

Structural insight into inhibitors of flavin adenine dinucleotide-dependent lysine demethylases

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ABSTRACT

Until 2004, many researchers believed that protein methylation in eukaryotic cells was an irreversible reaction. However, the discovery of lysine-specific demethylase 1 in 2004 drastically changed this view and the concept of chromatin regulation. Since then, the enzymes responsible for lysine demethylation and their cellular substrates, biological significance, and selective regulation have become major research topics in epigenetics and chromatin biology. Many cell-permeable inhibitors for lysine demethylases have been developed, including both target-specific and nonspecific inhibitors. Structural understanding of how these inhibitors bind to lysine demethylases is crucial both for validation of the inhibitors as chemical probes and for the rational design of more potent, target-specific inhibitors. This review focuses on published small-molecule inhibitors targeted at the two flavin adenine dinucleotide-dependent lysine demethylases, lysine-specific demethylases 1 and 2, and how the inhibitors interact with the tertiary structures of the enzymes.

Abbreviations: AEL, acute erythroid leukemia; AMKL, acute megakaryoblastic leukemia; AML, acute myeloid leukemia; AOD, amine oxidase domain; AOF, amine oxidase flavin-containing domain; BHC, BRAF–histone deacetylase complex; CoREST, corepressor for RE-1 silencing transcription factor; FAD, flavin adenine dinucleotide; HDAC, histone deacetylase; KDM, lysine (K) demethylase; LSD1, lysine-specific demethylase 1; LSD2, lysine-specific demethylase 2; MAO, monoamine oxidase; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; NPAC, no preservation of anterior capsule; 2-PCPA, (\pm)-*trans*-2-phenylcyclopropylamine; PDB, Protein Data Bank; SCLC, small cell lung cancer; SWIRM, Swi3/Rsc8/Moira; THF, tetrahydrofolate

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Introduction

In a eukaryotic cell, the activities of chromatin and its associated proteins are often modulated by posttranslational modifications.¹ For example, acetylation and methylation of lysine residues are typical modifications observed in the N-terminal flanking tails of chromatinized histones. Possible evidence for the active erasure of histone acetylation in a cell was revealed as early as 1978 by a series of chemical biology studies using non-specific histone deacetylase (HDAC) inhibitors (e.g., sodium butyrate) or specific HDAC inhibitors (e.g., trichostatin A).^{2–5} Identification of histone deacetylases Rpd3 and Hda1 in 1996 conclusively confirmed that histone acetylation is dynamically regulated in eukaryotic cells.^{6,7} In contrast, the reversibility of protein methylation at lysine or arginine residues was not unambiguously confirmed until 2004.⁸ Because methylated lysines in histones are generally more static than other known chromatin modifications (as exemplified by heritable histone H3K9 methylation, which is associated with heterochromatin),^{9–11} protein methylation had been assumed to be irreversible. However, identification of nuclear histone demethylase lysine-specific demethylase 1 (LSD1) by Shi and colleagues in 2004 completely changed this view.⁸

LSD1, also known as AOF2 or BHC110, uses flavin adenine dinucleotide (FAD) as a coenzyme for the removal of one or two methyl groups from a specific mono- or di-methylated lysine side chain of several nuclear proteins, including histone H3. Mammalian LSD1 has only one homolog, designated LSD2 or AOF1, which also uses FAD as a coenzyme and demethylates histone H3 at K4.¹² In addition to these two FAD-containing lysine demethylases, another type of lysine demethylases was reported in 2006.^{13,14} These enzymes, which contain a Jumonji C domain, belong to an Fe(II)-dependent and α -ketoglutarate-dependent dioxygenase family, and they catalyze the removal of one to three methyl groups from mono-, di-, or trimethylated lysine side chains. Currently, there are thought to be at least 20 different human α -ketoglutarate-dependent lysine demethylases.¹⁵ In the nomenclature of histone demethylase families,¹⁶ the FAD-dependent enzymes, LSD1/AOF2/BHC110 and LSD2/AOF1, are designated as lysine (K) demethylases 1A and 1B (KDM1A and KDM1B), respectively. They belong to the class I demethylase category. Other α -ketoglutarate-dependent demethylases belong to the class II demethylase category, which is composed of six families, designated KDM2, KDM3, KDM4, KDM5, KDM6, and KDM7.

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Because the KDMs in each class share the same catalytic domain, the development of a small-molecule compound or a peptidic compound that inhibits at least some of these enzymes may be possible. Structural information about the KDMs is particularly important not only for designing target-specific inhibitors but also for understanding their mechanism of action and target selectivity at atomic resolution. In particular, the development of specific inhibitors for LSD1 is highly desirable because this enzyme has emerged as a key epigenetic modifier in the self-renewal of several cancer stem cells, such as leukemic stem cells in acute myeloid leukemia (AML) and glioblastoma stem-like tumor-propagating cells.¹⁷⁻¹⁹ In fact, treatment of cells with LSD1 inhibitors can result in induction of differentiation and/or loss of cancer stemness,¹⁷⁻²⁰ and some irreversible LSD1 inhibitors have entered clinical trials for the treatment of AML and other diseases,^{21,22} as summarized in Table 1 and described below. In addition, no LSD2-specific inhibitors have yet been developed, which hinders the study of the chemical biology of LSD2. This review focuses on published small-molecule inhibitors of the FAD-dependent lysine demethylases, LSD1/KDM1A and LSD2/KDM1B, with emphasis on their tertiary structures.

Structures and functions of LSD1/KDM1A and its isoforms

LSD1 orthologues are found in organisms ranging from fission yeast to humans.⁸ The domain architecture of human LSD1 is shown in Fig. 1A. The tertiary structure of LSD1 except for approximately 170 residues of the N-terminal disordered region has been elucidated^{23,24} (Fig. 1B). The enzyme consists of a SWI3/Rsc8/Moira (SWIRM) domain, a Tower domain, and the catalytic amine oxidase domain (AOD). The SWIRM domain is a compact helical domain composed of approximately 85 residues and is found in several chromatin-modulating protein complexes.^{25,26} It packs against the catalytic domain, and this arrangement presumably contributes to the

Table 1. Summary of LSD1/KDM1A inhibitors in clinical trials and clinical use.^a

Drug designation	Organization	Phase	Disease
ORY-1001 ^b	Oryzon (Originator); Roche	I	Small cell lung cancer (SCLC)
		I/II	Acute myeloid leukemia (AML)
ORY-2001	Oryzon (Originator)	I	Alzheimer's disease
		I	Parkinson's disease
		I	Huntington's disease
GSK-2879552	GlaxoSmithKline (Originator)	I	SCLC
INCB-059872	Incyte (Originator)	I	AML
		I/II	Cancer
IMG-7289	Imago Biosciences (Originator)	I/II	AML
		I/II	Myelodysplastic syndrome (MDS)
CC-90011	Celgene (Originator)	I	Solid tumors
		I	Non-Hodgkin's lymphoma (NHL)
Tranylcypromine ^{c,d}	GlaxoSmithKline (Originator);	Launched	Depression

^aAs of January 20, 2017. ^bOther designations: RG-6016, RO-7051790. ^cOther designation: Parnate. ^dMonoamine oxidase (MAO) inhibitor.

