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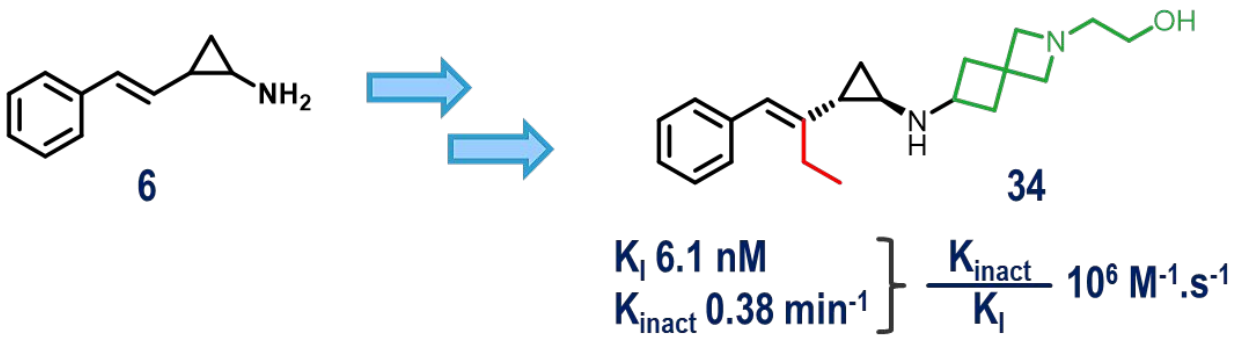

Title: Design and synthesis of styrenylcyclopropylamine LSD1 inhibitors.

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Abstract:

Leveraging the catalytic machinery of LSD1 (KDM1A), a series of covalent styrenylcyclopropane LSD1 inhibitors were identified. These inhibitors represent a new class of mechanism-based inhibitors that target and covalently label the FAD cofactor of LSD1. The series was rapidly progressed to potent biochemical and cellular LSD1 inhibitors with good physical properties. This effort resulted in the identification of **34,** a highly potent (<4 nM biochemical, 2 nM cell, and 1 nM GI_{50}), and selective LSD1 inhibitor. In-depth kinetic profiling of **34** confirms its covalent mechanism of action, validates the styrenylcylopropane as an FAD-directed warhead, and demonstrates that the potency of this inhibitor is driven by improved non-covalent binding (K_I). **34** demonstrates robust cell-killing activity in a panel of AML cell lines and robust anti-tumor activity in a KASUMI-1 xenograft model of AML when dosed orally at 1.5 mg/kg once daily.

Keywords:

LSD1, KDM1A, histone demethylase, irreversible inhibition, styrenylcyclopropane, AML

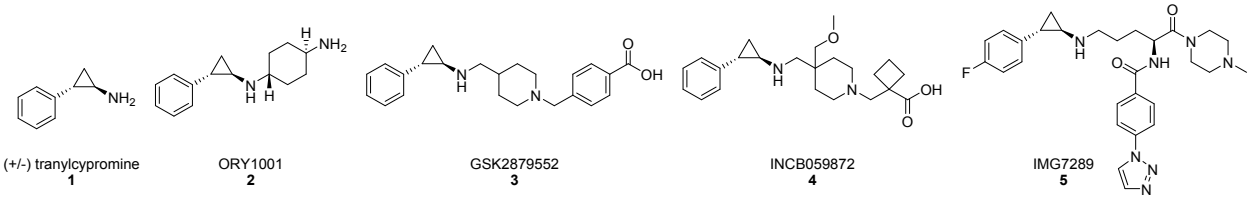
Introduction:

The dynamic organization of the genome in eukaryotic cells is regulated in large part by diverse post-translational modifications of histones, which control access to DNA and ultimately alter gene expression.[1,2] The identification of recurrent genetic alterations in genes encoding histone-modifying

enzymes in cancer cells of all types[3] suggests that manipulation of chromatin structure is a strategy commonly employed by cancer cells to invoke transcriptional programs that prevent differentiation and promote proliferation. Lysine-specific demethylase LSD1 (also known as KDM1A) acts on histone H3 both as a transcriptional co-repressor through demethylation of lysine-4 (H3K4) and as a co-activator by targeting the methylated lysine-9 residue (H3K9)[4,5]. These activities have been shown to play an essential role in a variety of normal physiological processes, including cell proliferation[6], hematopoietic differentiation[7], chromosome segregation[8], spermatogenesis[9], stem cell pluripotency[10], and embryonic development[11]. LSD1 is highly expressed in many cancer types[12], where it plays a key role in oncogenic processes, including compromised differentiation, proliferation, migration and invasion, as well as metabolic reprogramming[13,14].

