔ257 was tested using the same protocol as successful LSD1 expression. The clones were transformed into BL21(DE3) Star cells, grown at 23 °C in TB media and induced using 500 mM IPTG. Protein expression was visualized by SDS-PAGE (Figure 22). Overexpression of the LSD2 NΔ257 was successful but expression of full length LSD2 was not achieved under these conditions. Ni²⁺ affinity chromatography was performed as an initial purification step of LSD2 NΔ257 (Figure 23). Following concentration absorbance at 452 nm was not observed, indicating no FAD present in the protein. A crude activity assay confirmed the protein was not active.

Although LSD2 NΔ257 overexpressed, it did not provide active protein. Further expression of full length LSD2 should be optimized and cloning of LSD2 NΔ25 should be continued for further studies.
Figure 22. 10% SDS-PAGE gel visualizing expression tests of LSD2 and LSD2 NΔ257 in BL21(DE3) Star cells at 23 °C in TB media before and after induction with 500 mM IPTG.
Figure 23. Purification of LSD2 NΔ257. Akta FLPC trace (left) and 10% SDS-PAGE gel visualizing fractions 17, 19, 21, 23, 25, 37 and 48 (right).
3.5.3 Future directions

Here, we have shown that a selective inhibitor of LSD1 may not be feasible if it targets the FAD because of the potential of inhibiting the entire class of FAD-dependent amine oxidases, or at least the homologous LSD2 protein. However, these compounds can act as probes of LSD1 function in cellular environments when knockdown experiments are performed simultaneously (see Chapter 4). Additionally, we believe that selective inhibition of LSD1 could potentially arise from targeting the protein-protein interactions essential for the enzymatic function; this method would be ideal because it could be targeted to the transcriptional complexes that include LSD1 and are implicated in cancer proliferation (see Chapter 5).

3.6 Experimental section

General considerations: Horseradish peroxidase, Ampiflu red, tyramine and all other reagents and buffers were purchased from Sigma. MAO A (150 units/mg) and MAO B (45 units/mg) were obtained as membrane preparations from Sigma and used without further purification. Fmoc amino acids and Rink resin were obtained from Novabiochem, Applied Biosystems, or Advanced ChemTech.

Peptide substrate synthesis: A peptide corresponding to the first 21 amino acids of human histone H3 with a dimethylated lysine at the fourth residue
(ARTK(diMe)QTARKSTGGKAPRKQLA) was synthesized via classical Fmoc-peptide chemistry using an Applied Biosystems 433 peptide synthesizer or a CEM Liberty microwave peptide synthesizer with Rink resin which yields a C-terminal amide group. The residues GYG were added to the C-terminal end of the peptide to aid in quantification of the peptide concentration using tyrosine’s extinction coefficient of 1440 cm$^{-1}$M$^{-1}$ at 280 nm. The peptides were cleaved from the resin with trifluoroacetic acid for 2 h, precipitated with ice-cold diethyl ether, suspended in water, and lyophilized. Crude peptide was purified on a C$_{18}$ reversed-phase semi-prep column (Phenomenex) using a Thermo or Agilent HPLC and a linear gradient of water to either acetonitrile or methanol, each of which contained 0.1% TFA. Absorbance was monitored at 214 nm and 280 nm. Peptide mass was verified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Applied Biosystems).

**LSD1 expression and purification:** Human LSD1, lacking the first 150 amino acids was expressed and purified as previously described with minor modifications [94]. Codon optimized LSD1 in pET15b (Invitrogen) was obtained from Genscript and transformed into BL21 Star (DE3) *E. coli* cells (Invitrogen); cells were grown at 23 °C in TB media, containing 100 μg/ml ampicillin, to an OD$_{600}$ of 0.6 before induction with 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were induced overnight and harvested centrifugation. Cells were resuspended in 50 mM sodium phosphate, pH 7.6,
containing 350 mM NaCl, 0.4 mM phenylmethysulphonyl fluoride (PMSF) and 5% glycerol prior to lysis with an EmulsiFlex-C5 homogenizer (Avestin). After centrifugation at 35000 x g for 40 minutes to remove cell debris, protein, which contained an N-terminal histidine tag, was purified via nickel affinity chromatography [Chelating Sepharose FF (Amersham) charged with nickel sulfate], using a gradient of 10-175 mM imidazole in 50 mM sodium phosphate, pH 7.4, with 5% glycerol followed by gel filtration chromatography (Sephacryl S-200, GE Life Sciences) in 50 mM sodium phosphate, pH 7.4 with 5% glycerol and finally, anion exchange chromatography (Q Sepharose Fast-Flow, GE Life Sciences), using a gradient of 0-1 M NaCl in 50 mM sodium phosphate, pH 7.4 with 5% glycerol. Purification was performed on an AKTA FPLC. The protein was concentrated using a Centricon-10 centrifugal concentrator (Millipore) and stored at -20 °C in 50 mM sodium phosphate, pH 7.4, containing 40% glycerol. The concentration of LSD1 was determined spectrophotometrically using the extinction coefficient of FAD (11600 cm⁻¹M⁻¹) at 458 nm.

Assays for LSD1 and MAO time dependent inactivation inhibition: Assays were performed as previously published[58,95]. Briefly, peroxide productive by LSD1 or MAO was monitored using a HRP-coupled assay in 50 mM Tris, pH 7.85, containing 0.01% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Amplex red (50 μM) was used as the fluorogenic electron acceptor. Final concentrations of 1 and 2
units/mL HRP were used to assay LSD1 and MAO, respectively. Fluorescence assay was performed in a final volume of 60 μL in a 96-well white plate format and monitored with 560 nm excitation and 590 nm emission in a Molecular Devices SpectraMax Gemini EM plate reader. The linear range for each enzyme was determined, resulting in the use of final concentrations of 0.3 μM LSD1, 7.8 units/mL MAO A, and 2.8 units/mL MAO B. For assessment of inhibition of LSD1, the $K_{m}^{pp}$ for the dimethylated peptide substrate was first assayed and found to be 9.8 μM. The use of $K_{m}^{pp}$ reflects the conversion of dimethylated substrate to unmethylated product via two demethylation events. These substrate concentrations were then used in all subsequent inhibition assays. Similarly, the $K_m$ values of MAO A and MAO B for tyramine were found to be 130 μM and 180 μM, respectively, and these concentrations were used in subsequent inhibition assays as well.

Inhibitors were prepared at 100 mM stocks in DMSO and stored at aliquots at -20 °C. In control experiments, DMSO was found to have no effect on activity at concentrations up to 10% and as a result, assays were performed with 3% DMSO to insure solubility of the inhibitors. To determine the rates of inactivation of LSD1 or MAO by the compounds, reactions were initiated via addition of enzyme to assay solutions containing substrate and varying concentrations of inhibitor. The resulting progress curves were fit directly to eq 1 describing time-dependent inactivation [85] using Grafit 4.0 software package (Erithacus Software):

$$\text{product} = \left( \frac{v}{k_{\text{obs}}} \right) \left( 1 - \exp^{-k_{\text{obs}}t} \right)$$  (1)
where \( v_i \) is the initial rate prior to inactivation, \( t \) is time, and \( k_{\text{obs}} \) is the observed rate of inactivation. The resulting values of \( k_{\text{obs}} \) were plotted as a function of inhibitor concentration to obtain values of \( K_i \) and \( k_{\text{inact}} \) according to eq 2 in GraFit [85]:

\[
k_{\text{obs}} = \frac{k_{\text{inact}}[I]}{K_i + [I]}
\]

where \( k_{\text{inact}} \) is the maximal rate of inactivation and \( K_i \) is the inhibitor concentration that yields half that rate of inactivation.

Assays for reversibility of LSD1 inhibition: To test for reversibility of inhibition of LSD1, 25 \( \mu \)M enzyme was incubated with 250 \( \mu \)M or 3% DMSO as a positive control at 23 °C overnight. An aliquot (7.6 \( \mu \)L) was used to initiate a 60 \( \mu \)L reaction containing peptide substrate and coupling reagents. The residual enzymatic activity was assayed as described above.

Expression tests of LSD1 NΔ122: Human LSD1, lacking the first 122 amino acids, in pET151 vector, was transformed into various \( E. \ coli \) cells. 100 mL cultures were grown under the indicated conditions. The expression of LSD1 was determined by visualization of 1 mL aliquots of uninduced and induced cells by SDS-PAGE and Coomassie staining.

Purification of LSD1 from inclusion bodies: Human LSD1, lacking the first 122 amino acids, in pET151 vector, was transformed into BL21(DE3) Star \( E. \ coli \) cells. Cells were grown at
23 °C in LB media, containing 100 μg/mL ampicillin, to an OD600 of 0.6 before induction with 500 μM IPTG. Cells were induced overnight and were collected by centrifugation, resuspended in lysis buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 0.1% NaN3, 0.5% Triton X-100, 0.1 mM PMSF, 1 mM DTT), and lysed by sonication. After centrifugation, the pellet was washed 3X with lysis buffer supplemented with 10 mM MgSO4 and 50 mg lysozyme. The pellet was resuspended in unfolding buffer (50 mM sodium phosphate, pH 7.8, 50 mM glycine, 8 mM guanidine) and stirred for 22 h at 4 °C. To refold on the Ni2+-affinity column, the unfolded protein in 8 M guanidine was loaded onto 3 mL of chelating Sepharose resin charged with NiSO4. The resin was washed with 5 mL each of decreasing unfolding buffer (8 M – 0 M guanidine) and increasing refolding buffer (50 mM sodium phosphate, pH 7.8, 0.4 M L-Arg, 300 mM NaCl, 500 μM FAD). The protein was washed with wash buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 10 mM imidazole) and then eluted with elution buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 500 mM imidazole). The protein was visualized by SDS-PAGE and Coomassie staining. For the rapid dilution method, 2 mL of unfolded protein was added dropwise to a stirring refolding buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 0.4 M L-Arg, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 1 μg/mL leupeptin and pepstatin, 0.2 mM PMSF, 500 μM FAD) at 4 °C. It was stirred for 30 min, clarified by centrifugation and concentrated using a Centricon-10 centrifugal concentrator (Millipore).
Cloning of LSD2 constructs: PCR amplified constructs were inserted into ZeroBlunt TOPO vector (Invitrogen) and confirmed by DNA sequencing (Integrated DNA Technologies). They were digested with XhoI and NdeI and ligated overnight at 16 °C with cut and dephosphorylated pET15b empty vector. The primer sequences for PCR are seen in Table 18.

**Table 18. Primer sequences for PCR of LSD2 constructs.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOF1_Nd25_F</td>
<td>TATACATATGGGCCTAGCGCAAAAGC</td>
</tr>
<tr>
<td>AOF1_Nd257_F</td>
<td>TATACATATGAGCGATGCGCATGTGCCGG</td>
</tr>
<tr>
<td>AOF1_R</td>
<td>TATACTCGAGTTAAAAACGCCGCAATTTTG</td>
</tr>
</tbody>
</table>

LSD2 NΔ257 expression and purification: Human LSD2, lacking the first 257 amino acids in a pET15b vector was transformed into BL21(DE3) Star cells; cells were grown at 23 °C in TB media, containing 100 μg/mL ampicillin, to an OD₆₀₀ of 0.6 before induction with 500 μM IPTG. Cells were induced overnight, collected by centrifugation, resuspended in lysis buffer (50 mM sodium phosphate, pH 7.8, containing 300 mM NaCl, 10 mM imidazole, 40% glycerol and 0.4 mM PMSF) and lysed with an EmulsiFlex-C5 homogenizer (Avestin). After centrifugation at 35000 x g for 40 minutes to remove cell debris, protein, which contained an N-terminal histidine tag, was purified via a nickel affinity chromatography [Chelating Sepharose FF (Amersham) charged with nickel sulfate], using a gradient of 10-500 mM imidazole in 50 mM sodium phosphate, pH 7.8.
4. Evaluation of small molecule inhibitors as probes of LSD1 function in breast cancer cells

4.1 Background information

4.1.1 Breast cancer

In 2011, it is predicted that there will be over 230,000 new cases of breast cancer in the United States, resulting in an estimated 40,000 deaths [96]. Of the ten leading cancers afflicting women, breast cancer is the most diagnosed and the second highest cause of death in the US (Figure 24). This disease is inherently heterogeneous, with several distinct subtypes that vary in clinical behavior and therapeutic implications.
Figure 24. Ten leading female cancer types estimated for 2011 in the United States. A. New cancer diagnoses. B. Cancers causing casualties.
4.1.2 Estrogen Receptor-positive breast cancer

The estrogen receptor α (ERα, Figure 25) is a ligand-inducible transcription factor that belongs to the nuclear receptor super family and has been the major target of breast cancer therapeutics [97].