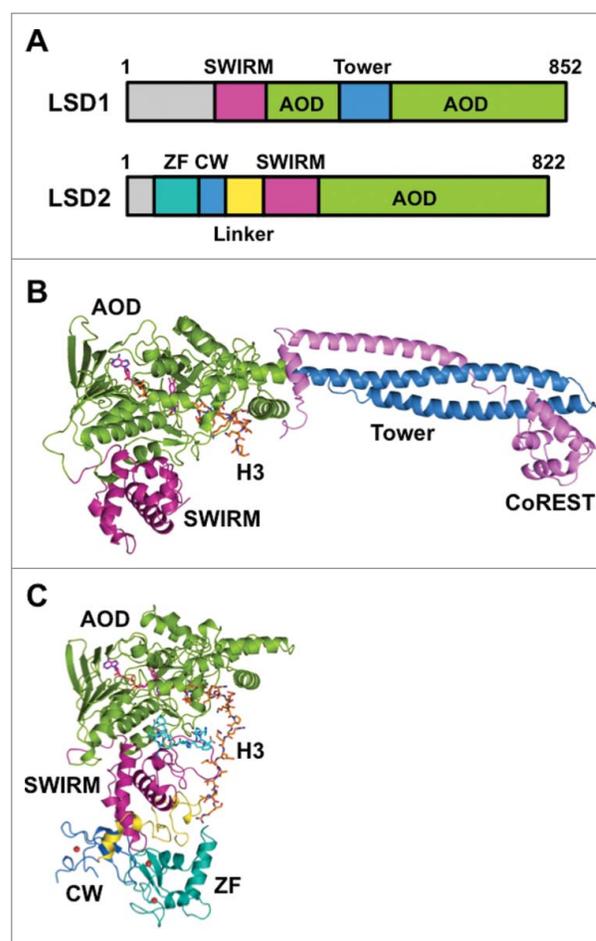


Figure 1. Structures of LSD1/KDM1A and LSD2/KDM1B. (A) Domain architectures of human LSD1 and LSD2. (B) Tertiary structure of LSD1 in complex with CoREST and an H3-like peptide inhibitor (PDB ID: 2V1D). Domains are colored as in panel (A), CoREST is depicted in pink, and the H3-like peptide is depicted in orange. (C) Tertiary structure of LSD2 (PDB ID: 4HSU) in complex with an H3 peptide (residues 1–26) (orange) and the NPAC linker region (cyan).

stability of the protein and the formation of a substrate-binding groove^{23,24} (Fig. 1B). The AOD possesses sequence similarity to the catalytic domain of small-molecule amine oxidases, such as monoamine oxidases A and B (MAO-A and MAO-B). The catalytic cavity of LSD1 is larger and more open than the cavities of MAOs. The SWIRM domain and the AOD reportedly interact with a G-quadruplex RNA and a long noncoding RNA, respectively.²⁷ Unlike MAOs, LSD1 has the Tower domain inserted in the middle of the AOD. The Tower domain is a slender, 90-Å-long coiled-coil structure protruding from the AOD. It provides the binding site for CoREST, a protein that presumably helps LSD1 tether a nucleosome.²⁸⁻³⁰ Accordingly, in the absence of the Tower domain, LSD1 cannot associate with CoREST and thus loses its ability to demethylate nucleosomes.^{24,31}

The crystal structure of LSD1 in complex with an H3(1–21)-derived peptide in which the K4 residue has been replaced by methionine (H3K4M(1–21)) reveals that the N-terminal 1–3 residues of H3 adopt a helical conformation that is accommodated in a negatively charged pocket in the catalytic center of LSD1.³² In the pocket, H3R2 forms hydrogen bonds with the D553 and D556 residues of LSD1. The side chain of H3K4 to

be demethylated is directed toward FAD along the side chain of Y761 of LSD1. The H3 peptide is compactly folded and interacts extensively with LSD1 residues on the rim of the catalytic cavity. The C-terminal residues after H3P16 are not visible in the electron density map, presumably owing to lack of structural restraints.³² The structure of LSD1 in complex with another H3(1–21)-derived peptide, in which K4 is replaced by a covalent-bond-forming *N*-methylpropargyl lysine, shows similar enzyme–substrate interactions, although the conformation of the H3 peptide seems to be more constrained owing to the covalent bond to FAD.³³ These structures demonstrate that there is little room for extension of the N-terminus of the H3 peptide, which explains the specificity of LSD1 for its major substrate residue, that is, H3K4.^{32,33} There may be an additional H3-binding site, as indicated by the fact that full-length H3 can tightly bind to LSD1.³⁴

Although LSD1 was first identified as an H3K4 demethylase, it also reportedly demethylates H3K9 when interacting with the androgen receptor.³⁵ The mechanism of demethylation at H3K9 remains unclear, but recent studies have indicated that splicing isoforms of LSD1 exhibit altered substrate specificity with a preference for H3K9 or H4K20.^{36–39} Thus, there may be another binding mode in which the H3K9 or H4K20 side chain is in close proximity to FAD in the catalytic center of LSD1. The neuron-specific LSD1 isoform has an in-frame insertion of four amino acid residues (DTVK) between A369 and D370. This tetrapeptide forms a short antiparallel β -turn that sticks out from the body of the protein without changing the overall conformation of the LSD1•CoREST complex.³⁶ In addition, phosphorylation of the threonine residue in the insertion sequence triggers a local structural change in LSD1, which may in turn disrupt interactions of LSD1 with CoREST, HDAC1, and HDAC2.⁴⁰

In addition to histone H3, nonhistone nuclear proteins, including p53, Dnmt1, E2F1, and MYPT1, are also substrates for LSD1.^{41–44} The functions of these nonhistone proteins are regulated by residue-specific lysine demethylation by LSD1. There is also an LSD1-binding protein that inhibits the demethylation activity of LSD1. Specifically, the N-terminal SNAG domain of the transcription factor SNAIL1, which mediates E-cadherin repression during the epithelial–mesenchymal transition, binds to the AOD of LSD1, thereby recruiting LSD1 to the targeted gene promoter of SNAIL1 for transcriptional repression.⁴⁵ The first nine residues of SNAG, which are well conserved among species and resemble the first nine residues of H3, tightly bind to the catalytic cavity of LSD1 by mimicking the LSD1-binding mode of H3, resulting in competitive inhibition of LSD1 demethylase activity.⁴⁶ Therefore, the structure of the SNAG domain has been explored to develop peptidic LSD1 inhibitors (described below).