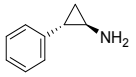
LSD1 has drawn considerable and increasing attention as a therapeutic target in human malignancies due to its involvement in a variety of disease states, including acute myelogenous leukemia (AML)[15], small-cell lung cancer[16], and myelofibrosis[17]. This interest has resulted in the advancement of multiple LSD1 inhibitors into clinical trials (See figure 1). Many of these inhibitors are derived from tranylcypromine (**1**), a mechanism-based, covalent inhibitor of monoamine oxidase A and B (MAO A and B), which demonstrates weak activity against LSD1. These clinical-stage inhibitors support LSD1 inactivation via selective reduction of the LSD1-bound flavin adenine dinucleotide (FAD) cofactor.

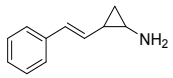
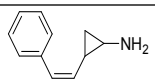
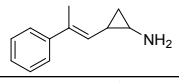
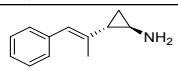
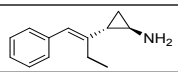
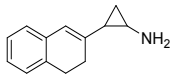
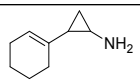
Figure 1. Tranylcypromine and related LSD1 inhibitors.



Given our interest in targeting disease-relevant, chromatin-regulatory mechanisms for therapeutic applications we began a hit finding campaign with the goal of identifying novel covalent inhibitors of LSD1. LSD1 has a catalytic domain that is structurally homologous with those of monoamine oxidases, which utilize a non-covalently bound FAD as a cofactor[18]. The similarity in the catalytic and structural properties of LSD1 and MAO A and B prompted the investigation of anti-MAO chemotypes as potential LSD1 inhibitors[19]. A survey of the MAO literature identified propargylamines and cyclopropylamines as functional groups capable of labeling the FAD cofactor of the MAOs. Using these cyclopropylamines and propargylamines to inform our design, we generated an initial hit-finding collection that included MAO inhibitors, commercially available cyclopropylamine and propargylamine compounds, and *de-novo* designs. Screening of this collection using a LSD1 enzymatic assay (see supporting information) identified several interesting cyclopropylamine derivatives as starting points, including spirocyclopropylamines[20,21] and styrenylcyclopropylamine **6**. Herein we report our efforts on optimizing the styrenylcycloproplyamine **6** due to its impressive potency compared to tranylcypromine (**1**).

Table 1: Exploration of the styrene.

CPI#	Structure	LSD1 TR-FRET IC_{50} ± SD µM (n)	ClogD[a]
1[b]		22.9 ± 6.9 (42)	-0.5

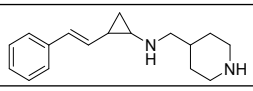
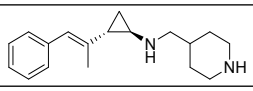
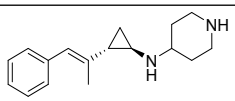
6[b]		0.444 ± 0.165 (3)	0.1
7[b]		20.4 ± 2.2 (2)	0.1
8[b]		4.28 ± 0.83 (4)	0.3
(±)-9		0.898 ± 0.199 (3)	0.0
(±)-10		0.412 ± 0.146 (2)	0.4
11[b]		0.788 ± 0.201 (2)	0.1
12[b]		9.40 ± 0.488 (2)	-0.4

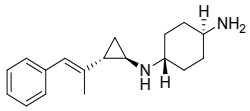
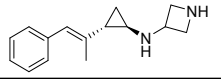
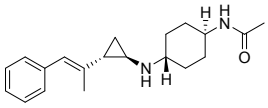
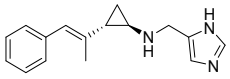
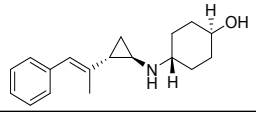
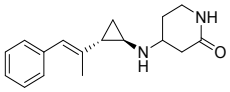
[a]Calculated using Stardrop 6.6.