![Figure 25. Estrogen Receptor α domain structure.](image)

The inspiration of developing therapeutic antagonists to estrogen action in order to combat breast cancer dates back to before the discovery of ER to 1936 when Antoine Lacassagne observed overstimulating estrogen in mice resulted in breast cancer [98]. From there, tamoxifen and raloxifene (Figure 26) were among the first antiestrogens identified. Today, they are more commonly known as selective estrogen receptor modulators (SERMs) and both are used clinically [97]. Crystallization studies of the ligand-binding domain of ERα with estradiol and raloxifene have shown that conformational changes occur in the receptor when bound to agonist and antagonist, respectively [99]. When a SERM such as raloxifen binds to ERα, it prevents the receptor from closing around the native ligand of estradiol and therefore neutralizes the estrogenic
properties. A large number of coactivator and corepressor proteins participate in the construction of a transcriptional complex containing ERα and understanding these proteins will reveal partners that are essential for ERα regulation (Figure 27) [100].

Figure 26. Structures of SERMs, tamoxifen (left) and raloxifene (right).
Figure 27. ERα function as a transcriptional complex.
4.1.3 Estrogen Receptor-negative breast cancer

Despite the successes in treating ERα-positive breast cancers, up to one-third of breast carcinomas lack ERα at the time of diagnosis and a fraction of cancers that are initially ERα positive lose ERα during tumor progression. Binding of tamoxifen to ERα is critical for its antiestrogenic effect in tumors, and indeed, tamoxifen treatment of patients with ER-negative tumors does not reduce recurrence rates or death rates [101]. Triple negative breast cancers, malignancies that are ERα, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) negative, lack established therapeutic targets and cytotoxic chemotherapy is currently the only treatment option [102]. Given the limited therapeutic options for ER-negative cancers and the poor outcome associated with this phenotype, the development of novel therapies specifically targeted to genes and proteins actively engaged in driving the underlying pathophysiology of the disease are desperately needed, irrespective of ER.

4.1.4 Epigenetics as a target for cancer therapy

The complexity of gene regulation can be attributed not only to primary transcription factors such as ER but also to the large number of co-regulatory complexes that bind to and manipulate the chromatin landscape. The core histones, subject to many post-translational modifications including methylation, acetylation, phosphorylation, and ubiquitination, supply binding sites for numerous transcription factors. These modifications, collectively known as the ‘histone code,’ are present in different
combinations and have a powerful effect on chromatin structure allowing the cell to spatially direct transcriptional activity [12,103]. Understanding the role(s) of the enzymes involved in establishing and maintaining the histone code is an essential step in defining the role of epigenetic gene regulation in disease pathology and will be instructive with respect to new drug discovery.

The likely impact of this class of targets has been highlighted by the success of inhibitors of histone deacetylases. Currently, Vorinostat (SAHA, Zolinza™) is FDA-approved for the treatment of cutaneous T-cell lymphoma [104]. Vorinostat is a nonselective HDAC inhibitor that increases global acetylation of histones H2B, H3 and H4. The changes in histone acetylation result in increased expression of p21 and Bax and decreased expression of STAT6, ultimately leading to apoptosis [104]. Histone deacetylation has been linked to ER silencing in aggressive breast cancers. Interestingly, the HDAC inhibitor, Trichostatin A (TSA) is capable of reactivating expression of ER and may open a new avenue for therapy of hormonal resistant breast cancer [105].

Methylation of lysine residues within chromatin leads to dichotomous responses, in that depending on the specific histone modified and the extent to which modification occurs (mono, di, tri-methylation) transcriptional activity can be enhanced or decreased [106,107]. Histone methylation is catalyzed by histone methyltransferases (HMTs) and reversed by histone lysine-specific demethylases (LSD1/KDM1 and LSD2) and JmjC domain-containing histone demethylases [21,108,109]. LSD1 activity is essential for
mammalian development and implicated in many important cellular processes [33]. By associating with various transcription complexes containing the androgen receptor, estrogen receptor and corepressors, LSD1 impacts transcription by demethylating histone H3 lysine 4 (H3K4) and/or lysine 9 (H3K9) and non-histone substrates such as p53 and DMNT1 [25,28,35,37,110,111]. Furthermore, the high expression of LSD1 in breast cancer, coupled with known roles of the enzyme in transcriptional activation, has heightened interest in LSD1 as a potential therapeutic target for breast cancer [38].

4.2 Small molecules as probes of LSD1 function in breast cancer.

LSD1 has been shown to be overexpressed in some breast cancers and may function as a biomarker of the aggressiveness of the disease [38]. Because LSD1 is a FAD-dependent amine oxidase and a variety of MAO inhibitors have been used to probe LSD1 function in cells, we were motivated to explore the consequence of LSD1 inhibition with small molecules that could be evaluated at concentrations lower than 1 mM. Previously, we have shown that the concentration of propargylamines (pargyline and clorgyline) necessary to inhibit the specific demethylation of histone substrates in vitro are not likely to be achievable in vivo or in cell culture [44]. However, there are several recent examples in the literature where propargylamines are used at very high
concentrations to probe LSD1 function in a variety of cellular environments [25,112,113]. As off-target actions of these compounds are likely at concentrations above 1 mM, we set out to use our 2-phenylcyclopropylamine LSD1 inhibitors (2-PCPA and compounds 4.1-4.3, Table 19) at lower concentrations in order to take a first step in defining the utility of this enzyme as a cancer therapeutic. Additionally, we used pargyline and two derivatives (pargyline and compounds 4.4-4.5, Table 19) to investigate potential off-target effects.

Table 19. Structures of 2-phenylcyclopropylamines and propargylamines used in breast cancer studies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-PCPA</td>
<td><img src="image1" alt="2-PCPA Structure" /></td>
</tr>
<tr>
<td>4.1</td>
<td><img src="image2" alt="4.1 Structure" /></td>
</tr>
<tr>
<td>4.2</td>
<td><img src="image3" alt="4.2 Structure" /></td>
</tr>
<tr>
<td>4.3</td>
<td><img src="image4" alt="4.3 Structure" /></td>
</tr>
<tr>
<td>pargyline</td>
<td><img src="image5" alt="Pargyline Structure" /></td>
</tr>
<tr>
<td>4.4</td>
<td><img src="image6" alt="4.4 Structure" /></td>
</tr>
<tr>
<td>4.5</td>
<td><img src="image7" alt="4.5 Structure" /></td>
</tr>
</tbody>
</table>
4.3 LSD1 expression and importance in breast cancer cells and tumors

We first investigated the expression levels of all of the FAD-dependent amine oxidases in breast cancer to determine which members of this class of enzymes may be useful targets and to define the best model system(s) to study LSD1 action. To this end, the relative expression levels of nine different FAD-dependent amine oxidases were assessed in published array data derived from a breast cancer tumor dataset of 347 primary invasive breast tumors (GSE4922 combining both U133A with the U133B chips [114]). The data obtained in this manner are presented as a heatmap (Figure 28) and show that LSD1 and LSD2 are steadily the most highly expressed amine oxidases across all tumors. A similar analysis was performed in a panel of 51 established cellular models of breast cancer (dataset GSE12777 [115]). As observed in tumors, LSD1 and LSD2 were consistently expressed at much higher levels than the other FAD-dependent amine oxidases (Figure 29). The high expression levels of LSD1 and LSD2 across all types of breast cancer suggest that, if proven effective, inhibitors of these enzymes may be useful in the treatment of both ER-positive and ER-negative breast cancers. Most significant was the observation that LSD1 was highly expressed in cellular models of the difficult to treat triple negative breast cancers.
(MDA-MB-231, HCC1143, and HCC1937 cells). Experimentally, we examined the mRNA levels of LSD1 in ten breast cancer cell lines (four ERα-positive and six ERα-negative); overall the expression levels were fairly consistent with the highest expression in MDA-MB-648 cells (Figure 30). These expression data indicate that LSD1 is likely to be a useful therapeutic target, and considering expression alone, significant off-target activities on the homologous protein, LSD2, may be observed.
Figure 28. Expression levels of FAD-dependent amine oxidases in breast cancer tumors as evaluated by mRNA levels. Red indicates high expression and blue indicates low expression.
Figure 29. Expression of FAD-dependent amine oxidases in 51 breast cancer cell lines as evaluated by mRNA levels. Red indicates high expression and blue indicates low expression.
Figure 30. LSD1 mRNA levels in a panel of breast cancer cell lines as analyzed by qRT-PCR. All cells are normalized relative to MCF7 cells.
The roles of LSD1 and LSD2 in the proliferation of ERα-positive and triple negative breast cancer cells was assessed following knockdown of their expression using small interfering RNAs (siRNAs). Using this approach, we were able to accomplish a quantitative knockdown of LSD1 and LSD2 in both MCF7 and MDA-MB-231 cells using two distinct siRNAs (Figure 31A-B and Figure 33A, respectively). This correlated to decreased levels of LSD1 protein as visualized by Western blot analysis (Figure 32). Knockdown of LSD1 dramatically inhibited proliferation of both MCF7 and MDA-MB-231 cells (Figure 31C-D). This was primarily a cytostatic activity as no signs of cell death or apoptosis were observed at any point during the 10 day experiment. Conversely, knockdown of LSD2 expression using the same approach had no effect on proliferation (Figure 33B-C). These data demonstrate that LSD1, but not LSD2, is required for proliferation in these cell models; a result that highlights the utility of targeting this enzyme in breast cancer.
Figure 31. Results after knockdown of LSD1 in breast cancer cells using siRNA. A. mRNA levels of LSD1 in MCF7 cells. B. mRNA levels of LSD1 in MDA-MB-231 cells. C. Proliferation of MCF7 cells. D. Proliferation of MDA-MB-231 cells.
Figure 32. LSD1 protein levels in MCF7 cells after knockdown of LSD1 using siRNA. GAPDH was used as a loading control.
Figure 33. Results after knockdown of LSD2 in breast cancer cells using siRNA. A. mRNA levels of LSD2 in MCF7 cells. B. Proliferation of MCF7 cells. C. Proliferation of MDA-MB-231 cells.
4.4 LSD1 as a coactivator of estrogen-dependent gene transcription

4.4.1 Changes in transcription levels

Previous studies have shown that LSD1 is an essential mediator of the interchromosomal interactions necessary for estrogen (E2)-dependent ERα-mediated transcription [37]. Initially, we set out to elucidate if this mechanism was dependent on the physical presence of the protein as a scaffold or could be expanded to include a requirement for its demethylase enzymatic activity. For these experiments, we chose MCF7 cells, breast cancer cells that are ERα-positive and respond well to estrogen-treatment.