Structures and functions of LSD2/KDM1B

When LSD1 was discovered as the first lysine demethylase,⁸ LSD2 was identified as the only mammalian homolog of LSD1. The domain architecture of human LSD2 is shown in Fig. 1A. Like LSD1, LSD2 demethylates mono- and di-methylated H3K4 in an FAD-dependent manner.^{12,47–49} Mouse LSD2 reportedly demethylates H3K9 at a comparatively higher

concentration.⁵⁰ Unlike the catalytic activity of LSD1, the catalytic activity of LSD2 still seems to be the subject of discussion. The domain architectures of both enzymes (Fig. 1) share the AOD as the catalytic center in the C-terminal region, and they also have the SWIRM domain on the N-terminal side of the AOD. However, the AOD of LSD2 lacks the Tower domain that is present in the AOD of LSD1. LSD2 demethylates nucleosomal histone H3 *in vivo*.^{48,49,51} Because LSD2 does not have the nucleosome-binding Tower•CoREST architecture (Fig. 1B), the demethylation of nucleosomal histone H3 by LSD2 must occur in a structurally distinct manner from demethylation by LSD1.

LSD2 has two types of zinc fingers in the N-terminal region (Fig. 1A): a C₄H₂C₂-type zinc finger followed by a CW-type zinc finger. Some of the CW-type zinc fingers function as a reader for K4-methylated histone H3.^{52–54} The CW domain typically forms an aromatic cage with a preference for hosting the methylated lysine, and the cage-forming residues and four Zn(II)-coordinating cysteine residues are conserved in LSD2. Nevertheless, the CW domain of LSD2 does not bind to the H3 peptide, even though the domain is required for the demethylase activity of LSD2.^{48,55} Structural comparison of the CW domain of LSD2 with other K4-methylated H3-binding CW domains reveals that L340 and I343 of the neighboring SWIRM domain occupy the binding site of the methylated lysine, and a helix-loop-helix moiety of the SWIRM domain and a helix within the CW domain seem to hinder H3 from binding to LSD2.^{54–56}

As expected from the distinct domain architectures of LSD1 and LSD2, they have distinct binding partners. Specifically, LSD1 associates with HDAC1 and HDAC2,²⁹ whereas LSD2 does not associate with HDACs.^{48,57} In addition, only LSD1 associates with CoREST, and this association may tether LSD1 to a nucleosome.^{28–30} Furthermore, NPAC/GLYR1 is an LSD2-specific cofactor that enhances the catalytic activity of LSD2 toward histone H3.⁵⁶ NPAC binds to LSD2 through the NPAC linker region between the N-terminal putative H3K36me₃-binding PWWP domain and the C-terminal dehydrogenase domain. The structure of LSD2 in complex with an NPAC linker region and the H3(1–21) peptide revealed that residues 214–225 of NPAC interact with LSD2 and that this dodecapeptide is sufficient for enhancement of the catalytic activity of LSD2.⁵⁶ Specifically, F217 of NPAC and Y273, E277, and R285 of LSD2 form a hydrophobic pocket that interacts with L20 of H3⁵⁶ (Fig. 1C). In contrast to the crystal structure of the LSD1•H3(1–21) complex in which only H3(1–16) was visible in the electron density map,³² H3(1–20) was visible in the crystal structures of LSD2•H3(1–21)•NPAC, presumably owing to the interactions between Q19 and L20 of H3 and LSD2•NPAC.⁵⁶ The LSD2•H3(1–26)•NPAC complex further demonstrated that H3 residues 19–26 bind to a second site on LSD2, which is formed by the linker region between the CW domain and the SWIRM domain. In this complex, NPAC seems to facilitate the interactions between LSD2 and the H3 peptide⁵⁸ (Fig. 1C). There have been fewer studies of LSD2 than of LSD1, but the studies to date implicate LSD2 in several biological contexts that differ from those of LSD1.^{47,59}

Categories of LSD1/KDM1A inhibitors

Small-molecule inhibitors are useful tools for identifying the functional roles of a target protein. However, the potency and specificity of an inhibitor for the protein of interest are important. When a promiscuous inhibitor is used, drawing valid conclusions from the results can be difficult.⁶⁰ In early chemical biology studies of LSD1, researchers often used the MAO inhibitor tranylcypromine ((±)-*trans*-2-phenylcyclopropylamine; abbreviated as 2-PCPA in this review), which exhibited better LSD1-inhibitory activity than the other MAO inhibitors known at the time.^{61,62} However, 2-PCPA is now regarded as a promiscuous FAD-inactivating inhibitor, and studies in which 2-PCPA was used as an LSD1 inhibitor must be evaluated carefully. Many research groups have pursued the development of LSD1 inhibitors more specific and potent than 2-PCPA, as described below. In this review, we categorize these new LSD1 inhibitors as irreversible covalent inhibitors and reversible non-covalent inhibitors, and we group the irreversible inhibitors into three subcategories: 2-PCPA derivatives, *N*-alkylated 2-PCPA derivatives, and inhibitors other than 2-PCPA derivatives.

2-PCPA derivatives that inhibit LSD1/KDM1A

Some typical irreversible LSD1 inhibitors are shown in Fig. 2, and kinetic parameters and structural information (where available) are summarized in Table 2. Most of the irreversible LSD1 inhibitors developed to date are based on the 2-PCPA scaffold (1, Fig. 2).⁶³ Researchers initially focused on chemical modification of the phenyl ring of 1. For example, Ueda et al. attached a lysine-mimicking group at the *meta*-position (NCL-1, 2, Fig. 2) or the *para*-position (NCL-2) of the ring, and the resulting compounds show enhanced affinity for LSD1 and are selective for LSD1 over MAOs.⁶⁴ Mimasu et al. designed a *meta*-fluoro compound with a benzyloxy group at the *ortho*-position (designated S1201; the complex with LSD1 is assigned PDB ID 3ABU).⁶⁵ First, they determined the struc-

ture of LSD1 in complex with pentafluorinated 2-PCPA (PDB ID: 3ABT), and then they synthesized various *ortho*-substituted 2-PCPA derivatives based on the structures of LSD1•2-PCPA complexes^{66,67} and MAO-B.⁶⁸ Eventually, they developed a compound, designated S2101 (3, Fig. 2), with one more fluorine atom than S1201. This difluoro compound exhibits submicromolar potency and better selectivity for LSD1 over MAOs. S2101 is also selective for LSD1 over LSD2 (unpublished). S2101 and NCL-1 were shown to be cell active as LSD1-selective inhibitors. In particular, compact cell-permeable compound S2101 has been used in many studies to assess the biological roles of LSD1.^{19,69-76}