[b]Mixtures of diastereomers.

The initial medicinal chemistry efforts explored substitution and modification of the styrenyl group. The *Z*-styrene analog (**7**) lost significant activity (>40 fold) against LSD1 demonstrating that the geometry of the styrene is critical for productive binding. Subsequently, substitution of the styrene by incorporating a methyl group at the 1- or 2-position, afforded **8** and **9**. There was clear preference for substitution at the 2-position over the 1 position (see, compounds **8** and **9**). The 2-substituted compound **9** demonstrated similar inhibition of LSD1 relative to the unsubstituted starting point **6** while being ClogD neutral. Extension from the methyl group to an ethyl group resulted in **10**, a compound with comparable activity to the methyl substituted analog **9** but with a modest impact on the physical properties (ΔClogD = 0.4) relative to **6**. In addition to substitution more dramatic modifications to the styrenyl group were investigated, including cyclization to form dihydronaphthyl derivative **11**, and removal of the phenyl ring to afford cyclohexene **12**. The dihydronaphthyl derivative retained similar activity to the ethyl substituted compound, however removal of the aryl ring, as in **12**, resulted in a >10-fold loss in activity. Further optimization of this series focused upon exploration of the amine substitution on styrenylcyclopropylamines **6** and **9**.

Table 2. N-substitution of early styrenylcyclopropylamine analogs.

CPI#	**Structure**	**LSD1 TR-FRET IC_{50} ± SD µM (n)**	***LY96* mRNA EC_{50} ± SD µM (n)**	**ClogD[a] / LLE[b]**
13		0.0072 ± 0.003 (3)	n.t.[c]	0.5 / NC[d]
(±)-14		0.0042 ± 0.001 (7)	n.t.[c]	0.5 / NC[d]
(±)-15		<0.0028 ± NA (3)	n.t.[c]	0.4 / NC[d]

(±)-16[e]		0.0029 ± 0.001 (5)	n.t. [c]	-0.1 / NC[d]
(±)-17		0.028 ± 0.016 (4)	0.312 (1)	0.3 / 6.2
(±)-18 [e]		0.056 ± 0.016 (5)	0.54 ± 0.11 (3)	0.7 / 5.6
(±)-19		0.0088 ± 0.002 (4)	0.362 (1)	0.5 / 5.9
(±)-20 [e]		0.025±0.006 (5)	0.327 ± 0.057 (3)	0.3 / 6.2
(±)-21[f]		0.020 ± 0.004 (4)	0.173 (1)	0.5 / 6.3

[a] Calculated using Stardrop 6.6.

[b]LLE = - log (*LY96* EC_{50}) – ClogD.

[c] n.t. = not tested

[d] NC = not calculated.

[e]Single unknown diastereomer.

[f]Mixture of diastereomers.

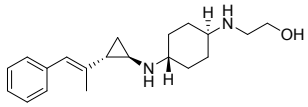
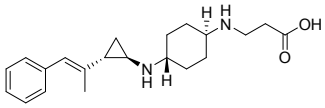
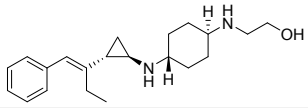
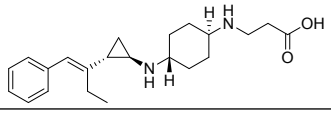
Our initial N-substituted analogs appended a piperidinyl group to the unsubstituted styrenyl, as seen in compound **13** (Table 2)**.** These initial compounds demonstrated good potency (< 10 nM) in our biochemical assay. Further profiling of **13** identified poor plasma stability as a potential liability. This observation was made when attempting to collect plasma protein binding information (PPB), as it was observed that ≤ 5% of parent compound remained after incubation in plasma**.** Efforts to address this issue focused upon substitution of the styrene as we hypothesized this group was contributing to the poor stability. The methyl substituted analog **14** was synthesized to test this hypothesis and encouragingly, **14** had comparable biochemical potency to **13** and demonstrated increased plasma stability across species.