Cell viability experiments using concentrations of 2-PCPA and 4.1-4.3 between 0.2 μM and 1 mM indicated that within 24 h of treatment with the inhibitors, MCF7 cells are viable (Figure 34); therefore, we could examine the mRNA levels during these time periods without the confounding influence of cell death or apoptosis.
Figure 34. MCF7 cell viability experiments after treatment for 24 h with indicated concentrations of 2-phenylcyclopropylamines. A. 2-PCPA. B. Compound 4.1. C. Compound 4.2. D. Compound 4.3. Data is presented as ±SEM for three separate treatments.
In order to confirm the role of LSD1 in E2-induced transcription, ERα-positive MCF7 cells were treated with the inhibitors of LSD1 and compared to the data from siRNA knockdown of LSD1. As seen from the knockdown experiments (Figure 35), lack of LSD1 protein leads to decreased transcription of a number of E2-target genes. Of note are pS2, a marker for hormone-dependent breast cancer, GREB1, a gene important in hormone-responsive cancer, and PR, the gene encoding the progesterone receptor. Others impacted by LSD1 knockdown include MCM2, CatD, WISP2, AMyB, SDF1, and SIAH2. Many of these genes contribute to gene replication, cell proliferation and differentiation, and cancer pathogenesis [116]. By contrast, other ERα target genes including Erbb4, IL1-R1, and Notch3 were not affected. Additionally, we examined three genes not dependent on E2 for transcription, EGR1, SMAD2, and MYC, and found that in all cases, there were not significant changes in mRNA levels after knockdown of LSD1.
Figure 35. RT-qPCR results after knockdown of LSD1 using siRNA. Data is presented as ±SEM for triplicate wells.
Before examining all of the E2-dependent genes described above, the length of
time for treatment with the inhibitors had to be determined. We performed a time
course experiment where we treated the cells for 3 h, 7 h, and 25 h with 250 μM
compound 4.1 before inducing transcription with E2 for 18 h (resulting in 21 h, 25 h, and
43 h of total treatment, respectively). As seen in Figure 36, both pS2 and PR mRNA
levels were decreased at the earliest timepoints maxing out at 25 h. From here, we used
24-25 h total treatment with the inhibitors.
Figure 36. RT-qPCR results after time course experiment of treatment with 250 μM compound 4.1. Data is presented as ±SEM for triplicate wells.
We next examined compounds 4.1-4.3 using the E2-dependent genes as described above during the knockdown of LSD1 experiments. We saw very similar effects on E2-target genes (Figure 37, Figure 38, Figure 39) indicating that it is LSD1 enzymatic activity that is essential for gene expression and not just the presence of the protein. All of the genes investigated and the results observed for knockdown and inhibition studies are summarized in Table 20.
Figure 37. qRT-PCR results after treatment of MCF7 cells with 250 μM compound 4.1 for 24 h. Data is presented as ±SEM for triplicate wells.
Figure 38. qRT-PCR results after treatment of MCF7 cells with 250 μM compound 4.2 for 24 h. Data is presented as ±SEM for triplicate wells.
Figure 39. qRT-PCR results after treatment of MCF7 cells with 250 μM compound 4.3 for 24 h. Data is presented as ±SEM for triplicate wells.
Table 20. Genes investigated by qRT-PCR in this study and representative observations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Name</th>
<th>Encodes</th>
<th>E2 response</th>
<th>Effects of LSD1 knockdown</th>
<th>Effects of LSD1 inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMyb</td>
<td>v-myb myeloblastosis viral oncogene homolog (avian)-like 1</td>
<td>Transcription factor</td>
<td>up</td>
<td>decreased E2-induced transcription</td>
<td>decreased E2-induced transcription</td>
</tr>
<tr>
<td>CatD</td>
<td>cathepsin D</td>
<td>Lysosomal aspartyl protease, implicated in breast cancer pathogenesis</td>
<td>up</td>
<td>decreased E2-induced transcription</td>
<td>decreased E2-induced transcription</td>
</tr>
<tr>
<td>EGR1</td>
<td>early growth factor 1</td>
<td>C2H2-type zinc-finger transcriptional regulator, cancer suppressor</td>
<td>none</td>
<td>some decreased transcription</td>
<td>no change</td>
</tr>
<tr>
<td>ERBB4</td>
<td>v-erb-a erythroblastic leukemia viral oncogene homolog 4</td>
<td>Membrane protein that induces cellular responses such as mitogenesis and differentiation</td>
<td>down</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>GREB1</td>
<td>growth regulation by estrogen in breast cancer 1</td>
<td>Early response gene in ER-regulation, important in cancer</td>
<td>up</td>
<td>decreased E2-induced transcription</td>
<td>decreased E2-induced transcription</td>
</tr>
<tr>
<td>IL1R1</td>
<td>interleukin 1 receptor, type 1</td>
<td>Cytokine receptor that mediates immune and inflammatory responses</td>
<td>down</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>MCM2</td>
<td>minichromosome maintenance complex component 2</td>
<td>Protein involved in initiation of genome replication</td>
<td>up</td>
<td>decreased E2-induced transcription</td>
<td>decreased E2-induced transcription, increased basal transcription levels</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Effect 1</td>
<td>Effect 2</td>
<td>Effect 3</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>MYC</td>
<td>v-myc myelocytomatosis viral oncogene homolog</td>
<td>none</td>
<td>no change</td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td>NOTCH3</td>
<td>notch 3</td>
<td>down</td>
<td>no change</td>
<td>no change</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
<td>up</td>
<td>decreased E2-induced transcription</td>
<td>decreased E2-induced transcription</td>
<td></td>
</tr>
<tr>
<td>pS2</td>
<td>trefoil factor 1</td>
<td>up</td>
<td>decreased E2-induced transcription</td>
<td>decreased E2-induced transcription</td>
<td></td>
</tr>
<tr>
<td>SDF1</td>
<td>chemokine (C-X-C motif) ligand 12</td>
<td>up</td>
<td>decreased E2-induced transcription</td>
<td>decreased E2-induced transcription, increased basal transcription levels</td>
<td></td>
</tr>
<tr>
<td>SIAH2</td>
<td>seven in absentia homolog 2</td>
<td>up</td>
<td>slight decreased E2-induced transcription</td>
<td>slight decreased E2-induced transcription</td>
<td></td>
</tr>
<tr>
<td>Smad2</td>
<td>SMAD family member 2</td>
<td>none</td>
<td>no change</td>
<td>increased basal transcription</td>
<td></td>
</tr>
<tr>
<td>WISP2</td>
<td>WNT1 inducible signaling pathway protein 2</td>
<td>up</td>
<td>decreased E2-induced transcription</td>
<td>decreased E2-induced transcription, increased basal transcription levels</td>
<td></td>
</tr>
</tbody>
</table>
Alternatively, when MCF7 cells were depleted of LSD2 using siRNA, the mRNA levels of estrogen-dependent genes were not changed; two representative examples are *pS2* and *PR* (Figure 40). This data implies that the LSD1 (and not LSD2) is an essential demethylase for transcription of some but not all estrogen-regulated genes in breast cancer.
Figure 40. qRT-PCR results after knockdown of LSD2 in MCF7 cells using siRNA. Data is presented as ±SEM for triplicate wells.
4.4.2 Simultaneous recruitment of LSD1 and ERα to gene promoters

Upon binding estrogen, ERα interacts with specific estrogen response elements (EREs) located within the regulatory regions of target genes where it nucleates the assembly of large protein complexes that influence gene transcription. Thus, we used chromatin immunoprecipitation (ChIP) assays to determine if recruitment to ERα target genes was influenced by the activity or physical presence of LSD1. Using this assay we were able to show that after E2 treatment, we were able to see LSD1 (Figure 41) recruitment to promoters of the pS2 and PR genes; this recruitment is specific to the ERE binding sites and not observed on distal sites of the promoters. This once again suggests cooperation between LSD1 and ERα to initiate transcription on these specific genes.
Figure 41. LSD1 recruitment is specific for ERE binding sites as indicated by chromatin immunoprecipitation. A. *pS2* promoters. B. *PR* promoters. Data is presented as ±SEM for triplicate wells. IgG is used as a negative control.
As expected, ERα is recruited to the ERE binding sites on pS2 and PR. However, when LSD1 is inhibited by compounds 4.1 and 4.3 or knocked down using siRNA, recruitment of ERα to the pS2 ERE decreases (Figure 42) resulting in diminished transcription of the target gene. This phenomenon is also observed at two PR EREs (Figure 43).
Figure 42. Chromatin immunoprecipitation experiments reveal that ERα recruitment is decreased at pS2 promoters when LSD1 is inactive or not present. A. ERα levels at inhibition with compounds 4.1 or 4.3. B. ERα levels at after knockdown using siRNA. Data is presented as ±SEM for triplicate wells. IgG is used as a negative control.
Figure 43. Chromatin immunoprecipitation experiments reveal that ERα recruitment is decreased at PR promoters when LSD1 is inactive or not present. A. ERα levels at inhibition with compounds 4.1 or 4.3. B. ERα levels at after knockdown using siRNA. Data is presented as ±SEM for triplicate wells. IgG is used as a negative control.
4.4.3 Histone methylation levels at estrogen-dependent gene promoters

The data above suggest that LSD1 regulates the DNA binding activity of the ERα-transcription complex and/or modifications to the chromatin at the target ERE are subject to LSD1 modification. In order to investigate the latter hypothesis, we set out to visualize the methylation status at the pS2 and PR EREs. The estrogen-dependent transcriptional regulation of PR by ERα is well established; however, the manner in which the PR gene is regulated in regards to specific binding sites of ERα is not as well understood. Therefore, we initially looked at eight regions of the PR gene, from 311 kb upstream to 4 kb downstream of the transcription start site, recently discovered as ERα binding sites [117]. We mapped the dimethyl mark at H3K4 and H3K9 at these promoters (Figure 44). At almost all sites, the H3K4-Me2 mark was decreased upon E2 treatment. However, the H3K9-Me2 mark was almost completely unchanged. Therefore, for our future investigations of histone methylation for PR expression, we focused on promoter region 311 kb upstream.
Figure 44. Methylation marks at eight PR ERE binding sites. A. H3K4-Me2. B. H3K9-Me2. Data is presented as ±SEM for triplicate wells.
We have been able to show that the methylation status at the pS2 and PR EREs were influenced by LSD1. Specifically, using ChIP, when the function of LSD1 is inhibited by the small molecule inhibitors or siRNA knockdown, E2-induced demethylation of H3K4 does not occur, suggesting that LSD1 may be the primary demethylase acting at these sites (Figure 45). Thus, although a role for LSD1 as a scaffold protein has been suggested to be important in ER action, these results confirm that the catalytic activity of LSD1 is also required for the transcriptional activity of ERα at some target genes. This likely reflects a requirement for H3K4 methylation at or around the ERα enhancer.
Figure 45. Histone methylation results determined by ChIP after treatment with inhibitor 4.3 or after knockdown of LSD1 using siRNA. Data is presented as ±SEM for triplicate wells.
4.5 Global effects on breast cancer cells

4.5.1 Proliferation of breast cancer cells

While LSD1 is important for estrogen-dependent gene transcription, preliminary experiments using siRNA-mediated knockdown resulted in a decrease in the proliferation of both ER-positive and ER-negative cell lines, a result that highlighted a more fundamental role of this enzyme in cancer biology (Figure 31). This result provided the impetus to explore whether or not 2-PCPA and derivatives impacted cell growth and proliferation of multiple breast cancer cell lines. To this end, MCF7, MDA-MB-231, HCC1143, and HCC1937 breast cancer cells lines were treated with compounds 4.1-4.3 and 2-PCPA at 250 μM every other day for 10 days. Similar to what was observed in MCF7 and MDA-MB-231 cells treated with siRNAs directed against LSD1 it was observed that cell proliferation was significantly decreased (Figure 46). Significantly, we observed that longer term treatment with compounds 4.2 and 4.3 resulted in cell death by day 6 or 8. In addition, the reduced rate of cellular proliferation was shown to be inhibitor dose-dependent (Figure 47, Figure 48) when examined at concentrations between 10 μM and 250 μM. Inactivation of LSD1 by the small molecule inhibitors significantly influences the proliferation of breast cancer cells. It is interesting that compound 3 has the greatest impact on proliferation in all cases.
Figure 46. Cellular proliferation of four breast cancer cells lines after treatment with 250 μM 2-PCPA or 4.1-4.3 over the course of 10 days. A. MCF7 cells, ERα-positive cell line. B. MDA-MB-231, triple negative cell line. C. HCC1143, triple negative cell line. D. HCC1937, triple negative cell line. Data is presented as ±SEM for three individual treatments.
Figure 47. MCF7 cell proliferation after treatment with indicated concentrations 2-PCPA and 4.1-4.3. Data is presented as ±SEM for three individual treatments.
Figure 48. MDA-MB-231 cell proliferation after treatment with indicated concentrations of 2-PCPA and 4.1-4.3. Data is presented as ±SEM for three individual treatments.
4.5.2 Cell cycle analysis and apoptosis studies

Treatment of either MCF7 or MDA-MB-231 cells for 24 h with 2-PCPA or compounds 4.1-4.3 at inhibitor concentrations of up to 500 μM (Figure 34, Figure 49) did not influence cell viability. However, MCF7 cells treated with 250 μM 2-PCPA or compounds 4.1-4.3 for 24 h, were growth arrested as evidenced by the accumulation of cells in G1 and G2/M phases and a decrease in the number of cells in S phase (Figure 50, Figure 51A). This same pattern, albeit less robust, was observed in MCF7 cells following siRNA mediated knockdown of LSD1 (Figure 51B).
Figure 49. MDA-MB-231 cell viability after treatment for 24 h with inhibitors at indicated concentrations. A. 2-PCPA. B. Compound 4.1. C. Compound 4.2. D. Compound 4.3. Data is presented as ±SEM for three separate treatments.
Figure 50. Cell cycle raw data after 48 h treatment with DMSO, 2-PCPA and 4.1-4.3 as analyzed by flow cytometer.
Figure 51. Compiled cell cycle analysis results after 48 h treatment with 2-PCPA or compounds 4.1-4.3 or knockdown of LSD1 using siRNA.
In order to confirm cell cycle arrest as the basis for decreased cellular proliferation, the ability of 2-PCPA and compounds 4.1-4.3 to induce apoptosis was examined. MCF7 cells were tested for annexin V binding, an early marker of apoptosis on the cell surface [118]. Binding of annexin V was measured following treatment with 250 μM of the inhibitors for 24 h and 72 h. Cells undergoing apoptosis were analyzed by flow cytometry (Figure 52). There were no significant changes in the number of healthy or alive cells in the treated cells compared to the DMSO control (Figure 53). By contrast, apoptosis was efficiently induced in these cells by 24 h staurosporin treatment, as indicated by 40-50 % decrease in healthy, live cells. These results indicate that the cell cycle arrest induced by LSD1 may be primarily responsible for the anti-proliferative effects of 2-PCPA and derivatives 4.1-4.3 in MCF7 cells.
Figure 52. Apoptosis raw data after treatment with DMSO, 2-PCPA or 4.1-4.3 for 24 and 72 h as analyzed on flow cytometer. Staurosporine is used as a positive control for apoptosis.
Figure 53. Compiled apoptosis results after 24 and 72 h treatment with 2-PCPA and compounds 4.1-4.3. No change is observed between DMSO control and treated cells. Staurosporine is used as a positive control for apoptosis.
Interestingly, the 2-PCPA derived inhibitors of LSD1 appear to have anti-proliferative effects similar to those observed when cells are treated with HDAC inhibitors which also induce G1 and G2 cell cycle arrest [16]. Given that LSD1 and HDAC1/2 are found together in transcriptional complexes [21], it is not unreasonable to hypothesize that inhibitors of either protein may work in cooperation toward a specific substrate. Accordingly, it has recently been observed that treatment of breast cancer cells with HDAC inhibitors leads to an increase in methylation marks at some LSD1 target genes; a result that supports the functional link between these two enzymes [112]. Other studies however, have shown that the catalytic activities of HDAC1/2 and LSD1 on some genes are distinct. This is important in light of our finding that inhibition of LSD1 using 2-PCPA was not associated with changes in the acetylation state of H3 in bulk nucleosomes [44]. Thus, the role of LSD1 may differ between cell types and on different promoters.