Several *para*-substituted 2-PCPA derivatives have been developed, including MC2584/13b and MC2580/14e (structures of their respective complexes with LSD1•CoREST are assigned PDB IDs 2XAQ and 2XAS), which exhibit selectivity for LSD1 over MAO-B and approximately 20-fold enhanced potency against LSD1 relative to that against LSD2⁷⁷ (Table 2). In the LSD1•MC2580/14e structure, one phenylalanine in the linear extension occupies the position of T3 in the LSD1•H3 structure, and the other branched phenyl ring occupies the same position as the *ortho*-benzyloxy group of S1201. Other *para*-substituted inhibitors include the following: OG-L002 (Table 2), which inhibits viral infection;⁷⁸ pan-histone demethylase inhibitors that are hybrids of 2-PCPA and Jumonji C-containing KDM inhibitors;⁷⁹ compounds coupled with hydroxycinnamic acid;⁸⁰ and biphenyl derivatives.⁸¹

Although 2-PCPA inactivates both LSD1 and MAOs by covalent bonding of the cyclopropylamine group to the FAD, the structures of the resulting adducts are different. In the MAO-B•2-PCPA complex, the covalent bond forms at the C4a atom of FAD.⁸² In contrast, in the LSD1•2-PCPA complex, the adduct has been modeled as a 5-membered ring in which both the C4a atom and the N5 atom are covalently bonded to the inhibitor⁶⁶ (PDB ID: 2UXX). There may be an N5 adduct in the LSD1•2-PCPA complex as well⁶⁷ (see PDB IDs 2Z5U and 2Z3Y for a 5-membered-ring adduct model and an N5 adduct, respectively). The adduct structure is affected by differences in

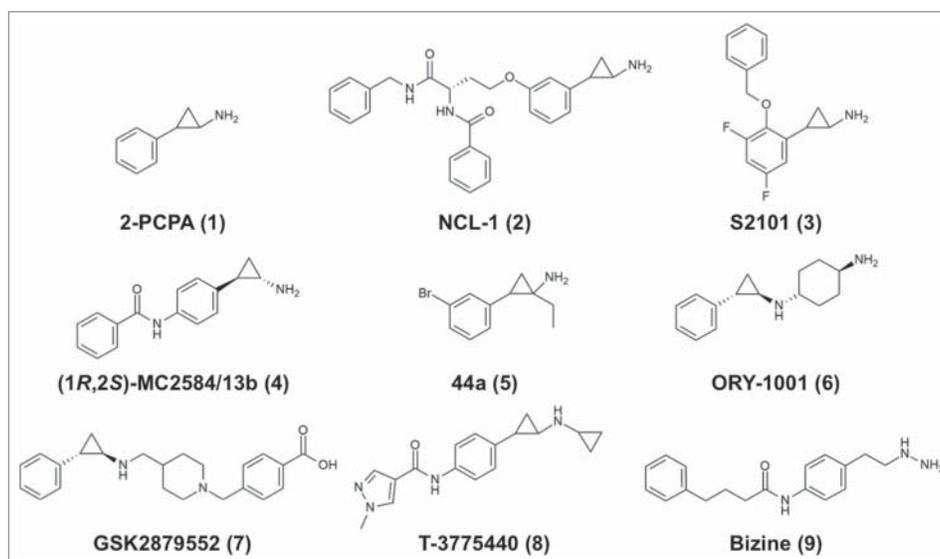


Figure 2. Chemical structures of representative covalent LSD1/KDM1A inhibitors.

Table 2. Summary of covalent-binding LSD1/KDM1A inhibitors.

Compound name	LSD1 inhibition (nM)	PDB ID	Resolution (Å)	Reference
2-PCPA derivatives				
2-PCPA (1)	500,000 ^a 100,000 ^a	2UXX 2Z5U, 2Z3Y	2.75 2.25	66 65,67
2-PCPA stereoisomers	284,000 ^a , 168,000 ^a	2XAH, 2XAJ	3.1, 3.3	77
Br-2-PCPA stereoisomers	28,000 ^a , 23,000 ^a	2XAG, 2XAF	3.1, 3.2	77
NCL-1 (2)	2,500 ^b			64
(1S,2R)-NCL-1	1,600 ^b			84
S1201	2,400 ^a	3ABU	3.1	65
S2101 (3)	610 ^a			65
MC2584/13b	1,100 ^a	2XAQ	3.2	77
(1R,2S)-MC2584/13b (4)	13 ^b			85
MC2584/13b derivative	84 ^b			87
MC2580/14e	1,300 ^a	2XAS	3.2	77
(S,1S,2R)-MC2580/14e	30 ^b			86
MC2580/14e derivative	61 ^b	4UXN	2.85	88
OG-L002	20 ^b			78
1-substituted derivatives	131 ^b	4UVC ^d	3.1	90
1-substituted "44a" (5)	31 ^b			91
N-alkylated 2-PCPA derivatives				
RN-7	31 ^b			92
NCL-1 derivative	380 ^b			95
ORY-1001 (6)	18 ^b			21
GSK2879552 (7)	1,700 ^c			94
T-3775440 (8)	2.1 ^b , 20 ^b			20
Other inhibitors				
Bizine (9)	59 ^a			103
Propagyl-K4-H3 (1–21)	120 ^a	2UXN	2.72	33

^aK_i; ^bIC₅₀; ^cK_i^{app}; ^dRelated structures: 4UVA, 4UVB, 4UV8, 4UV9.

the steric environment near the flavin ring, such as structural differences between the LSD1 and MAO-B cavities and structural differences between the various inhibitors (e.g., 2-PCPA substituents and the configuration of the cyclopropyl carbons; described below).

Because 2-PCPA has two chiral centers on the cyclopropylamine ring, there are four possible stereoisomers. Racemic *trans*-2-PCPA is in clinical use as a MAO-inhibiting antidepressant (brand name Parnate; Table 1), but investigation of the effects of its chirality is important in the pursuit of more potent and selective inhibitors.⁸³ Binda et al. examined the potencies of the four stereoisomers of 2-PCPA and found that *trans*-(1R,2S)-PCPA is a more potent inhibitor of LSD1 than *trans*-(1S,2R)-PCPA and that the two *trans*-enantiomers are more potent than the two *cis*-enantiomers.⁷⁷ These investigators also found that the structures of the FAD•2-PCPA adducts of the two *trans*-enantiomers differ from each other. *trans*-(1R,2S)-PCPA forms an N5 adduct in which the phenyl ring extends "vertically" (PDB ID: 2XAJ), whereas *trans*-(1S,2R)-PCPA forms an N5 adduct in which the phenyl ring extends "laterally" (PDB ID: 2XAH). The structure of the FAD adduct with racemic *trans*-2-PCPA in the LSD1 complex^{66,67} corresponds to the "vertical" N5 model of *trans*-(1R,2S)-PCPA, which is consistent with the higher potency of *trans*-(1R,2S)-PCPA relative to the other *trans*-isomer.⁷⁷