With promising activity demonstrated by **14,** we began a systematic investigation of amine substituents on the methyl substituted scaffold (Table 2). Our goal in the optimization process was to improve the biochemical and cellular potency while ensuring that these compounds remained in drug-like chemical space. A variety of saturated heterocycles were investigated including piperidine **15**, *trans*-cyclohexylamine **16**, and azetidine **17**. These initial analogs were highly potent biochemical LSD1 inhibitors. Interestingly, analogs containing a linker consisting of 4 to 5 bond lengths between the two amine atoms (such as **14**, **15**, **16**) demonstrated superior potency in the biochemical assay compared to analogs with shorter linkers between the two amine atoms (such as **17**).

Azetidine **17** was subjected to a cell target engagement assay. Compound-induced changes in expression of the LSD1 target gene lymphocyte antigen 96 (*LY96*) were measured in human MV4-11 AML cells (see supporting information)[22]. In the *LY96* target engagement assay compound **17** demonstrated good activity (< 1 μM), simultaneously validating our triage scheme and confirming the activity of the styrenylcycloprane scaffold in cellular settings. While the LSD1 TR-FRET assay was initially useful in SAR analysis, many of the N-substituted analogs could not be differentiated with this assay. Thus, we transitioned to driving the program based on the *LY96* assay data including using this data to calculate the lipophilic ligand efficiency (LLE)[23] and track optimization of this series.

Next, non-basic substituents were investigated, including amide **18**, imidazole **19**, alcohol **20**, and lactam **21**. These non-basic substituents were designed to probe whether an explicit hydrogen bond donor could replace the basic amine heterocycles present in earlier analogs. While the mono-basic compounds afforded promising activity in the biochemical assay, these analogs did not significantly improve upon the initial cellular activity seen with azetidine **17** and were net neutral with respect to LLE.

Table 3: Comparison of methyl and ethyl styrenylcyclopropylamines

CPI#	Structure	LSD1 TRFRET IC_{50} ± SD μM (n)	*LY96* mRNA EC_{50} ± SD μM (n)	ClogD[a] / LLE[b]
(±)-22[c]		0.0026 ± 0.0001 (6)	0.052 ± 0.60 (4)	-0.1 / 7.4
(±)-23[c]		0.017 ± 0.009 (4)	0.55 ± NA (1)	-1.4 / 7.6
(±)-24[c]		<0.0018 ± NA (2)	0.006 ± NA (1)	0.2 / 8.0
(±)-25[c]		0.020 ± 0.007 (3)	0.43 ± 0.091 (2)	-1.1 / 7.5

[a] Calculated using Stardrop 6.6.

[b] LLE = - log (*LY96* EC_{50}) – ClogD.

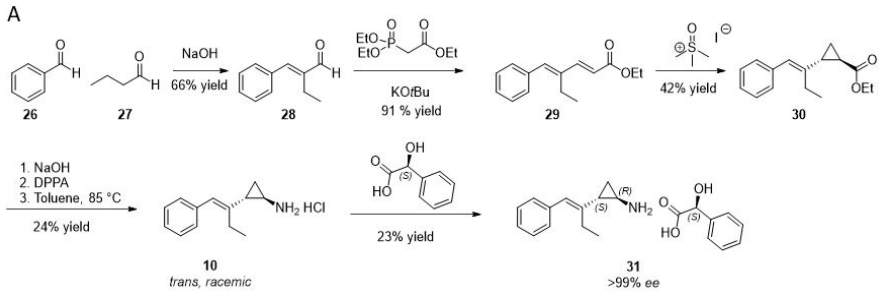
[c] Single unknown diastereomer.