4.5.3 Histone methylation levels

Having demonstrated substantial effects of 2-PCPA and derivatives and siRNA-mediated knockdown on transcription and proliferation, we set out to establish the impact of these manipulations on H3K4 and H3K9 methylation. To
this end, the breast cancer cell lines MCF7 and MDA-MB-231 were treated with 2-PCPA and the derivatives 4.1-4.3, and the global levels of H3K4 and H3K9 dimethylation were examined by Western blot analysis. In the ERα-positive cell line (MCF7) the level of H3K4-Me2 was increased after treatment with 2-PCPA and 4.1-4.3 (Figure 54A). However, under the same conditions we did not observe significant changes in the levels of H3K9-Me2. In contrast, we observed robust increases in global dimethylation of both H3K4 and H3K9 in MDA-MB-231 cells following treatment with the 2-PCPA and 4.1-4.3 (Figure 54B). Interestingly, siRNA mediated knockdown of LSD1 expression resulted in an increase in global H3K4-Me2 levels in both cell lines although the level of H3K9-Me2 levels were unchanged in either (Figure 54C-D).
Figure 54. Global dimethylation levels H3K4 and H3K9 in breast cancer cells after removal of LSD1 functionality. A. MCF7 cells after 24 h treatment with 250 μM 2-PCPA and 4.1-4.3. B. MDA-MB-231 cells after 24 h treatment with 250 μM 2-PCPA and 4.1-4.3. C. MCF7 cells after knockdown of LSD1 using siRNA. D. MDA-MB-231 cells after knockdown of LSD1 using siRNA.
These results highlight a heretofore unappreciated complexity in the mechanisms that impact the activity and or target gene specificity of LSD1 action. One of the most intriguing results observed is that inhibition of LSD1 using either 2-PCPA or siLSD1 resulted in an increase in H3K4-Me2 but not H3K9-Me2. However, increases in both marks were observed in MDA-MB-231 cells. It must be stressed that these differences were observed in several independent experiments. One interpretation of these data is that the methyl transferase responsible for the H3K9-Me2 mark in MCF-7 cells is highly active and/or the ability of the H3K9-Me2 mark to recruit LSD1 in these cells is compromised. It will be interesting to extend these studies to other cells to see which specific processes are associated with and/or responsible for regulating the specificity of LSD1 (H3K4-Me2 vs H3K9-Me2).

4.5.4 Effects on non-transformed cells

In order to investigate the therapeutic potential of 2-PCPA and compounds 4.1-4.3, we performed similar cellular proliferation assays in two non-transformed cell lines, hMEC/hT, human mammary epithelial cells, and MCF10A, non-tumorigenic mammary gland cells. The inhibitors were able to slow the growth of these cells similar to the breast cancer cell lines (Figure 55). More exploration to determine if this effect is due to
LSD1 inhibition or off-target effects of this class of compounds is essential to fully evaluate the potential of these small molecules as useful drugs. Additionally, the next step will be to examine the effect of these compounds on mouse tumor growth. This can be done using xenograft mouse studies with both ERα-positive and ERα-negative tumors.
Figure 55. Cellular proliferation of nontransformed cells after treatment with 250 μM of 2-PCPA and 4.1-4.3.
4.6 Effects of propargylamines on breast cancer cells

Interestingly, pargyline and derivatives 4.4-4.5, although exhibiting no inhibition of LSD1 in vitro at concentrations below 5 mM, slightly slowed proliferation of MCF7 and MDA-MB-231 cells (Figure 56A-B). Like 2-PCPA and derivatives, pargyline is not toxic to the cells within 24 h or treatment (Figure 56C-D). The exact mechanism of anti-proliferative effects of pargyline is unknown but is clear that at concentrations below 5 mM it is not due to LSD1 inactivation. It is possible that it is the result of the irreversible inhibition of another amine-oxidase containing enzyme or reversible inhibition of various other proteins or enzymes found in the cells. We arrived at this conclusion by evaluating its activity in a manner similar to that which was described above for the 2-PCPA and its derivatives. In cells treated with up to 250 μM of each drug we did not observe changes in E2-dependent transcription (pS2 or PR) (Figure 56E). Pargyline has been used as a probe of LSD1 function in breast cancer cells [38], prostate cancer cells [25], and herpes infection [113]; however, as highlighted by our results the activity of this class of compounds are less pronounced than that which was observed with LSD1 knockdown or with the 2-PCPA derived compounds.
Figure 56. Effects of pargyline and derivatives 4.4-4.5 on breast cancer cells. A. MCF7 cellular proliferation. B. MDA-MB-231 cellular proliferation. C. MCF7 cell viability after 24 h treatment. D. MDA-MB-231 cell viability after 24 h treatment. E. mRNA levels of pS2 and PR. Data presented as ±SEM of triplicate wells.
4.7 Conclusions and future directions

We have determined that LSD1 is essential for the proliferation of both ERα-positive and ERα-negative breast cancer cells. While the specific mechanisms underlying sensitivity to LSD1 inhibition remain to be defined, it is clear from the results of our studies that the anti-proliferative activities of the compounds we have developed is not only due to inhibition of ERα-transcriptional activity but that this enzyme is involved in additional processes fundamental for proliferation. Our studies demonstrate that, despite having similar substrate specificities, LSD2, an LSD1 ortholog, is not essential for breast cancer proliferation. This may indicate that the proteins found in complex with LSD1 and LSD2 help regulate their target specificity within certain cell types.

It appears that 2-PCPA derivatives have a similar but slightly more profound effect on the proliferation of the breast cancer cells than the knockdown of LSD1 by siRNA. This could be attributed to a few factors. First, the knockdown approach we have developed does not completely deplete the cellular levels of LSD1 protein, and therefore, active enzyme may be present, albeit at lower levels. Thus, the residual levels could be expected to carry out the essential functions of LSD1. Second, the 2-PCPA derived compounds may be inhibiting other enzymes that are also crucial to breast cancer cell proliferation and survival. However, among the many breast cancer cell lines we found to be sensitive to 2-PCPA, LSD1 and LSD2 were the only two FAD dependent
amine oxidases highly expressed. Whereas, it is possible that there may be additional “off-target” effects of these drugs we think that it is their ability to inhibit LSD1 that accounts for most of their antiproliferative activity. Regardless, the question still remains as to whether or not a specific LSD1 inhibitor or one with less specificity is the optimal drug for breast cancer treatment.

4.8 Experimental section

Bioinformatic Analysis: The breast cancer cell line dataset, GSE12777 [115] was downloaded from the Gene Expression Omnibus (GEO) at “http://www.ncbi.nlm.nih.gov/geo/”. For expression analysis the CEL files were normalized using R/Bioconductor [119-121] with RMA and individual probe expression values for each gene were obtained. The FAD-dependent Amine-Oxidase probes were then subset from this dataset, and the expression data was converted into a heatmap using the gplots package. To analyze the expression of FAD-dependent Amine-Oxidase genes in tumor datasets, we used GSE4922 [114], combining both U133A with the U133B chips into a single dataset and normalized as above. For purposes of identifying relative expression within each tumor, the rows consisting of tumor samples were scaled and displayed as a heatmap.
Cell Culture: MCF7 cells were maintained in DMEM/F12 (Gibco) supplemented with 8% fetal bovine serum (FBS) (Sigma), 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. MDA-MB 231 cells were maintained in DMEM (Cellgro) supplemented with 8% FBS, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. HCC1937 and HCC1143 cells were maintained in RPMI 1640 (Gibco) supplemented with 8% FBS, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. All cells were grown in a 37 °C incubator with 5% CO₂.

Transfection assays: For siRNA transfections, MCF7 cells were plated in phenol red-free media containing 8% charcoal-stripped FBS (Hyclone laboratories), 1 mM sodium pyruvate and 0.1 mM non-essential amino acids into either 150 mm dishes (for ChIP), 12-well plates (for mRNA levels) or 6-well plates (for Western blot) and were transfected with DharmaFECT 1 (Invitrogen) according to the supplier’s protocol. MDA-MB-231 cells were plated in DMEM (Gibco) supplemented with 8% FBS, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids into either 12-well plates (for mRNA levels) or 6-well plates (for Western blot) and were transfected with DharmaFECT 1 (Invitrogen) according to the supplier’s protocol. siRNA were obtained from Invitrogen.

