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Identification of Cell-Active Lysine Specific Demethylase 1-Selective Inhibitors

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The methylation status of histone lysine residues, which is tightly controlled by two counteracting enzyme families, the histone methyl transferases and the histone demethylases, plays a pivotal role in the regulation of gene expression.[1] Lysine specific demethylase 1 (LSD1), the first histone demethylase to have been discovered, removes the methyl groups from mono- and dimethylated Lys4 of histone H3 (H3K4me1/2) through flavin adenine dinucleotide (FAD)-dependent enzymatic oxidation (Figure 1a).[2]

LSD1-selective inhibitors are useful as tools for elucidating in detail the biological functions of the enzyme.[3] To date, only a few types of LSD1 inhibitors have been identified. Monoamine oxidase (MAO) inhibitors such as *trans*-2-phenylcyclopropylamine (PCPA) (Figure 1b) and pargyline have been reported to inhibit LSD1, although their inhibitory activity and selectivity for LSD1 are very low.[4] Among MAO inhibitors, PCPA is the most potent LSD1 inhibitor. LSD1 inhibition by PCPA occurs via formation of a covalent adduct at the N5 and C4a positions of the flavin ring following one-electron oxidation and cyclopropyl ring opening (Figure 1b). *N*-Propargyl lysine-containing H3 peptides (Figure 1c) have been reported to be LSD1-selective inhibitors.[5] The mechanism of LSD1 inhibition by the peptides involves conjugate addition of the flavin N5 to the γ carbon of the electrophile following two-electron oxidation of the iminium ion (Figure 1c). The propargyl lysine peptides are selective for LSD1 over MAO-B and can be used as biochemical tools for in vitro study of LSD1. However, it is difficult to use peptide inhibitors for cellular studies because of their poor membrane permeability. In addition to the inhibitors mentioned above, polyamine analogues have been reported,[6] although their selectivity for LSD1 over other flavin-containing oxidases was not examined. Therefore, LSD1-selective inhibitors that show activity in in vivo assays are still required for elucidation of the cellular functions of LSD1. Herein we report the LSD1-inhibitory activity, selectivity, inhibitory mechanism, and cellular activity of small molecules designed on the basis of the reported X-ray crystal structures of LSD1.

In designing small-molecule LSD1-selective inhibitors, we focused on the X-ray crystal structures of the FAD−PCPA adduct[4a] and the FAD−*N*-propargyl lysine peptide adduct[5b] in the active site of LSD1. Figure 2a shows the superimposition of the two structures. The FAD parts of the two adducts are well-superimposed, and the benzene ring of the FAD−PCPA adduct overlaps with the ε-N and δ-C of the FAD−*N*-propargyl lysine peptide adduct. On the basis of these superimposed structures, we designed PCPA−lysine hybrid compounds **1** and **2** (Figure 2b) in which the side chain of the amino acid is linked with the phenyl ring of PCPA through an ether bond at the meta and para positions, respectively. We chose benzylamino and benzoyl groups as substituents of the carbonyl and amino groups of the amino acid, respectively, since they are expected to be recognized by hydrophobic amino acid residues (Val 333, Ile 356, Phe 382, Leu 386, Leu 536, Ala 539, Thr 566, and Leu 677) at the entrance to the N-methylated lysine binding channel of LSD1 (Figure S1a in the Supporting Information). In addition, the attachment of these small, hydrophobic groups should enhance the membrane permeability. Furthermore, compounds **1** and **2** were expected to selectively inhibit LSD1 over MAO-A and MAO-B, as the X-ray crystal structures of MAO-A[7] and MAO-B[8] indicate that their active-site cavities are not capacious enough to accommodate the large group attached to the phenyl ring of PCPA in compounds **1** and **2** (Figure S1b,c). Compounds **3** and **4** (Figure