Specific stereoisomers of other compounds that were initially tested as racemic mixtures have also been prepared, including (1S,2R)-NCL-1⁸⁴, (1R,2S)-MC2584/13b (4, Fig. 2),⁸⁵ and (S,1S,2R)-MC2580/14e⁸⁶ (Table 2). A specific stereoisomer

of an MC2584/13b derivative with a 4-methylpiperazine substituent was prepared and found to be orally active, with an IC₅₀ of 84 nM against LSD1.⁸⁷ In addition, the stereochemistry of MC2580/14e was simplified by preparation of derivatives in which the chiral amino acid moiety was replaced with an *N*-carbobenzyloxy-proline residue (PDB ID: 4UXN) and with pyrrole or indole derivatives. The resulting compounds showed higher LSD1-inhibitory activity compared with MC2580/14e; specifically, a pyrrole derivative showed an IC₅₀ of 32 nM against LSD1.⁸⁸

The atoms on the cyclopropyl core moiety of 2-PCPA can also be modified.^{89,90} A structure-activity relationship study of α -substituted cyclopropylamines identified a compound (5, Fig. 2) with an IC₅₀ of 31 nM.⁹¹ It is noteworthy that the structure of the FAD adducts depends on the substituents on the cyclopropyl ring carbon atoms and on their chirality, as indicated by crystal structures.⁹⁰

N-alkylated 2-PCPA derivatives that inhibit LSD1/KDM1A

Oryzon Genomics reported that LSD1 inhibition could be substantially improved by using *N*-alkylated 2-PCPA derivatives, which exhibit nanomolar LSD1-inhibitory potency (WO 2010/043721). ORY-1001 (6, Fig. 2) has an IC₅₀ of 18 nM and greater than 1000-fold selectivity for LSD1 over LSD2 and MAOs.²¹ Two Oryzon inhibitors have been proven to be efficacious for the treatment of MLL-AF9 AML cells.¹⁸ Other *N*-alkylated compounds, RN-1 and RN-7, have been demonstrated to be brain penetrant, and inhibition of LSD1 by high concentrations of these compounds reportedly impairs long-term memory formation.⁹² Selectivity for LSD1 may be critical to this result, as indicated by the fact that MAOs are highly expressed in the brain. RN-1 is also selective for LSD1 over LSD2 and reportedly suppresses the growth of AML cells.⁹³

GSK2879552 (7, Fig. 2) and two of its analogues, GSK-LSD1 and GSK2699537, are LSD1-inhibiting *N*-alkylated compounds that were developed through biochemical screening of a library of 2.5 million compounds by researchers at GlaxoSmithKline.⁹⁴ A kinetics study showed that the mechanism involves reversible binding followed by time-dependent irreversible inactivation. GSK2879552 is selective for LSD1 over LSD2 and MAOs and is orally bioavailable. The crystal structure of the LSD1•GSK2699537 complex shows the formation of a C4a-type adduct similar to that of the MAO-B•2-PCPA complex.⁸² AML cell lines and small cell lung cancer (SCLC) cell lines are sensitive to LSD1 inhibition by these compounds.⁹⁴ Several *N*-alkylated inhibitors, including ORY-1001, ORY-2001, GSK2879552, INCB-059872, IMG-7289, and CC-90011, are currently in clinical trials for treatments of cancers, such as AML, SCLC, myelodysplastic syndrome (MDS), and non-Hodgkin's lymphoma (NHL), and neurodegenerative diseases (Table 1).^{21,22}

Recently, Takeda Pharmaceuticals reported another *N*-alkylated 2-PCPA derivative as an LSD1 inhibitor.²⁰ T-3775440 (8, Fig. 2) inhibits LSD1 irreversibly *in vitro* with an IC₅₀ of 2.1 or 20 nM in the presence or absence of CoREST, respectively (Table 2), and exhibits rapid antiproliferative and pro-apoptotic activities against acute erythroid leukemia (AEL) and acute megakaryoblastic leukemia (AMKL) cells.²⁰

The reason for the enhanced potency of *N*-alkylated 2-PCPA analogues is not clearly understood. *N*-alkyl substitution without modification of the aryl substituents enhances inhibitory activity and selectivity for LSD1 over MAOs (WO 2010/043721).^{92,95,96} The adduct produced by the reaction between FAD and the *N*-alkylated 2-PCPA analogues is lacking the amine group,^{94,95,97} which is in accordance with the proposed reaction mechanism.⁹⁸ Further studies will be necessary to shed light on the role of the *N*-substituent in conferring high potency against LSD1.

Covalent LSD1/KDM1A inhibitors other than 2-PCPA derivatives

In the early stages of the research on LSD1 inhibitors, the MAO inhibitor pargyline was found to also exhibit LSD1-inhibitory potency,³⁵ and hence it has been used as an LSD1 inhibitor. However, pargyline is a nonselective inhibitor of LSD1, and in most of the early research, pargyline was used at high doses, which makes interpretation of the results problematic.⁹⁹ An H3 peptide-based inhibitor bearing a propargylated K4 side chain is an effective irreversible inhibitor ($K_i = 120$ nM).^{33,100} Using the propargyl group, Schmitt et al. pursued the development of cell-active LSD1-specific inhibitors.¹⁰¹ Another MAO inhibitor that also inhibits LSD1 is phenelzine, which has a hydrazine that covalently binds to FAD.^{61,102} Structure-activity relationship studies on phenelzine analogues resulted in the development of bizine (**9**, Fig. 2), which inactivates LSD1 with a K_i of 59 nM and selectivity for LSD1 over LSD2 and MAOs.¹⁰³

H3 peptide-based inhibitors have specificity for LSD1 over MAOs. Peptide-based irreversible inhibitors have been developed using propargylamine, hydrazine, or 2-PCPA as the moiety that reacts with FAD.^{100,102,104} Because the SNAIL1 can inhibit the binding of the H3 N-terminal peptide,⁴⁶ SNAIL1 peptide-based inhibitors have been explored. These peptidic inhibitors exhibit specificity for LSD1 over not only MAOs but also LSD2,¹⁰⁵ which is consistent with the fact that SNAIL1 binds to LSD2 with lower affinity than to LSD1.⁴⁶

Noncovalent inhibitors of LSD1/KDM1A

In terms of clinical utility, reversible inhibitors would be preferred because they are considered to have a safer metabolic profile than irreversible inhibitors. However, only a limited number of noncovalent LSD1 inhibitors have been developed thus far. Typical examples of reversible LSD1 inhibitors are shown in Fig. 3, and kinetic parameters and structural information (where available) are summarized in Table 3.