RNA isolation and Real-Time PCR: For RNA analysis, MCF7 cells were seeded in 12-well plates in phenol red-free media containing 8% charcoal-stripped serum, 1 mM sodium
pyruvate, and 1 mM non-essential amino acids. After 4 d, the cells were treated with the inhibitors (250 μM). After 6 h, the cells were treated with ethanol (no treatment) or 100 nM E2 for 18 h and then were harvested. Total RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad) according to the supplier’s protocol. One half microgram of total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). The Bio-Rad iCycler Realtime PCR System was used to amplify and quantify levels of target gene cDNA. qRT-PCR reactions were performed with 8 μL cDNA, 0.4 μM specific primers and iQ SYBR Green Supermix (Bio-Rad). Data are normalized to the 36B4 housekeeping gene and presented as fold induction over control. Data are presented as the mean ± SEM for triplicate amplification reactions from one representative experiment. Each experiment was repeated at least three independent times with nearly identical results. PCR reagents were obtained from Bio-Rad. PCR oligos (Table 21) were purchased from Integrated DNA Technologies and Sigma-Aldrich.
Table 21. RT-qPCR primer sequences used for analysis of mRNA levels in MCF7 cells.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
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<tr>
<td>36B4 F</td>
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</tr>
<tr>
<td>36B4 R</td>
<td>GGGCCGAGACCAGTGTT</td>
</tr>
<tr>
<td>LSD1 F</td>
<td>GTGACGTACCCAGCCAAAG</td>
</tr>
<tr>
<td>LSD1 R</td>
<td>CCGAGCCAGGGATCAG</td>
</tr>
<tr>
<td>LSD2 F</td>
<td>GCGTGCTGATGTCTGATTT</td>
</tr>
<tr>
<td>LSD2 R</td>
<td>GACCTCCTGCTCCTGAACA</td>
</tr>
<tr>
<td>pS2 F</td>
<td>TCCCCCTGCTTCTCATTCTATAATAC</td>
</tr>
<tr>
<td>pS2 R</td>
<td>GCCTCCTGGCTTCTGGAGCC</td>
</tr>
<tr>
<td>PR F</td>
<td>GCTCGTGTGATAATCAGCAT</td>
</tr>
<tr>
<td>PR R</td>
<td>AATCTGCTGGCTTGCTCGCC</td>
</tr>
<tr>
<td>GREB1 F</td>
<td>GCACGCAAGCAGCTTCTGA</td>
</tr>
<tr>
<td>GREB1 R</td>
<td>GCTCTGTTCACCACCACCTTG</td>
</tr>
<tr>
<td>MYC F</td>
<td>CAGCGCTGTTGACGCTTTG</td>
</tr>
<tr>
<td>MYC R</td>
<td>GTAGAAATACGGCTGCACCA</td>
</tr>
<tr>
<td>EGR1 F</td>
<td>CACCTGACCCAGAGAGTCTT</td>
</tr>
<tr>
<td>EGR1 R</td>
<td>AGCGGCCATATAGTGATG</td>
</tr>
<tr>
<td>MCM2 F</td>
<td>TGCAAGCCAGGAGACGAAG</td>
</tr>
<tr>
<td>MCM2 R</td>
<td>CCATTCGGCAGTGTTGAGGG</td>
</tr>
<tr>
<td>AMyb F</td>
<td>CACAAATAGGAGCCATAC</td>
</tr>
<tr>
<td>AMyb R</td>
<td>CTAGAAATACGGCTGCACCA</td>
</tr>
<tr>
<td>CatD F</td>
<td>ACAACAGCAGCAAGTCCAGCC</td>
</tr>
<tr>
<td>CatD R</td>
<td>TGCGATGAAAGGTGATG</td>
</tr>
<tr>
<td>WISP2 F</td>
<td>ATGAGGAGCAGAGACAGAG</td>
</tr>
<tr>
<td>WISP2 R</td>
<td>GGTACCGACCTTTAGAGGA</td>
</tr>
<tr>
<td>SDF1 F</td>
<td>GTGATGGCAGTGCTCTC</td>
</tr>
<tr>
<td>SDF1 R</td>
<td>GATGCTGGACGTGGCTGT</td>
</tr>
<tr>
<td>Siah2 F</td>
<td>TCCCTGATAATTGATGCCAC</td>
</tr>
<tr>
<td>Siah2 R</td>
<td>CTCCCTGAAAGGTGATG</td>
</tr>
<tr>
<td>Notch3 F</td>
<td>TGCGGCTGGATGTATAG</td>
</tr>
<tr>
<td>Notch3 R</td>
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<td>ERBB4 R</td>
<td>GGATGATCCATACTTGCC</td>
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<tr>
<td>SMAD3 F</td>
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<tr>
<td>SMAD3 R</td>
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<tr>
<td>CoREST F</td>
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<tr>
<td>CoREST R</td>
<td>TCCACAGTCACACTCATCTTG</td>
</tr>
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</table>
ChIP assays: MCF7 cells were grown to 90% confluence in 150-mm dishes in phenol red-free media containing 8% charcoal-stripped FBS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids for 3 d, after which the cells were serum starved for 24 h. After treatment with vehicle or E2 (100 mM) for 45 min, the cells were fixed with 1% formaldehyde for 10 min at rt. The reaction was stopped with glycine (250 nM final concentration) by incubation at rt for 5 min. The cells were washed with ice-cold PBS, harvested in PBS, and centrifuged for 1 min. The cells were frozen (-80 °C) until ready to lyse. The cells were lysed in 1 mL sonication buffer (50 mM HEPES, pH 7.8, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1X protease inhibitor) by sonication (13 X 13 sec at 9-10 W). The lysate was clarified by centrifugation (15 min, 4 °C, 17000 g) and the supernatant collected, diluted with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 1X protease inhibitor), and precleared in 100 μL Protein A/G Agarose beads (50% slurry in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 200 μg sonicated salmon sperm DNA, and 500 μg Bovine serum albumin) for 30 min at 4 °C. Immunoprecipitation was performed for 4-6 hr at 4 °C with antibodies as described below. After immunoprecipitation, 100 μL Protein A/G Agarose beads (50% slurry in PBS) was added and allowed to incubate overnight at 4 °C. Precipitates were washed sequentially twice with sonication buffer, buffer A (50 mM HEPES, pH 7.8, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 1X protease inhibitor), buffer B (20 mM Tris, pH 8.0, 1
mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1X protease inhibitor), and TE (10 mM Tris, pH 8.0, 1 mM EDTA). The precipitates were eluted twice with 50 mM Tris, pH 8.0, 1 mM EDTA, 1% SDS at 65 °C for 10 min. Cross-linking was reversed by addition of NaCl (final concentration 230 mM) and incubation overnight at 65 °C. Protein was removed by incubation with EDTA (final concentration 4.5 mM) and proteinase K (final concentration 45 μg/mL) for 1 h at 42 °C. DNA was isolated with a QIA-quick PCR Purification kit (Qiagen, Valencia, CA). qRT-PCR reactions were performed with immunoprecipitated DNA, specific primers, and iQ SYBR Green Supermix (Bio-Rad). Data were normalized to the input for the immunoprecipitation. PCR oligos (Table 22 were purchased from Integrated DNA Technologies and Sigma-Aldrich.)
Table 22. RT-qPCR primers used for ChIP analysis in MCF7 cells.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>pS2 ERE F</td>
<td>TTAGGCCTAGACGGAATGGGCTTCTCAT</td>
</tr>
<tr>
<td>pS2 ERE R</td>
<td>TGAAGATTCAGAAGTCCCTCTTTCCC</td>
</tr>
<tr>
<td>pS2 distal F</td>
<td>CCAGAGGCCTGGCAGGAAAC</td>
</tr>
<tr>
<td>pS2 distal R</td>
<td>CGTCCCTCTCCACACACCCTC</td>
</tr>
<tr>
<td>PR distal F</td>
<td>TTAGTTCTGCTTCCGAATCTG</td>
</tr>
<tr>
<td>PR distal R</td>
<td>CCTCCCTCCTACACTTGG</td>
</tr>
<tr>
<td>PR 48 F</td>
<td>AAATAGGGAAGGGAACAG</td>
</tr>
<tr>
<td>PR 48 R</td>
<td>CCCACACTTAACCCCAATCC</td>
</tr>
<tr>
<td>PR 168 F</td>
<td>GATGACAGAAGGAGAAGTAAG</td>
</tr>
<tr>
<td>PR 168 R</td>
<td>ATATGGCATTGAAGCAACAG</td>
</tr>
<tr>
<td>PR 205 F</td>
<td>AAAGAGATGACTATTGTG</td>
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<tr>
<td>PR 205 R</td>
<td>CAGGAGATCGTGAGTT</td>
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<tr>
<td>PR 221 F</td>
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<tr>
<td>PR 221 R</td>
<td>CCAAGGATAGGCGATTCAGAAG</td>
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<tr>
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<tr>
<td>PR 306 R</td>
<td>CAATTTGAAATGAGAGGATAG</td>
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<tr>
<td>PR 311 F</td>
<td>ATGACATCAGCAGCAGT</td>
</tr>
<tr>
<td>PR 311 R</td>
<td>GAAAGAACACACCAACCTG</td>
</tr>
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**Cell Viability Assays:** MCF7 cells were seeded at 8000 cells per well in 96 well plates in phenol red-free media containing 8% charcoal-stripped FBS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. MDA-MB-231 cells were seeded at 8000 cells per well in 96 well plates in DMEM media containing 8% FBS, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. After 4 d, cells were given fresh media and treated with inhibitors at various concentrations (0.2 μM – 1 mM) for 24 h. Cells were incubated for 2 h after addition of CellTiter-Blue reagent (Promega), and then the fluorescence was measured (excitation 535 nm, emission 630 nm) using SpectraMax Gemini EM microplate reader (Molecular Devices). The data were normalized to a no-cell control.
Cell Cycle Analysis Assays: MCF7 cells were seeded at 400,000 cells per well in 6 well plates in DMEM/F12 (Invitrogen) containing 8% FBS, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. After 24 h, the cells were treated with fresh media containing 250 μM inhibitor. After 48 h of treatment, the cells were pulsed for 2 h with BrdU (10 μg/mL). The cells were trypsinized and collected using cold IFA buffer (3 mL) (4% charcoal stripped FBS, 150 mM sodium chloride, 10 mM HEPES, pH 7.5). They were washed with PBS, resuspended in 1 mL of PBS and fixed with 70% ethanol. The cells were incubated for 30 min on ice and then stored at -20 °C. The cells were washed with PBS containing 0.5% BSA. The pellet was denatured with 2 M hydrochloric acid containing 0.5% BSA. The residual acid was neutralized using 0.1M sodium borate, pH 8.5. The cell pellet was resuspended in dilute anti-BrdU-Alexa Fluor-488 antibody (Molecular Probes) (PBS + 0.5% Tween 20 + 0.5% BSA + 5% antibody). After washing excess antibody away, the cell pellets were resuspended in PI + RNase A (10 μg/mL propidium iodide and 10 μg/mL RNase A in PBS). The cells were vortexed and analyzed using flow cytometry (Accuri C6).

Apoptosis Assays: MCF7 cells were seeded at 300,000 cells per well in a 6-well plate in DMEM/F12 (Invitrogen) containing 8% FBS, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. After 24 h, the media was changed to serum starve. Then the cells
were treated with the inhibitors (250 μM) for 24 or 72 h. Control cells were forced into apoptosis by treatment with staurosporin (1 mM) for 24 h. All cells, including dead cells in media, were collected after trypsinization and were washed with cold PBS. The live cells were resuspended in Annexin-V binding buffer (600 μL, 10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) and then incubated with Alexa Fluor 488 Annexin-V conjugate (5 μL, Invitrogen) and Sytox® Red (1 μL, Invitrogen) for 20 min at rt protected from light. The cells were vortexed and analyzed using flow cytometry (Accuri C6).

**Cell Proliferation Assays:** MDA-MB 231, MCF7, HCC1143 and HCC1937 cells were seeded at 3000 cells per well in 96-well plates. After 2 d, the cells were treated with fresh media containing the inhibitors at the concentrations indicated. Every 2 d, for a total of 6 or 10 d, respectively, the cells were similarly treated. Total DNA content was measured by fluorescence using Hoechst 33258 dye (Sigma, ex 360 nm, em 460 nm) using a SpectraMax Gemini EM microplate reader (Molecular Devices). Data are presented as the mean ±SEM for triplicate wells in one experiment. Each experiment was repeated at least two independent times with nearly identical results.

**Western Blot Analysis:** MCF7 cells or MDA-MB 231 cells were seeded in six-well plates. Cells were treated after 48 h with inhibitor for 24 h. Whole-cell extracts were isolated using RIPA buffer [50 mM Tris (pH 8.0), 200 mM NaCl, 1.5 mM MgCl2, 1% Nonidet P-40]
(NP40), 1 mM EGTA, 10% glycerol, 50 mM NaF, 2 mM Na3VO4 and 1x protease inhibitor mixture]. Crude histones were extracted from the lysate pellet by resuspending in water and precipitating with 25% TCA. The pellets were washed with acetone and then resuspended. Concentration of whole-cell lysate or resuspended histones was determined using Bio-Rad Bradford reagent using BSA for standard curve production. For each sample, proteins were resolved by SDS-PAGE and transferred to a PVDF membrane (Biorad).

Antibodies: H3K4-Me2 was detected using polyclonal rabbit antibody (Millipore 07-030 or Abcam ab32356). H3K9-Me2 was detected using a monoclonal mouse antibody (Abcam ab1220). Total H3 was detected using a polyclonal rabbit antibody (Abcam ab1791). LSD1 was detected using a monoclonal rabbit antibody (Millipore 05-939) or polyclonal rabbit antibody (Abcam ab17721). ERα (D12) was detected using monoclonal mouse antibody (Santa Cruz sc-8005). GAPDH was detected using polyclonal goat antibody (Santa Cruz sc-20357). Secondary antibodies were purchased from Bio-Rad.
5. Investigation of the protein-protein interactions essential for LSD1 function

5.1 Background information

5.1.1. Targeting protein-protein interactions

Protein-protein interactions are central to most biological processes from intercellular communication to cell death. These dynamic interactions represent a diverse and expansive group of targets for therapeutic intervention. Although there has been recent advancement in identifying small molecules that potently inhibit protein complexes, more success has utilized peptides and peptidomimetics to mimic the surface at which two proteins bind [122]. For example, β-peptides and peptoids are capable of folding into stable secondary structures that represent organized protein-binding surfaces. They have been used to disrupt g41-mediated cell-cell fusion of HIV-1 [123] and to inhibit the interaction between HDM2 and p53 tumor repressor and cause cancer cell apoptosis [124]. Because of the ever-growing arsenal of molecules that mimic protein-protein interactions [125,126], it is important to identify targets to disrupt that will give favorable outcomes.

5.1.2 The importance of protein-protein interactions for gene regulation

Chromatin structure is neither homogenous nor inert. Instead, the dynamic elements of chromatin provide regulatory flexibility for transcription and other
processes such as replication, recombination and repair. The post-translational modifications of DNA and histones allow for manipulation of the chromatin, and therefore much of the complexity of gene transcription can be accredited to the proteins involved transcriptional complexes [6]. Activated and repressed chromatin have intrinsically different protein compositions [127]. In order to fully understand the intricacies of gene regulation, it is essential to elucidate the mechanisms by which the proteins that manipulate chromatin act. Although some information can be gained by looked at the proteins individually, complete appreciation can only occur when the proteins are examined in their native transcriptional complexes.

LSD1 has been found in a number of large transcriptional complexes containing proteins such as CoREST [42], histone deacetylases (HDAC) [110], p53 [35], Groucho2 [128], Snail1 [36], metastasis tumor antigen (MTA) [129], androgen receptor (AR) [25], and estrogen receptor (ER) [28]. Our group has focused much research on the collaboration between LSD1 and CoREST and LSD1 and ER. Recently, our group reported the thermodynamic characterization of binding between LSD1 and CoREST [130]. Additionally, in this thesis we have elaborated the role of LSD1 in governing ERα mediated transcription. We have now made efforts to determine if CoREST is essential for ERα mediated transcription in order to further characterize the transcriptional complex that contains LSD1 and ER. We have also attempted to disrupt the interaction
between CoREST and LSD1 in order to orphanize the latter protein and ultimately inhibit its demethylase activity.