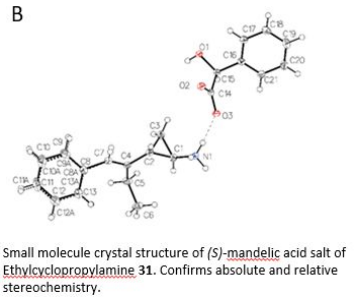
In search of improved activity in the *LY96* assay, we focused additional effort around substitution of the distal amine. Initially, we introduced polar groups, such as the ethanol group in **22** or the acid as in **23**. The ethanol group in **22** increased potency in both our biochemical and *LY96* gene induction assays relative to earlier compounds. This change also resulted in an impressive lipophilic ligand efficiency (LLE) of 7.4 for compound **22**. Introduction of an acid, as in **23**, resulted in a large potency loss in the *LY96* assay (10-fold) but the dramatic decrease in the ClogD resulted in a net improvement in the LLE (see, **22** and **23**).

Further exploration of the scaffold focused on ethyl substituted styrene analogs of **22** and **23** in an effort to modestly increase their ClogD. Synthesis of the ethyl analogs of these compounds afforded ethanol **24** and acid **25.** Introduction of the ethyl group afforded **i**mproved activity in the cellular assay for ethanol analog **24** (~9 fold, **22** vs **24**). The improvement in cellular activity offset the increased lipophilicity, as demonstrated by an increase in LLE from **22** to **24**. Interestingly, introduction of the ethyl group did not have the same effect on the carboxylic acid analog, as compound **25** and compound **23** demonstrate similar activity in the *LY96* assay.

With the impressive *LY96* activity and LLE observed with compound **24,** we focused our efforts on the ethyl cyclopropylamine scaffold. To enable this effort, we developed a scalable synthesis of the enantioenriched cyclopropylamine building block. The synthesis begins with the condensation of butanaldehyde (**26**) and benzaldehyde (**27**) to afford enal **28**. Reaction of the Horner-Wadsworth-Emmons reagent with enal **28** afforded the diene **29** in excellent yield. This diene was then treated with the Corey-Chaykovsky reagent to afford the cyclopropane **30** in modest yield. Hydrolysis of the ester, followed by a Curtius rearrangement and workup afforded the racemic, *trans*-cyclopropane **10**. Resolution of **10** with *(S)*-mandelic acid afforded the enantioenriched (*1R,2S*)-ethyl cyclopropylamine **31**. The absolute stereochemistry of **31** was confirmed by small molecule x-ray crystallography.

Scheme 1: Synthesis of *(1R*, *2S*)-**31**



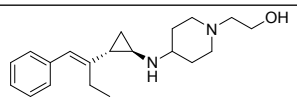
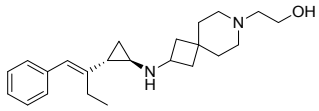
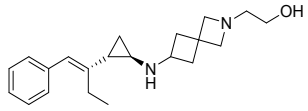
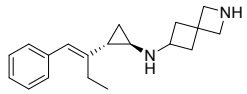
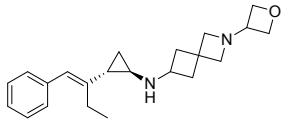
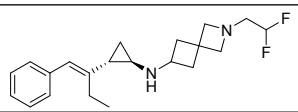
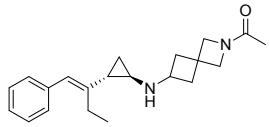
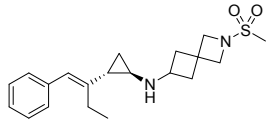


Small molecule crystal structure of *(S)*-mandelic acid salt of Ethylcyclopropylamine **31**. Confirms absolute and relative stereochemistry.

Continuing our SAR efforts on the ethylcyclopropylamine scaffold, we re-examined the impact of the linker between the two amines on activity and ADME properties. We kept the ethanolamine fragment in place and surveyed a variety of saturated heterocyclic linkers, including piperidine **32**, 4,6-*spiro* **33**, and 4,4-*spiro* **34**. These heterocyclic linkers (compounds **32** to **34**) afforded highly active LSD1 inhibitors with TR-FRET IC_{50}'s less than 3 nM with *LY96* EC_{50}'s less than 10 nM and LLE's ≥ 7.