Polyamine analogues such as bisguanidine and biguanide have been reported to noncovalently inhibit LSD1.¹⁰⁶ Their bisurea and bithiourea derivatives also reportedly inhibit LSD1, with an IC_{50} of approximately 5 μ M for the most potent compound.¹⁰⁷ Guanidinium-based compounds such as CBB1007 have been designed to mimic R2 and R8 of H3 in the LSD1•H3(1–21) structure. CBB1007 reportedly inhibits LSD1 with an IC_{50} of approximately 5 μ M but does not inhibit LSD2.¹⁰⁸ Apart from the polyamine analogues and guanidinium compounds, Namoline is a small-molecule compound reported to inhibit LSD1 selectively over MAOs, although its potency is not high ($IC_{50} = 51$ μ M).¹⁰⁹ Several research groups have developed other noncovalent LSD1 inhibitors.^{110–118} The IC_{50} values of some of these compounds are only in the micromolar range, however, inhibitors with submicromolar inhibitory potency have recently started to appear (Table 3).¹¹⁹

Noncovalent LSD1 inhibitors with submicromolar potency include a compound designated SP2509/HCl2509, which contains a benzohydrazide scaffold, inhibits LSD1 at an IC_{50} of 13 nM and has specificity for LSD1 over MAOs.¹²⁰ This compound has been shown to impede the growth of Ewing sarcoma tumor cells¹²¹ and AML cells.¹²² Optimization of the structure by means of the introduction of conformational restraints resulted in a benzohydrazide derivative **5a** (**10**, Fig. 3) with an IC_{50} of 1.4 nM against LSD1 and antiproliferation effects against LSD1-overexpressing cancer cells, including A2780 ovarian cancer cells.¹²³ GSK354/GSK690 (referred to in ref. 119), which inhibits LSD1 with an IC_{50} of 80 nM and shows selectivity for LSD1 over LSD2 and MAOs, has recently been demonstrated to be effective against AML.⁹³ Wu et al.

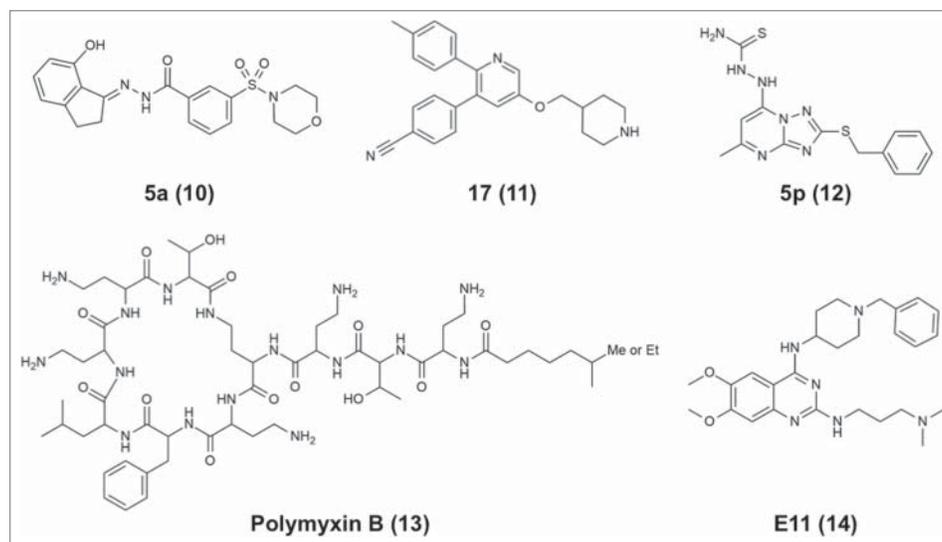


Figure 3. Chemical structures of representative noncovalent LSD1/KDM1A inhibitors.

Table 3. Summary of noncovalent-binding LSD1/KDM1A inhibitors.

Compound name	LSD1 inhibition (nM)	PDB ID	Resolution (Å)	Reference
Polyamine analogues				
Verlindamycin	13,000 ^b			106,107
Bisthiourea "6d"	4,800 ^b			107
Small-molecule inhibitors				
CBB1007	5,270 ^b			108
Namoline	51,000 ^b			109
Amidoxin "22"	16,800 ^b			110
Phenyl oxazole "9a"	9,500 ^b			111
Aminothiazole "16k"	7,500 ^b			112
SP2509/HCI2509	13 ^b			120
Benzohydrazide "5a" (10)	1.4 ^b			123
GSK354/GSK690	90 ^b			119d
Pyridine containing "17" (11)	29 ^a			96
XZ09	2,410 ^b			116
Pyrimidine-thiourea "6b"	650 ^b			117
Triazole-based "26"	2,100 ^b			113
Triazole-based "8k"	390 ^b			114
Triazole-based "6"	1,192 ^b			115
Triazole-based "5p" (12)	154 ^b			118
E11 (14)	440 ^a	5L3E	2.8	126
MC3767	not determined ^a	5LBQ	3.3	126
Natural products				
Resveratrol	15,000 ^b			128
Geranylgeranoic acid	22,220 ^b			129
Polymixin B (13)	157 ^a	5L3F	3.5	126
Polymixin E	193 ^a	5L3G	3.1	126
Baicalin	3,010 ^b			130
Peptidic inhibitors				
H3K4M (1–21)	40 ^a , 50 ^a	2V1D	3.1	32
SNAIL peptide (1–20)	210 ^a	2Y48	3.0	46
SNAIL peptide PRSFLV	28,400 ^a	3ZMT ^c	3.1	132

^a K_i ; ^b IC_{50} ; ^cRelated structures: 3ZMS, 3ZMU, 3ZMV, 3ZMZ, 3ZN0, 3ZN1; ^dReview.

independently developed an almost identical pyridine-containing molecule (11, Fig. 3) with a piperidine ring at one end instead of the pyrrolidine ring of GSK354/GSK690, and this new molecule inhibits LSD1 with a K_i of 29 nM and shows anti-tumor activity against four tumor cell lines.⁹⁶ Finally, Wang et al. recently reported that a [1,2,4]triazolo[1,5-a]pyrimidine-based derivative 5p (12, Fig. 3) inhibited LSD1 with an IC_{50} of 154 nM.¹¹⁸

The structure of LSD1 in a noncovalent complex with tetrahydrofolate (THF) is interesting. THF binds to LSD1 with moderate affinity ($K_D = 19.5 \mu\text{M}$ for (6S)-THF), and when THF is polyglutamated, K_D increases to $2.77 \mu\text{M}$. However, THF does not exhibit inhibitory activity against LSD1.¹²⁴ The crystal structure of LSD1•CoREST complexed with THF (PDB ID: 4KUM)¹²⁵ shows that the THF molecule is located deep in the substrate-binding cavity, occupying the place of the H3K4 side chain. Luka et al. suggest that THF may scavenge formaldehyde molecules generated during demethylation of LSD1, because these investigators found other THF-bound enzymes. They also argue that the natural substrate peptide such as the N-terminal tail of histone H3 may bind to LSD1 in a different conformation from that observed in the crystal structure of LSD1 with an H3(1–21)-derived peptide in which the K4 residue has been replaced by methionine (H3K4M(1–21)).¹²⁵