5.2 CoREST, a protein essential for LSD1 demethylation of nucleosomes

5.2.1 Interaction between LSD1 and CoREST is localized to the linker region

LSD1 is consistently found in various transcriptional repressor complexes that include CoREST, C-terminal binding protein (CtBP) and histone deacetylases 1 and 2 (HDAC 1 and 2). LSD1 alone is sufficient to demethylate H3K4 in peptides of bulk histones, but its activity toward nucleosomal substrates is regulated by its interaction with CoREST. CoREST is a 66 kDa protein consisting of an ELM2 domain, two SANT domains and a linker region between the two SANT domains (Figure 57). Studies have shown that the linker and SANT2 domain (residues 286-482) are essential for LSD1-catalyzed demethylation of nucleosomes and the linker region alone is sufficient for binding to LSD1.
Figure 57. Domain structure of CoREST (top) and crystal structure of CoREST linker (blue) and SANT2 (red) domains in complex with LSD1 (grey). Picture generated from PDB 2UXX.
Sun Hwang has characterized the dissociation constant ($K_d$) between LSD1 and CoREST$^{286-482}$ to be in the nM range and determined that the linker region (CoREST$^{293-380}$) is responsible for the binding energy [130]. Presumably, the binding energy is favorable because of the helical character of linker region and the tower domain of LSD1. Furthermore, the linker region is able to disrupt the binding interaction between CoREST and LSD1 (Figure 58). As expected, when using an *in vitro* pull down assay between GST-CoREST, LSD1 and the linker domain, the linker is capable of disrupting the interaction between CoREST and LSD1. Instead it binds to LSD1, detaching it from the beads in a dosage dependent manner with complete inhibition at concentrations equal to the amount of LSD1 present.
Figure 58. Western blot probing for LSD1 binding to GST-CoREST$^{286-482}$. Linker domain is capable of disrupting the interaction in a dosage dependent manner.
5.2.2 Efforts to disrupt the interaction between LSD1 and CoREST using peptides

Targeting the interaction between LSD1 and CoREST may be a way to circumvent simultaneous inhibition of other FAD-dependent amine oxidases. The interface between these two proteins is most likely specific, so inhibitors, whether small molecules, peptides, or peptidomimetics, designed exclusively for this interaction may be completely discriminatory. In particular, peptides are powerful tools in drug discovery, especially in validating targets on the surface between large macromolecular proteins. Previously, peptides have been used as intermediate leads for development of non-peptidic small molecule drugs as well as developed into therapeutic agents themselves. Peptides are advantageous because the synthetic technology allows for diversification and optimization of binding to specific targets. Therefore, we synthesize peptides corresponding to the linker region of CoREST; this domain was segmented into 20-mer peptides overlapping by five amino acids to insure full coverage (Figure 59).

Figure 59. Peptides corresponding to the linker region (red) of CoREST.
Initially, five peptides were synthesized using microwave assisted FMOC-peptide synthesis (Table 23). A tryptophan was added to the C terminal end for quantification of the peptides using the molar extinction coefficient of the residue [131]. Additionally, a β-alanine was added to the N terminal end for future attachment of a fluorophore for quantification of the binding using fluorescence anisotropy [132].

Table 23. Synthesized peptides corresponding to linker region of CoREST.

<table>
<thead>
<tr>
<th>Name</th>
<th>Residues</th>
<th>Sequence</th>
<th>Mass calculated (M⁺, m/z)</th>
<th>Mass observed (M⁺, m/z)</th>
</tr>
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<tr>
<td>CoR300</td>
<td>300-319</td>
<td>H-βATQAKNRAKRKPKGFLSQEW-NH₂</td>
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<td>CoR330</td>
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</table>

In order to examine the capability of the peptides to prevent the interaction between LSD1 and CoREST, a GST pulldown assay was employed. Using a glutathione resin, GST-CoREST, LSD1 and each peptide were incubated together. The resin was washed extensively to remove any unbound peptides and proteins and any bound LSD1 visualized after elution using Western blot analysis. As can be seen in Figure 60, only two peptides (CoR315 and CoR330) were able to prevent the subsequent binding between LSD1 and CoREST; this was only observed at the extremely excessive concentration of 500 μM peptide.
Figure 60. Western blot probing LSD1 after binding to GST-CoREST$^{293-462}$. Some inhibition of interaction is seen with 500 µM CoR315 and CoR330 peptides.
The insignificant disruption of the interaction between LSD1 and CoREST could be attributed to a few factors. First, it may be necessary for the entire linker region of CoREST in order to bind to LSD1 and targeting a smaller region may not be possible. However, it is more likely that the peptides do not form α-helices and therefore may not have the favorable secondary structure for binding to LSD1. Much research has been focused on constricting the motion of peptides and, in particular, on the design and synthesis of α-helical peptides and mimetics [126]. In addition to traditional methods of incorporation of β-amino acids or α,α-dialkylamino acids into the peptide, synthetic backbone scaffolds, such as enaminones and pyridylamides, have been used to project sidechain functionality with similar distances and angular relationships to those found within α-helices. These compounds improve flexibility, solubility and synthetic accessibility. Alternatively, there has been success in using disulfide or amide bridges to constrain conformational flexibility and lock segments into helical conformations. Additionally, recent advancements have focused on the use of hydrocarbon-stapled peptides that not only improve the helicity of the peptides but also enhance the ability of the peptide to cross biological membranes and, in some cases, bind more tightly to the intended target [133]. We hypothesize that using one of the aforementioned methods would rigidify the helical nature of the CoREST peptides and may increase their ability to bind to LSD1. The incorporation of a rigid, hydrophobic cross-linker may not only
increase the peptide helicity but could lead to cell permeation which would allow us to use any successful peptides as probes of this interaction in cellular environments.

5.2.3 Knockdown of CoREST in breast cancer cells

In order to investigate the importance of CoREST as a scaffolding protein of LSD1 in breast cancer cells, we initially used small interfering RNA (siRNA) to CoREST to deplete the protein in MCF7 cells. Previously, CoREST has been seen to regulate endogenous LSD1 stability [110]. Agreeably, we observed this in MCF7 cells; CoREST knockdown did not alter LSD1 mRNA levels but decreased the protein levels (Figure 61).
Figure 61. LSD1 proteins levels visualized by Western blot analysis after knockdown of LSD1 and CoREST by siRNAs. Mock is untreated control and siMED is a scrambled control siRNA.
Only one siRNA resulted in successful knockdown of mRNA levels of CoREST and even in this case only to approximately 25% (Figure 62A). Despite incomplete knockdown of CoREST, we examined the effects of on the LSD1 and ERα-target genes identified in Chapter 4. As seen in Figure 62, the knockdown of CoREST is very similar to the knockdown of LSD1. However, in some cases such as MCM2, CatD and Erbb4, basal expression levels were changed considerably.
Figure 62. mRNA levels of ERα target genes after knockdown of CoREST in MCF7 cells. Data is presented at ±SEM of triplicate wells.
Previous studies showed that knockdown of LSD1 in both ERα-positive and ERα-negative cells resulted in decreased proliferation of the breast cancer cells. In order to investigate if CoREST has a similar affect, we repeated the experiments in MCF7 and MDA-MB-231 cells. Knockdown of CoREST did not slow the proliferation of the cells (Figure 63). This could simply be an artifact of incomplete depletion of CoREST protein levels due to insufficient knockdown by the siRNA. Alternatively, this could speak to the potential multiple functions of LSD1 in breast cancer. Although LSD1 plays an important role in transcription of estrogen-induced gene expression, it clearly has a role in breast cancer proliferation that is independent of ER. It could be that CoREST is essential in the transcriptional complex containing ER and LSD1 but not present in another, unidentified LSD1-containing complex that contributes more significantly to the proliferation of the cells.
Figure 63. Proliferation of breast cancer cells after knockdown of CoREST with siRNA. A. ERα positive MCF7 cells. B. ERα negative MDA-MB-231 cells.
5.2.4 Overexpression of CoREST\textsuperscript{293-380} in breast cancer cells

In addition to binding to LSD1, CoREST is also a scaffolding protein for HDAC1/2. Therefore, depletion of CoREST levels using siRNA is not specific to LSD1 function and may indeed affect HDAC function simultaneously with LSD1 function. Therefore, we set out to overexpress the linker region of CoREST (residues 293-380) in breast cancer cells and examine the global histone methylation levels. We hypothesized that overexpression of CoREST\textsuperscript{293-380} would allow for sequestration of LSD1 away from the nucleosome and therefore increase dimethylation of H3K4 as seen with LSD1 inhibitors. However, as quantified by cotransfection with ZsGreen and analysis using flow cytometry, transfection efficiencies that would allow for examination of global methylation levels were not consistently successful. We did see some increases in H3K4-Me2 in MCF7 and MDA-MB-231 cells, but only erratically. Current experimentation is underway to deliver purified CoREST\textsuperscript{293-380} as a TAT-fusion protein into the breast cancer cells. This will allow us to quantify the amount of linker present in the cells and more effectively visualize subtle changes in global histone methylation levels.

5.3 Probing the interaction between ER\textalpha, LSD1, and CoREST

5.3.1 ER\textalpha and transcriptional activators and repressors

The transcriptional activity of ER\textalpha is multifaceted; the binding of estrogen induces a conformational change, promoting dimerization and binding to specific
estrogen-response elements (EREs) located within regulatory regions of the target genes. From here, ERα communicates with the general transcription apparatus to positively or negatively regulate gene transcription. Since the discovery of the first two ER-interacting proteins in 1994 [134], there has been a focus on identifying cellular proteins that help to mediate ER functionality. Many of these transcriptional coactivators and corepressors bind specifically to domains of ERα either in the presence or absence of ligand. There exists other proteins that either enhance or repress ER activity by indirect methods. This complex network provides a balanced and sensitive control of ER-mediated target gene expression [100].
Figure 64. ERα function as a transcription factor is mediated by coregulatory proteins.
Because of the necessity of LSD1 function for transcription of some ERα-target genes explored in Chapter 4 and because the two proteins have been observed in the same complex [28], we set out to determine if there was a direct interaction between the two proteins. By analysis of the primary structure of LSD1, we hypothesized that if there was a physical interaction between the proteins, it may be attributed to the LXXLL motif contained in the tower domain (Figure 65). This sequence is found in a number of ER coregulators including steroid receptor coactivators 1-3 (SRC1-3) and receptor interacting protein of 140 kDa (RIP140) [100]. The LXXLL structural motif forms an amphipathic α-helix in which the leucines create a hydrophobic surface that fits into a hydrophobic pocket in the activation function-2 (AF-2) domain of ERα.
Figure 65. LXXLL motif in LSD1. Picture generated from PDB 2UXX.
5.3.2 *In vitro* pulldown assay to investigate interaction between LSD1 and ERα

In order to investigate the physical interaction between LSD1 and ERα, we employed an *in vitro* pulldown assay. Full length LSD1, truncated LSD1 containing only the SWIRM domain (residues 1-279), CoREST286-482 and control protein ZzGreen were expressed as GST-fusion proteins. After immobilization of the proteins to glutathione resin, \(^{35}\)S-methionine labeled full length human ERα was incubated with them and washed extensively. Any bound ERα eluted and visualized using autoradiography. Neither full length LSD1 or the SWIRM domain alone were able to bind to ERα in the absence or presence of estradiol (Figure 66). Although there is no interaction in the assay performed here, there could still be a direct interaction between LSD1 and ERα; it may be dependent on post-translational modification of ERα or be impeded by the GST in this assay. However, interestingly, CoREST286-482 is found to bind to ERα through this pulldown assay.
Figure 66. Results of GST binding experiment. A. 12% SDS/PAGE gel visualizing expression of GST fusion proteins. B. 12% SDS/PAGE gel visualizing normalized GST fusion proteins. C. Radiometric blot visualizing ERα after binding to GST fusion proteins (5 μg of GST proteins and in vitro synthesized ERα from 1 μg pcDNA).
Alternatively to the LXXLL motif found in some ERα binding partners, proteins containing a CoRNR box motif are also able to bind to the AF-2 domain. The CoRNR box typically forms an α-helix that binds to the hydrophobic surface. Both the nuclear receptor copresser (NCoR) and silencing mediator for retinoid and thyroid receptors (SMRT) bind ERα through this motif which is described by L/I-XX-V/I-I[135-137]. Interestingly, they both contain two CoRNR box motifs and have been seen to bind ERα in the presence and absence of tamoxifen [138]. Examination of the primary sequence and crystal structure of CoREST286-482 reveals two potential CoRNR box motifs, one that is α-helical (Figure 67). This could be the basis for the observed molecular binding between CoREST and ERα. In addition, it is possible that CoREST is mediating the interaction between LSD1 and ERα.
Figure 67. CoRNR motifs present in CoREST. Picture generated from PDB 2UXX.
5.4 Conclusions and future directions

LSD1 is a multifaceted protein and the complexity of substrate specificity, as well as function, could be attributed to the proteins with which it associates in particular cellular environments. Understanding these other proteins and the ways they interact with LSD1 will allow for more complete elucidation of integral role of LSD1 in gene expression and breast cancer proliferation. We have identified that similar to LSD1, CoREST is important for the transcription of particular ERα-target genes. In order to confirm the direct interaction observed between CoREST and ERα \textit{in vitro}, it is prudent to isolate the transcriptional complex from the cellular environment. This could be performed by a co-immunoprecipitation (IP) experiment isolating the endogenous ERα and probing for the presence of CoREST using Western blot analysis. This experiment will not completely confirm the interaction between the two proteins but using that in addition with other \textit{in vitro} binding assays, the residues important for binding can be determined. To confirm that CoREST is in the same transcriptional complex as LSD1 and ERα, recruitment of the protein to the specific ERE promoters of pS2 and PR will be examined using chromatin immunoprecipitation (ChIP) experiments. Lastly, the discovery of a peptide or peptidomimetic that is able to disrupt the interaction between LSD1 and CoREST would allow us to test our hypothesis about targeting this interaction as a means of LSD1 inhibition.
5.5 Experimental section