With the impressive potency of the ethylstyrenyl cyclopropane compounds in the *LY96* target engagement assay we began triaging compounds in our phenotypic assay (see supporting information). In the phenotypic assay we assessed for growth inhibition in the KASUMI-1 cell line upon 12-days of compound treatment. For these inhibitors there was very good correlation between all three assays, the TR-FRET IC_{50}, the *LY96* EC_{50} and the phenotypic GI_{50}. While multiple inhibitors afforded potent target engagement and phenotypic activity, we chose to focus on the 4,4-*spiro* system for further exploration due to the promising liver microsome (LM) data and high LLE of compound **34**.

Table 4: Structure activity relationships on *(1R, 2S)*-ethylcyclopropylamine **31**.

CPI#	Structure	LSD1 TR-FRET IC_{50} ± SD µM (n)	*LY96* mRNA EC_{50} ± SD µM (n)	KASUMI-1 GI_{50} (µM)	ClogD[a] / LLE[b]	Liver microsome clearance (m / r / d / h)[c] (µl/min/mg protein)
32		<0.0026 ± NA (3)	0.004 ± 0.001 (2)	0.003	0.7/7.7	46/27/106/41
33		<0.0030 ± NA (4)	0.005 ± 0.003 (3)	0.001	1.1/7.3	28/19/39/10
34		<0.0030 ± NA (4)	0.002 ± 0.001 (9)	0.001	0.8/7.9	25/15/41/9
35		0.0053 ± 0.0009 (4)	0.005 ± 0.002 (3)	0.011	0.8/7.5	17/19/14/11
36		0.0030 ± 0.0011 (5)	0.004 ± 0.002 (3)	0.001	1.6/6.8	85/43/283/49
37		<0.0030 ± NA (2)	0.007 ± 0.003 (4)	0.011	1.9/6.3	883/175/480/133
38		0.0077 ± 0.0014 (3)	0.014 ± 0.001 (2)	0.041	0.9/7.0	76/48/87/18
39		0.0040 ± 0.0019 (4)	0.006 ± 0.001 (2)	0.017	0.8/7.4	812/80/105/67
3	-	0.212 ± 0.130 (34)	0.054 ± 0.031 (48)	~0.020	0.2/7.0	n.t.

[a] Calculated using Stardrop 6.6.

[b] LLE = - log (*LY96* EC_{50}) – ClogD.

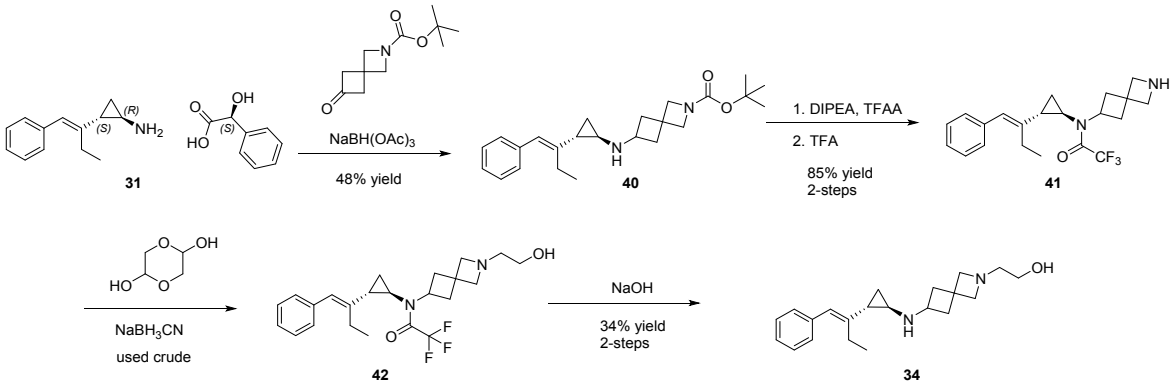
[c] m / r / d / h = mouse / rat / dog / human

[d] Calculated using Stardrop 6.6.