Recently, Sperazini et al. reported two groups of LSD1 inhibitors and their binding modes.¹²⁶ The first group is composed of polymyxin antibiotics, which have a circular peptide moiety with a branched linear aliphatic chain. Polymyxin B (13, Fig. 3)

and another similar compound, polymyxin E, inhibit H3K4M binding to LSD1 with K_i values of 157 and 193 nM, respectively. Crystallographic analysis (PDB IDs: 5L3F and 5L3G) demonstrated that these antibiotics bind at the entrance of the LSD1 substrate-binding cavity, possibly interacting with negatively charged regions comprising D555, D556, and E379. The molecules may be able to bind to the site in multiple orientations, as indicated by the lack of electron density for the linear branch of the molecules. The second group reported by Sperazini et al. is composed of quinazoline derivatives. One of the quinazoline derivatives E11 (14, Fig. 3) inhibits methyltransferase G9a and G9a-like protein with an IC_{50} of 778 nM against G9a-like protein.¹²⁷ E11 binds to LSD1 with a K_D of 243 nM and inhibits association of the H3K4M peptide with a K_i of 440 nM.¹²⁶ Crystallographic analysis demonstrated that a stack of E11 molecules binds at the entrance of the cavity. In the crystal structure with E11 (PDB ID: 5L3E), five layers of the molecule are modeled. MC3767, a truncated version of E11 with much lower affinity for LSD1 ($K_D = 7.78 \mu\text{M}$), still exhibits stacked binding (PDB ID: 5LBQ). Therefore, E11 may bind to LSD1 as a stack of oligomers, whereas it binds to G9a as a monomer. In addition to the above-mentioned antibiotics, other LSD1-inhibiting natural products, such as polyphenols,¹²⁸ geranylgeranoic acids,¹²⁹ and baicalin,¹³⁰ may be useful as scaffolds for developing potent reversible LSD1 inhibitors; however, polyphenols may affect the peroxidase assay used to detect LSD1 catalytic activity, so the results should be interpreted with caution.

Peptides can also inhibit LSD1, although their poor cell permeability is of concern. For example, an H3K4M(1–21) peptide increased LSD1-binding affinity relative to the native H3 peptide ($K_i = 40\text{--}50 \text{ nM}$ *in vitro*).³² A modified H3K4M(1–21) peptide in which K5 and E10 are macrocyclized inhibits LSD1 with an IC_{50} of $2.1 \mu\text{M}$ and is more stable than a linear peptide.¹³¹ As mentioned above, the SNAG domain of SNAIL1 (residues 1–20) binds to the substrate-binding site of LSD1 with a K_i of 210 nM, and the binding mode has been elucidated⁴⁶ (PDB ID: 2Y48). In addition, the SNAG domain of another SNAG-containing protein, INSM1, also binds to LSD1 with similar affinity ($K_i = 240 \text{ nM}$; PDB ID: 3ZMS). The N-terminal hexapeptide of SNAIL1 (PRSFLV) is sufficient to bind to LSD1 ($K_i = 28.4 \mu\text{M}$), and P1 and R2 are crucial for binding (PDB ID: 3ZMT). Mutation of F4 to methionine increases the affinity approximately 10-fold ($K_i = 2.6 \mu\text{M}$ *in vitro*).¹³²

Inhibitors of LSD2/KDM1B

Although many LSD1 inhibitors have been developed, the number of reported LSD2 inhibitors is limited. Because LSD2 is the only other known FAD-dependent demethylase, compounds have been assayed against it during the development of LSD1 inhibitors for the purpose of assessing inhibitor specificity. Inhibitors that potently and specifically inhibit LSD2 over LSD1 have not yet been reported.

Early studies demonstrated that the K_i of 2-PCPA against LSD2 ($180 \mu\text{M}$) *in vitro* is on the same order of magnitude as the K_i against LSD1 ($242 \mu\text{M}$).¹² Pargyline, deprenyl (Selegiline), and rasagiline do not inhibit LSD2,¹² but there is one report

indicating that LSD2 may be the target of pargyline inhibition.⁵⁰ Investigation of inhibition by stereoisomers of 2-PCPA and *para*-brominated 2-PCPA⁷⁷ demonstrated that these compounds have similar inhibitory potencies against LSD1 and LSD2, and that *para*-brominated PCPA isomers have higher potency than 2-PCPA isomers against both LSD1 and LSD2. However, there are subtle differences in the inhibitory behaviors of these compounds against LSD1 and LSD2. Against LSD1, the *cis*-2-PCPA isomers are generally weaker inhibitors than the *trans*-isomers, whereas against LSD2, the inhibitory ranges of the *cis*- and *trans*-isomers are the same. One enantiomer, (1*R*,2*R*)-(-)-*cis*-2-PCPA, shows higher inhibitory activity against LSD2 ($K_i = 68 \mu\text{M}$) than against LSD1 ($K_i = 506 \mu\text{M}$), and its *para*-brominated analogue remains more active against LSD2 ($K_i = 21 \mu\text{M}$) than against LSD1 ($K_i = 44 \mu\text{M}$).⁷⁷

When bulky substituents are attached to the *para*-position of the phenyl ring of *trans*-2-PCPA,⁷⁷ the resulting compounds are selective for LSD1 over MAO-B. Their inhibitory potencies against LSD1 are higher by one order of magnitude than those of the *para*-brominated derivatives, whereas no improvement in potency against LSD2 is observed. An interesting exception is MC2581/14l, in which a tryptophanyl group replaces the phenylalanyl group of MC2580/14e. MC2581/14l shows enhanced potency against LSD2 but not against LSD1; the K_i value for LSD2 ($12 \mu\text{M}$) is slightly better than that for LSD1 ($40 \mu\text{M}$).⁷⁷ *Meta*-substituted 2-PCPA analogue NCL-1 (**3**, Fig. 2) inhibits LSD2 with a K_i of $26 \mu\text{M}$ and LSD1 with a K_i of $2.5 \mu\text{M}$.^{133,134} A series of MC2584/13b benzamide-cyclopropylamine derivatives was also tested *in vitro* against LSD1, LSD2, MAO-A, and MAO-B.⁸⁷ Whereas all the tested derivatives exhibited submicromolar IC_{50} values for LSD1 (20–300 nM), their potencies for LSD2 ranged from 4.3 to $75 \mu\text{M}$; that is, they were specific for LSD1 over LSD2. However, some derivatives with bulky substituents show >10-fold enhanced potency against LSD2 compared with that of the parent inhibitor, resulting in IC_{50} values of approximately $5 \mu\text{M}$. Thus, bulky substituents on the 2-PCPA phenyl ring generally confer selectivity for LSD1. However, this approach may permit enhancement of LSD2-inhibitory activity and thus facilitate the development of pan-KDM1 inhibitors with selectivity over MAOs, or even the development of inhibitors with selectivity for LSD2.