LSD1 expression and purification: Human LSD1, lacking the first 150 amino acids was expressed and purified as previously described with minor modifications [94]. Codon optimized LSD1 in pET15b (Invitrogen) was obtained from Genscript and transformed into BL21 Star (DE3) E. coli cells (Invitrogen); cells were grown at 23 °C in TB media, containing 100 μg/mL ampicillin, to an OD$_{600}$ of 0.6 before induction with 500 μM IPTG. Cells were induced overnight and harvested centrifugation. Cells were resuspended in 50 mM sodium phosphate, pH 7.6, containing 350 mM NaCl, 0.4 mM PMSF and 5% glycerol prior to lysis with an EmulsiFlex-C5 homogenizer (Avestin). After centrifugation at 35000 x g for 40 minutes to remove cell debris, protein, which contained an N-terminal histidine tag, was purified via nickel affinity chromatography [Chelating Sepharose FF (Amersham) charged with nickel sulfate], using a gradient of 10-175 mM imidazole in 50 mM sodium phosphate, pH 7.4, with 5% glycerol followed by gel filtration chromatography (Sephacryl S-200, GE Life Sciences) in 50 mM sodium phosphate, pH 7.4 with 5% glycerol and finally, anion exchange chromatography (Q Sepharose Fast-Flow, GE Life Sciences), using a gradient of 0-1 M NaCl in 50 mM sodium phosphate, pH 7.4 with 5% glycerol. Purification was performed on an AKTA FPLC. The protein was concentrated using a Centricon-10 centrifugal concentrator (Millipore) and stored at -20 °C in 50 mM sodium phosphate, pH 7.4, containing 40%
glycerol. The concentration of LSD1 was determined spectrophotometrically using the extinction coefficient of FAD (11600 cm⁻¹M⁻¹) at 458 nm.

Expression and Purification of GST-CoREST286-482: CoREST gene (residues 286-482) was cloned into the pDEST15 vector, and the vector was then transformed into chemically competent BL21(DE3) Star E.coli cells. The cell pellets were lysed with an emulsiflex C-5 cell cracker in buffer containing 50 mM sodium phosphate, pH 7.3, containing 300 mM NaCl, 5 % glycerol, 0.4 mM PMSF, 50 mg lysozyme, and protease inhibitor cocktail. GST-CoREST286-482 was purified by glutathione glutathione affinity chromatography, which was pre-equilibrated a binding buffer containing 140 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate (dibasic), and 1.8 mM potassium phosphate (monobasic). The column was washed with 100 mL of a binding buffer and the protein sample was eluted with 200 ml of elution buffer containing 50 mM Tris, pH 8.0, containing 10 mM reduced glutathione. Then the protein was further purified by gel filtration chromatography (HiPrep 26/60 Sephacryl S100, GE life sciences) with a buffer containing 50 mM sodium phosphate, pH 7.4, containing 100 mM NaCl. All purification was performed on an AKTA FPLC. The protein was concentrated using a Centricon-10 centrifugal concentrator (Millipore). The concentration of CoREST286-482 was determined spectrophotometrically using the extinction coefficient of 68300 cm⁻¹M⁻¹ at 280 nm.
**Peptide Synthesis and Purification:** Peptides corresponding to the sequences described were synthesized via classical Fmoc-peptide chemistry using a CEM Liberty microwave peptide synthesizer with Rink resin which yields a C-terminal amide group. The residue W was added to the C-terminal end of the peptide to aid in quantification of the peptide concentration using tryptophan’s extinction coefficient of 5502 cm⁻¹ M⁻¹ at 280 nm. The peptides were cleaved with trifluoroacetic acid for 2 h, precipitated with ice-cold diethyl ether, suspended in water, and lyophilized. Crude peptide was purified on a C₁₈ reversed-phase semi-prep column (Phenomenex) using a Thermo or Agilent HPLC and a linear gradient of water to methanol with 0.1% TFA. Absorbance was monitored at 214 nm and 280 nm. Peptide mass was verified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Applied Biosystems).

**Pulldown assay for disrupting binding between LSD1 and GST-CoREST**: Purified GST-CoREST (50 nM) was rotated with glutathione-Sepharose resin (GE Healthcare) in binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) for 30 min at rt, and then the beads was washed extensively with binding buffer. Then either the linker or peptide (at the indicated concentrations) was added followed by LSD1 (50 nM). This was rotated for 1 h at rt. The unbound protein/peptides were washed off with binding buffer and then the bound proteins eluted by incubation with elution buffer (50
mM Tris, pH 8.0, 10 mM reduced glutathione) for 20 min at rt. The LSD1 was determined by performing SDS-PAGE and visualizing with Western blot analysis.

Cell Culture: MCF7 cells were maintained in DMEM/F12 (Gibco) supplemented with 8% fetal bovine serum (FBS) (Sigma), 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. MDA-MB 231 cells were maintained in DMEM (Cellgro) supplemented with 8% FBS, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. Cells were grown in a 37 °C incubator with 5% CO2.

Transfection assays: For siRNA transfections, MCF7 cells were plated in phenol red-free media containing 8% charcoal-stripped FBS (Hyclone laboratories), 1 mM sodium pyruvate and 0.1 mM non-essential amino acids into either 150 mm dishes (for ChIP), 12-well plates (for mRNA levels) or 6-well plates (for Western blot) and were transfected with DharmaFECT 1 (Invitrogen) according to the supplier’s protocol. siRNA were obtained from Invitrogen.

RNA isolation and Real-Time PCR: MCF7 cells previously transfected with siRNA for CoREST were treated with ethanol (no treatment) or 100 nM E2 for 18 h and then were harvested. Total RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad). One half microgram of RNA was reverse transcribed using the iScript cDNA synthesis kit.
The Bio-Rad iCycler Realtime PCR System was used to amplify and quantify levels of target gene cDNA. qRT-PCR were performed with 8 μL cDNA, 0.4 μM specific primers and iQ SYBR Green Supermix (Bio-Rad). Data are normalized to the 36B4 housekeeping gene and presented as fold induction over control. Data are presented as the mean ± SEM for triplicate amplification reactions from one representative experiment. PCR reagents were obtained from Bio-Rad. PCR oligos were the same as described in Chapter 4.

Cell Proliferation Assays: Transfected MCF7 cells were seeded at 3000 cells per well in 96-well plates. After 2 d, for a total of 10 d, the cells were given with fresh media. Total DNA content was measured by fluorescence using Hoechst 33258 dye (Sigma, ex 360 nm, em 460 nm). Data are presented as the mean ±SEM for triplicate wells in one representative experiment. Each experiment was repeated at least two independent times with nearly identical results.

Western Blot Analysis: Whole-cell extracts of MCF7 cells were isolated using RIPA buffer [50 mM Tris (pH 8.0), 200 mM NaCl, 1.5 mM MgCl₂, 1% Nonidet P-40 (NP40), 1 mM EGTA, 10% glycerol, 50 mM NaF, 2 mM Na₃VO₄ and 1x protease inhibitor mixture]. Concentration of whole-cell lysate was determined using Bio-Rad Bradford reagent using BSA for standard curve. Proteins were resolved by SDS-PAGE and transferred to a
PVDF membrane (Biorad). LSD1 was detected using a polyclonal rabbit antibody (Abcam ab17721). Secondary antibody was purchased from BioRad.

Expression of GST-fusion proteins for ERα binding studies: Full length LSD1, SWIRM domain (residues 1-279) and ZSGreen were all in the GST expression vector pGEX-6P-1. An empty pGEX-6P-1 plasmid was used as a control. CoREST^{286-482} was in pDEST15 as described above. Each GST-fusion plasmid was transformed into BL21(DE3) Star cells. For each, a single colony was grown at 37 °C in LB media containing 100 μg/ml ampicillin, while shaking (200 rpm), to an OD_{600} of 0.6 before induction with 100 μM IPTG. The cells were induced for 2.5 h at 37 °C and then collected by centrifugation. The cells were resuspended in 5 mL lysis buffer (20 mM Tris, pH 8.0, 0.5 % Nonidet P-40, 100 mM NaCl, 6 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 8 % glycerol and 1X protease inhibitors) and lysed by sonication. After clarification of the lysate, a 50 % slurry glutathione-Sepharose resin (GE Healthcare) beads prewashed with PBS were added and rotated for 2 h at 4 °C. After binding, the beads were washed with lysis buffer (4 X 1 mL). For normalization to 5 μg, the proteins were eluted from a small aliquot of beads (10 μL) by boiling in Laemmli loading buffer, performing SDS/PAGE on a 12 % gel and visualizing using Coomassie stain.
In vitro binding assay of ERα: [³⁵S]Methionine-labeled ERα (pcDNA3-ERα 1 μg) was synthesized using TNT® Quick Coupled in vitro transcription/translation kit (Promega). The resultant radiolabeled protein was incubated with normalized GST-fusion proteins immobilized on glutathione-Sepharose beads for 14-16 h at 4 °C. The resin was washed (4 X 1 mL, lysis buffer above) for 5 min. Proteins were eluted by boiling the beads in Laemmli loading buffer, subjected to SDS-PAGE, fixed, and visualized by autoradiography.
Appendix A. 2-PCPA derivatives as potential anti-Parkinsonian therapeutics

A.1 Introduction

A.1.1 Parkinson’s Disease

Parkinson’s disease (PD) is a debilitating neurological illness that affects an estimated 6 million people worldwide; in 2007, it was the 14th leading cause of death in the United States [139]. PD is largely characterized by the irreversible loss of brain dopamine (DA) neurons. DA neurotransmission is essential for normal locomotor functions and, in most cases, PD becomes clinically apparent when the loss of dopaminergic neurons reaches 60-70% leading to functional dysregulation of the related neuronal circuitry. Major motor and non-motor manifestations of DA deficiency in PD include tremors, rigidity, bradykinesia, cardiovascular and gastrointestinal abnormalities, cognitive dysfunction, and depression [140].

Currently, there is no known cure for PD. However, the symptoms can be controlled by therapeutic interventions. DA replacement therapy is the major medical approach to treating PD, and a variety of dopaminergic agents are available. The most powerful drug is the immediate precursor to dopamine, levodopa (L-DOPA). Although L-DOPA is the most effective drug to treat the symptoms of PD, after five years or less of treatment about 60% of patients develop complications including fluctuations in motor performance as well as psychotic reactions and dyskinesia [140]. DA agonists, as well as several other classes directly or indirectly affecting DA function (monoamine oxidase
(MAO) inhibitors and catechol-O-methyl transferase (COMT) inhibitors) have proven advantageous in PD patients but are typically effective only when administered at early stages of the disease or as supplementary medications to enhance the benefits of L-DOPA [141]. Due to the limitations of existing therapeutic approaches, the development of better anti-Parkinsonian drugs remains a major directive.

A.1.2 Dopamine synthesis and recycling

DA is synthesized from tyrosine by the rate-limiting enzyme tyrosine hydroxylase (TH) to produce L-DOPA that is subsequently decarboxylated by L-aromatic amino acid decarboxylase (L-AADC) to DA (Scheme 12) [142]. Intraneuronal DA is accumulated into synaptic vesicles by the vesicular monoamine transporter-2 (VMAT2).

![Scheme 12. Synthesis of DA from tyrosine.](image)

After DA is released into extracellular space, it exerts physiological functions by activation of G protein coupled D1-like or D2-like DA receptors. DA in the extracellular
space is subject to dilution by diffusion and metabolic degradation. However, the major
route of DA clearance is the rapid recycling back into dopaminergic terminals by the
NaCl-dependent plasma membrane dopamine transporter (DAT). The recycled DA is
then stored in the large pools and is available for re-release.

**A.1.3 Dopamine-Deficient Dopamine Transporter knockout (DDD)
mice as model of Parkinson’s disease**

A number of animal models have been developed to search for potential PD
therapeutics. These models mimic the loss of DA through pharmacological and genetic
manipulation or recapitulate the neurodegenerative process by mutation of critical
proteins and/or administration of selective neurotoxins [140]. Nevertheless, reproducing
the full spectrum of behavioral manifestations of DA deficiency has remained an elusive
goal for these model systems. For example, mice bearing mutations in genes coding for
α-synuclein and parkin, demonstrate only partial loss of striatal DA supply, essentially
eliminating their use for screening for novel anti-PD drugs. Virtually all neurotoxic
models of DA deficiency (6-OHDA, MPTP, or rotenone) suffer from a widely variable,
incomplete DA depletion, while the most affected animals are eliminated due to lethality
[143].