A subset of the 4,4-*spiro* analogs synthesized is presented in table 4**.** Unsubstituted spiro-analog **35** afforded a potent LSD1 inhibitor in the TR-FRET, *LY96*, and phenotypic assay. Additional substitutions aimed at modulating the basicity of the amine, as with oxetane **36** and difluoroethyl **37** afforded two highly potent LSD1 inhibitors with good activity in the phenotypic assay. These two compounds attenuate the basicity of the distal amine by >3 pKa[24] units and demonstrate that modulated bases can afford potent LSD1 inhibitors. Next, we tested the requirement for a second basic site in this *spiro*-scaffold by synthesizing acetamide **38** and sulfonamide **39.** Both mono-basic compounds afforded potent activity in

the *LY96* assay with good activity in the phenotypic assay. Additional profiling of the attenuated bases, **36** and **37,** and the mono-basic compounds, **38** and **39**, demonstrated a significant decrease in microsomal stability compared to compound **34**. Compound **34** was selected for further characterization due to its impressive combination of potency, LLE, and low molecular weight.

Scheme 2. Synthesis of **34**



The synthesis of **34** began from styrenyl cyclopropylamine **31**[25] via a reductive amination with *tert*-butyl 6-oxo-2-azaspiro[3.3]heptane-2-carboxylate. This afforded the spirocyclic cyclopropylamine **40** in good yield. Protection of the cyclopropylamine as the trifluoroacetamide followed by Boc deprotection afforded amine **41**. This material was then subjected to a reductive amination with glycoaldehyde dimer to afford the penultimate intermediate **42**. Removal of the trifluoroacetamide group under basic conditions, followed by purification afforded **34** in good yield.

Figure 2: Further characterization of **34**

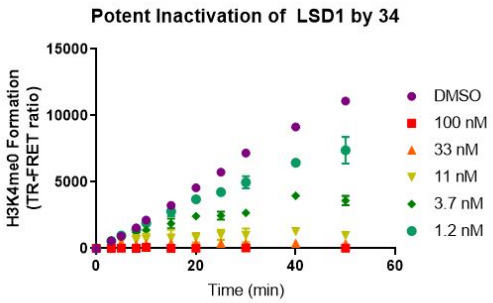
A

Assay	IC_{50} (μM)
LSD1 TR-FRET	0.002
LSD2 TR-FRET	>1
MAO-A	>80
MAO-B	>80

B

Assay	GI_{50} (μM)
KASUMI-1	0.001
MOLM-13	0.893
MV4-11	0.005
NOMO-1	0.078
OCI-AML3	0.271
OCI-AML5	0.332
SET-2	0.037
SKM-1	0.014

C



Compound	k_{inact} (min^{-1})	K_I (nM)	$\frac{k_{inact}}{K_I}$ ($M^{-1} \cdot S^{-1}$)
3	0.39	9,600	680
34	0.38 ± 0.10	6.1 ± 2.6	1,000,000

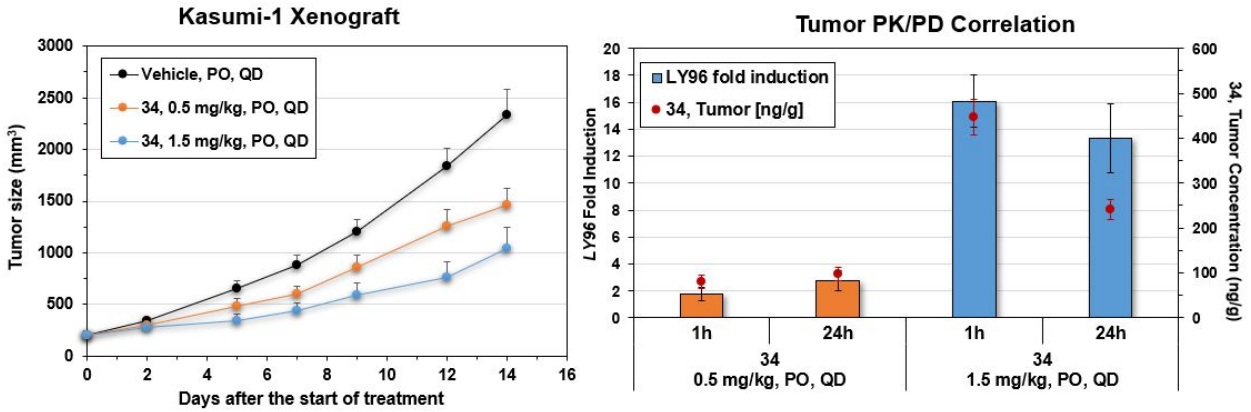
D

Mouse IV / PO Pharmacokinetic Data			
Parameter	1 mg/kg IV	Parameter	5 mg/kg PO
Cl (L/h/kg)	9.7	C_{max} (ng/mL)	40
Vd_{ss} (L/kg)	118	AUC_{inf} (ng*hr/mL)	189
$t_{1/2}$ (h)	25.3	F (%)	59