Kakizawa et al. showed that substitution of 2-PCPA derivatives with lysine-mimicking bulky groups on the nitrogen atom has a detrimental effect on LSD2 inhibition, making the resulting inhibitors more specific for LSD1 over LSD2.¹³⁴ This result indicates that substituents on the amine group also affect LSD2 specificity. It is noteworthy that when these investigators initially tried to conduct a mass spectrometry-based *in vitro* enzymatic assay using recombinant LSD2, the demethylase activity was so weak that the LSD2-inhibitory activities of the 2-PCPA derivatives could not be accurately evaluated.¹³⁴ When Kakizawa et al. used the demethylation activity-enhancing NPAC dodecapeptide in their assay, they were able to evaluate the LSD2-inhibitory activities; the IC_{50} value of 2-PCPA was 2.3 mM .¹³⁴ This result indicates that evaluation of the inhibitory activity of 2-PCPA against LSD2 may be hampered by the low catalytic activity of LSD2 as well as the low potency of the inhibitor.

Future directions for the development of LSD2/KDM1B-specific inhibitors

In the Protein Data Bank, there is an entry for LSD2 in complex with an NPAC peptide and 2-PCPA (PDB ID: 4GUU),⁵⁶ although to our knowledge the structure of this complex has not been described in the literature. The structure shows that LSD2•2-PCPA contains a 5-membered-ring adduct. However, superimposition of the LSD2•2-PCPA complex with the LSD1•2-PCPA complex (PDB ID: 2Z5U)⁶⁷ shows that the phenyl ring of the FAD•2-PCPA adduct in the former complex extends in a slightly different direction than that in the latter complex. This difference appears to be due to steric hindrance between the phenyl ring of 2-PCPA and N440 in LSD2, whereas the corresponding residue in LSD1 is T335, which has a smaller side chain than N440 (Fig. 4). The superimposition also shows amino acid differences between LSD1 and LSD2 in the vicinity of the isoalloxazine ring of FAD, which may also affect inhibitor binding. These residues include N440 in LSD2 and T335 in LSD1 (hereafter, the corresponding residues in LSD1 are shown in parentheses), I438 (V333), Y545 (F538), and Q803 (A809). It is noteworthy that in all cases, the residue in LSD2 has a bulkier side chain than the corresponding residue in LSD1, making the catalytic cavity of LSD2 smaller than that of LSD1. This structural feature is consistent with the assay results showing that 2-PCPA derivatives with bulky substituents generally have reduced inhibitory activities against LSD2 but maintain their LSD1-inhibitory activities.^{77,87,134}

Overall, the amino acid sequences and tertiary structures of the catalytic domains of LSD1 and LSD2 are similar. However, a few of the amino acid differences mentioned above may confer selectivity in the binding of inhibitors. Structural analyses of LSD2 in complexes with 2-PCPA derivatives with various shapes can be expected to facilitate the development of LSD2-specific inhibitors. The surface structures of the AOD in LSD1 and LSD2 differ considerably.⁵⁵ Furthermore, only LSD2 has

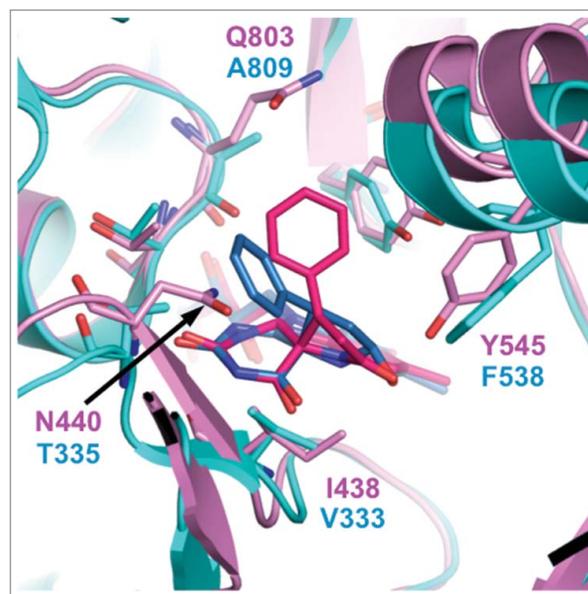


Figure 4. Superimposition of the catalytic cavities of LSD1/KDM1A (cyan) and LSD2/KDM1B (magenta) in complex with 2-PCPA. The FAD•2-PCPA adduct in the LSD1•2-PCPA complex (PDB ID: 2Z5U) is depicted in blue, and that in the LSD2•2-PCPA complex (PDB ID: 4GUU) is depicted in red.

the NPAC-binding site⁵⁶ and the second H3-binding site between the CW domain and the SWIRM domain.⁵⁸ These features may be useful for the development of LSD2-specific inhibitors.

Given that the crystal structures of LSD1 complexes with reversible inhibitors have been elucidated,¹²⁶ structural information on their binding modes and possible additional druggable sites will be useful in developing additional LSD1-specific inhibitors or in developing LSD2-specific inhibitors that are useful for studying chemical biology of LSD2. Pan-KDM1 inhibitors with potency and selectivity for both LSD1 and LSD2 over other FAD-containing enzymes may also be developed and can be expected to be useful for drug discovery.

Conclusions

The discovery of FAD-dependent demethylases and α -keto-glutarate-dependent demethylases enabled researchers to modulate epigenetic histone methylation by controlling the reverse (i.e., demethylation) process. Herein, we have reviewed published small-molecule inhibitors for LSD1/KDM1A and pan-KDM1 inhibitors. The inhibitors can be categorized as either irreversible or reversible, and the irreversible inhibitors can be classified as 2-PCPA derivatives, *N*-alkylated 2-PCPA derivatives, or non-2-PCPA-type derivatives, based on the chemical structures of their FAD-inactivating functional groups. Some of the irreversible inhibitors show high selectivity and potency toward LSD1, and several of them are currently in clinical trials for the treatments of cancers and neurodegenerative diseases. That is, the development of LSD1-specific inhibitors has generally been successful, and the prospects for drug discovery are promising. In contrast, few LSD2-specific inhibitors have been discovered, although some compounds have shown selectivity for inhibition of LSD2, as described herein. Additional information about LSD1 and LSD2 in complexes with their natural ligands or synthetic compounds can be expected to aid the development of more potent, ligand-efficient, and target-specific inhibitors for these FAD-dependent lysine demethylases.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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