Inhibition of the rate limiting enzyme of DA biosynthesis, TH by α-methyl-p-
tyrosine (α-MT) was thought to be a simple and straightforward way of producing an
acute PD mouse model. However, studies have shown that treatment of mice with α-MT
results only in partial depletion of DA is brain tissues and therefore is not adequate to generate PD-like symptoms [143]. This limited depletion is presumably due to the release of the large DA storage pool from vesicles (Figure 68A). Thus, in a normal mouse, complete depletion of DA is only achievable by dual inhibition of TH by α-MT and VMAT2 by reserpine [144]. However, inhibition of VMAT2 severely alters the levels of other neurotransmitters and the nonselective targeting results in complicated phenotypes that are not necessarily reflective of PD.
Figure 68. Dopaminergic neurons. A. Normal neurotransmission. B. Neurotransmission in DATKO mice
In Marc Caron’s research lab at Duke University Medical School, a novel model of acute, severe DA-deficiency has been developed by genetic alteration of mice resulting in the loss of a functional DA transporter gene [145]. The dopamine transporter (DAT) rapidly recycles extracellular DA into presynaptic neurons, thus controlling its diffusion and duration of action (Figure 68A). The lack of DAT-mediated inward transport in the DAT-KO mice results in elevated extracellular DA and at least 95% decreased intracellular DA stores (Figure 68B). These mice demonstrate dependence on DA synthesis and therefore inhibition of TH by α-MT induces profound depletion of DA [146]. Treatment with α-MT induces a transient recapitulation of essentially all PD symptoms for up to 16 h [141] making DA-deficient DAT-KO (DDD) mice an acute PD model useful for studying the efficacy of compounds that potentially can restore control of locomotion.

A.1.4 Previous experimentation to restore motor activity in DDD mice

Initial experiments in the Caron lab revealed that, as expected, L-DOPA and other non-selective DA agonists were effective at temporarily restoring normal locomotion to DDD mice.[141] Additionally, they tested approximately 100 non-DA compounds and identified only one compound, (+)-MDMA (Figure 69A), that was effective in restoring the major aspects of movement control required for forward locomotion in DDD mice[141]. Additionally, dual dosage with L-DOPA induced potent
synergistic effects (Figure 69B). The results are particularly promising because they allow for lower dosage treatment with L-DOPA which may combat long term sensitization and side effects of the drug. They also suggest that (+)-MDMA may affect movement control in a DA-independent manner.
Figure 69. A. (+)-MDMA B. Dual treatment with various concentrations of (+)-MDMA and L-DOPA/Carbidopa (10 mg/kg) induces forward locomotion in DDD mice.
Nevertheless, substituted phenylethylamines, such as (+)-MDMA are amphetamines that are psychostimulants, addictive, and potentially neurotoxic. Hence, use of them as PD treatment is significantly hampered by public perception. Although these amphetamines, and presumably all amphetamine derivatives, produce a favorable response in DDD mice, a different class of compound that may interact with the same target but without the negative side effects would be ideal. Phenylcyclopropylamines (Figure 70) are essentially conformationally constrained phenylethylamines. Because of the similarity to (+)-MDMA, we set out to test the effects of this class of compounds on restoring locomotion in DDD mice. Some members of this class have FDA approval for human use, are not addictive and have significantly reduced central nervous system activity and neurotoxicity as compared to amphetamines and (+)-MDMA.

![Phenylcyclopropylamine structure](image)

**Figure 70. Phenylcyclopropylamine structure.**

**A.2 Evaluation of small molecule derivatives in DDD mice**

**A.2.1 Phenylcyclopropylamines restore motion in DDD mice**

Eleven phenylcyclopropylamines (Table 24) were selected for testing in the DDD mouse model. After administration of the compounds alone, the overall movement of
the mice was observed and recorded (Figure 71, Figure 72). As seen in Figure 71, the experiment with compound A.2 was not completed because it was lethal after 30 mg/kg. Similarly, compound A.6 caused seizures and paralysis in the mice after 30 mg/kg treatment. However, compounds A.1, A.3, A.4, A.5, and A.7 resulted in active mice and reduced rigidity. The movement included shaking, tail straub, head bobbing and sniffing, similar to (+)-MDMA treatment [141]. These compounds exhibited marked anti-akinesia activity. Compound A.9, alone, was able to induce normal locomotion in DDD mice, making it the most promising derivative examined (Figure 73). A summary of the overall movement is presented in Figure 74.

Several compounds (A.3, A.4, A.5, and A.7) were also synergistic with L-DOPA/Carbidopa treatment (Figure 75). These compounds enhanced the low concentration of L-DOPA effects and induced locomotion and vertical activity. Compound A.9 was tested collectively with L-DOPA/Benserazide and the mice had increased locomotion and vertical activity (Figure 76). The results from treatment with the compounds and L-DOPA are summarized in Figure 77. This indicates that the compounds may have utility in dose-sparing L-DOPA, in turn preventing dyskinesias.
Table 24. Phenylcyclopropylamines tested in DDD mice.

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Figure 71. Results from unbiased screening of compounds in DDD mice (n=4 for each). Horizontal activity after treatment with: A. compound A.1, B. compound A.2, C. compound A.3, D. compound A.4, E. compound A.5, F. compound A.6 at indicated concentrations.
Figure 72. Continued results from unbiased screening of compounds in DDD mice (n=4 for each). Horizontal activity after treatment with: G. compound A.7, H. compound A.8, I. compound A.9, J. compound A.10, K. compound A.11 at indicated concentrations.
Figure 73. Normal locomotion observed after treatment with compound A.9 in DDD mice (n=4).
Figure 74. Summary of initial screening of compounds A.1-A.11 in DDD mice.
Figure 75. Results of treatment of DDD mice (n=6 for each) with phenylcyclopropylamines (5 mg/kg) and L-DOPA/Carpidopa (LD/CD, 10/10 mg/kg).
A. Total distance after treatment with A.3 and LD/CD. B. Vertical activity after treatment with A.3 and LD/CD. C. Total distance after treatment with A.4 and LD/CD.
D. Vertical activity after treatment with A.4 and LD/CD. E. Total distance after treatment with A.5 and LD/CD. F. Vertical activity after treatment with A.5 and LD/CD. G. Total distance after treatment with A.7 and LD/CD. H. Vertical activity after treatment with A.7 and LD/CD.
Figure 76. Results after treatment with compound A.9 (5 mg/kg) with L-DOPA/Benserazide (LD/BZ, 10/10 mg/kg) in DDD mice (n=6). A. Total distance traveled. B. Vertical activity.
Figure 77. Summary results of enhancement of anti-Parkinsonian effects of phenylcyclopropylamines (5 mg/kg) and L-DOPA/Carbidopa (10/10 mg/kg) or L-DOPA/Benserazide (10/10 mg/kg) in DDD mice.
These phenylcyclopropylamines (particularly compounds A.3, A.5 and A.9) are at least as effective as (+)-MDMA; the mice traveled further in one hour after treatment with these compounds than similar treatment with (+)-MDMA (Figure 69B).

**A.2.2 Propargylamines do not restore motion in DDD mice**

MAO inhibitors comprise some of the earliest drugs evaluated in PD[53]. Non-selective MAO inhibitors, such as 2-phenylcyclopropylamine, have been limited because of their side-effect/adverse reaction profile. On the other hand, MAO-B selective inhibitors such as selegiline (also known as deprenyl) and rasgiline have seen success in treating akinesia and motor fluctuations. In order to confirm that the promising results from our phenylcyclopropylamines above are indeed not a manifestation of MAO-B inhibition, we employed the use of propargylamines (Table 25) including pargyline (A.12) in the same studies with DDD mice.
Table 25. Propargylamines tested in DDD mice.

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As seen in Figure 78, these propargylamines do not elicit the same response as the phenylcyclopropylamines (Figure 71, Figure 72). The lack of positive response to MAO-B selective inhibitors by DDD mice had previously been observed by the Caron lab using selegiline [141]. Therefore, we conclude that the promising outcomes of treatment of DDD mice with phenylcyclopropylamines are irrespective of MAO-B inhibition. Despite the fact that these compounds are inhibitors of monoamine oxidases, the rapid response and the anti-akinesia properties observed clearly indicate that there is a unique mechanism of action.
Figure 78. Results from unbiased screening of propargylamines in DDD mice (n=4 for each). Horizontal activity after treatment with: A. compound A.12, B. compound A.13, C. compound A.14, and D. compound A.15 at indicated concentrations.
A.3 Conclusions and future directions

The DDD mice provide a unique model to assess the anti-Parkinsonian properties of potential therapeutics. However, this model is not yet clinically accepted in the PD field. Therefore, Dr. McCafferty has initiated a collaboration with Dr. T. Celeste Napier at Rush University to study the relief from dyskinesias, L-DOPA dose sparing, psychostimulatory effects, and addictive potential of the phenylcyclopropylamines, specifically compounds A.3, A.5, and A.9, in the 6-hydroxydopamine (6-OHDA)-treated rat model of PD [144]. We hypothesize that this model will confirm the ability of these compounds to dose-spare LD/CD and establish any abuse liabilities. Results from the rat studies will be compared to the DDD mouse model and evaluated as a predictor of experimental medications of PD and dyskinesia. These studies will continue to direct future medical chemistry efforts to tailor the physiochemical properties and pharmacological efficacy to reduce toxicity and off-target effects. Compounds that meet our expectations will then be considered for future evaluations in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned primate model of PD [144]. As the primate model is the closest system to human PD, it will allow us to evaluate the potential therapeutic utility of these phenylcyclopropylamines.

In tandem, with the Caron group at Duke and the Gainetdinov group at the Italian Institute of Technology (Gainetdinov is a former Caron lab member), we are making efforts to identify the target of these compounds in DDD mice. The system in
their lab, allows for the generation of DDD mice lacking other receptors. By subsequent treatment with the phenylcyclopropylamines, we can accurately determine when we have lost the desired response. We hypothesize that this class of compounds may exert their effects by interactions with trace amine-associated receptors (TAARs), a recently discovered class of G protein-coupled receptors. These receptors are located on both presynaptic and postsynaptic cells and are activated by a less well characterized group of endogenous amines derived from the metabolism of amino acids [147]. Trace amines, such as tyramine and octopamine, are as major neurotransmitters and are involved in many vital functions including movement, feeding and stress reactions. Although the functional role of the trace amines remains mostly unknown, trace amine levels are altered in PD [148]. Previously, (+)-MDMA and other closely related compounds have been shown to activate TAAR in vitro [148]. For these reasons, the interaction profiles and affinities of the phenylcyclopropylamines with TAAR class of proteins will be investigated first. Once the target receptor or enzyme is identified, we could optimize the structure to improve the selectivity of the drug.

A.4 Experimental section

Animals: DAT-KO mice were generated as previously described [149]. Animal care was in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health publication #865-23, Bethesda, Maryland, United States) with an
approved protocol from the Duke University Institutional Animal Care and Use Committee. DAT-KO mice, 3-5 month old, of both sexes were used.

Drugs: Compounds 1-15 or saline (0.9% NaCl) were administered intraperitoneally (i.p) in a volume of 10 mL/kg. L-DOPA, carbidopa and benzerazide were purchased from Sigma (St. Louis, Missouri).

Behavioral methods: Locomotor activity of DAT-KO mice were measure in an Omnitech CCDigiscan (Accuscan Instruments, Columbus, Ohio) activity monitor under bright illumination [150]. All behavioral experiments were performed between 10:00 am and 5:00 pm. Activity was measured at 5 min intervals. To evaluate the effects of the treatments on motor behaviors, the mice were placed into activity monitor chambers (20 X 20 cm) for 30 min and then treated with α-MT (250 mg/kg, i.p.). The compound were injected 1 h after α-MT administration and various parameters of locomotor activity were monitored for up to 3 h. In cumulative dosing experiments, animals were treated with increasing doses of drugs at 1 h intervals.

Data analysis: The data are presented as mean ± SEM and analyzed using a two-tailed Student’s t-test and one way analysis of variance (ANOVA).
References


Biography

Julie Ann Pollock was born July 20, 1984 in Indianapolis, IN. She graduated summa cum laude with an ACS certified B.S. in Chemistry and a minor in Mathematics from Hope College in May 2006. While there, she participated in NSF-REU and GlaxoSmithKline Undergraduate Fellowship funded research under Dr. Stephen Taylor that resulted in the published article: A mild biosynthesis of lactones via enantioselective hydrolysis of hydroxynitriles in Tetrahedron: Asymmetry.

In August 2006, Julie began work towards her doctoral degree in Chemistry at Duke University. During this period, he worked in the chemical biology lab of Dr. Dewey McCafferty. In her third year, she received the Burroughs Welcome Fellowship for Organic Chemistry. She was also a member of the graduate student honor society Phi Lambda Upsilon for the duration of her studies. In 2011, she was awarded an ACS Biological Division Travel Grant to attend the national meeting. She published the article: Facile synthesis of substituted trans-2-arylcyclopropylamine inhibitors of the human histone demethylase LSD1 and monoamine oxidases A and B in Bioorganic and Medicinal Chemistry Letters and will soon be publishing more related to LSD1 function in breast cancer and small molecules that restore function in Parkinsonian mice.