Further characterization of **34** in a panel of FAD-utilizing enzymes confirmed that this compound was highly selective for inhibition of LSD1, with no activity detected against the homologous enzyme LSD2 and related monoamine oxidases MAO-A and MAO-B (Figure 2A). Additional biochemical characterization of **34** demonstrated that this molecule displays time-dependent inhibition of LSD1 biochemical activity corroborating its hypothesized, covalent mechanism of inhibition (Figure 2C). This covalent inhibition occurs via a two-step mechanism consisting first of a reversible, non-covalent binding event followed by covalent inactivation of the FAD co-factor, in accordance with previous characterization of tranylcypromine-based LSD1 inhibitors.[14,26] Interestingly, ~1,500-fold improved inactivation efficiency ($\frac{k_{inact}}{K_I}$) of **34** over **3** is driven through the reversible, non-covalent binding event, while the observed rate of inactivation, k_{inact}, is comparable to that of the tranylcypromine-based **3**.[14,27]

Profiling compound **34** in a panel of human AML cell lines resulted in dose-dependent effects on cell growth with 5/8 cell lines as highly sensitive to inhibitor treatment (GI_{50} < 100 nM). *In vivo* characterization of **34** began with a single dose PK experiment in mice (Figure 2D). This PK experiment revealed that **34** is a high clearance, high volume compound with acceptable oral bioavailability to enable *in vivo* efficacy studies.

Figure 3. Kasumi-1 xenograft data for compound **34**.



In a Kasumi-1 xenograft model, **34** demonstrated significant and dose-dependent tumor growth inhibition (TGI) when administrated at 0.5 mg/kg and 1.5 mg/kg PO QD (Figure 3) for 14-days. Both doses were well-tolerated, with ≤5% body weight loss observed at 1.5 mg/kg PO and no significant body weight changes at the 0.5 mg/kg PO (see supporting information). Compound exposure and target engagement were measured in the plasma and tumor samples at 1 and 24 hours post last dose. Target engagement was monitored via induction of *LY96* in the tumor and a good correlation between induction of *LY96*, compound concentration in the tumor, and TGI was observed.

Herein we've described our efforts directed towards the discovery of novel covalent inhibitors of LSD1. This research program identified the styrenylcyclopropane structure as a previously unreported mechanism-based inhibitor of LSD1 which covalently labels the FAD cofactor. This novel chemotype diversifies upon the clinically relevant tranylcypromine-based scaffolds and potentially offers access to different physical properties, FAD-adducts, and biological effects. Optimization of this series culminated in the identification of compound **34**, a highly potent biochemical, cellular, and efficient (LLE) inhibitor. Further, we characterized the kinetics of its covalent inhibition and demonstrate that **34** has an improved inactivation efficiency compared to tranylcypromine-derived **3**. This covalent inhibitor is differentiated from the current tranylcypromine-based inhibitors by its highly potent non-covalent binding, KI, which results in an impressive inactivation efficiency roughly 3 orders of magnitude greater than tranylcypromine-based **3**. Additional characterization of **34** across a panel of AML cell lines demonstrate the broad potential utility of this mechanism in AML. Finally, we show that **34** provides dose-dependent tumor growth inhibition in an AML xenograft model at tolerated doses.

Supporting information

The supporting information is available free of charge via the internet at http://pubs.acs.org.

Methods and materials, experimental procedures, LSD1 biochemical assay, LSD1 K_{inact} / K_i studies, LSD1 LY96 assay, cell proliferation assays, *in vivo* Kasumi-1 xenograft experiment

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