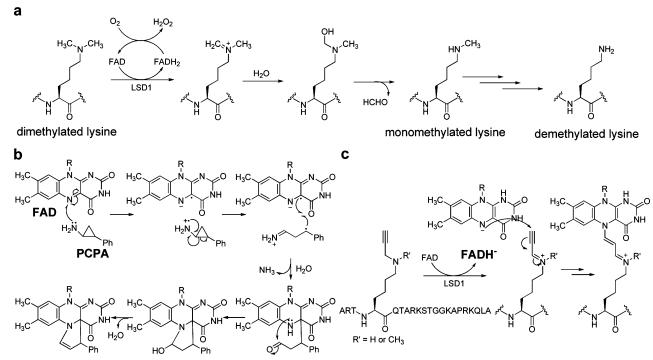

Figure 1. (a) Proposed catalytic mechanism for the demethylation of methylated lysine by LSD1. (b) Proposed mechanism of inactivation of LSD1 by PCPA. (c) Proposed mechanism of inactivation of LSD1 by *N*-propargyl lysine peptides.

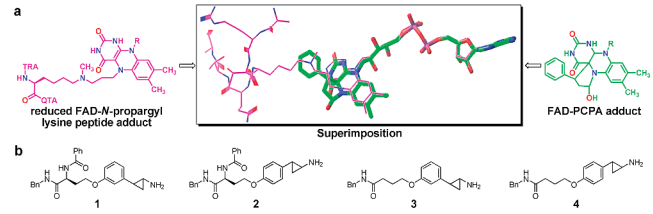

Figure 2. (a) Superimposition of the FAD−PCPA adduct (PDB entry 2UXX) (green tube) and the reduced FAD−*N*-propargyl lysine peptide adduct (PDB entry 2UXN) (magenta wire) in the active site of LSD1. Amino acid residues in the active site are not shown for the sake of clarity. (b) Structures of compounds **1**−**4**.

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Table 1. In Vitro LSD1-, MAO-A-, and MAO-B-Inhibitory Activities of Compounds **1** and **2** and PCPA

	IC_{50} (μM)			selectivity[a]	
cmpd	LSD1	MAO-A	MAO-B	MAO-A/LSD1	MAO-B/LSD1
PCPA	32	7.3	4.3	0.23 (1)	0.13 (1)
1	2.5	230	500	92 (400)	200 (1500)
2	1.9	290	>1000	150 (650)	>520 (>11000)

[a] Numbers in parentheses are the selectivity values divided by the (MAO IC_{50})/(LSD1 IC_{50}) value for tranylcypromine.

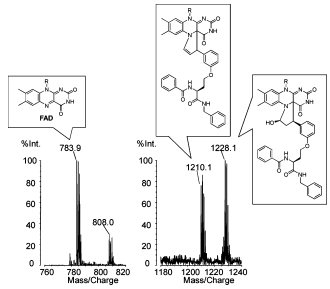

Figure 3. Mass spectrometric detection of the FAD−**1** adduct.

2b), which lack the α-amino moiety of compounds **1** and **2**, were designed as reference compounds.

Compounds **1**−**4** were synthesized, and their inhibitory activities toward human LSD1 and MAO-A and -B were evaluated. The results are summarized in Table 1 and Table S1 as IC_{50} values (also see Figure S2). The LSD1-inhibitory activities of compounds **1**−**4** were more potent than that of PCPA (IC_{50} values: PCPA, 32 μM; **1**, 2.5 μM; **2**, 1.9 μM; **3**, 22 μM; **4**, 9.7 μM). Furthermore, while PCPA inhibited MAO-A and MAO-B more potently than LSD1 [(MAO-A IC_{50})/(LSD1 IC_{50}) = 0.23; (MAO-B IC_{50})/(LSD1 IC_{50}) = 0.13], compounds **1**−**4** inhibited LSD1 more potently than MAO-A and MAO-B [(MAO-A IC_{50})/(LSD1 IC_{50}) = 2.8 to 150; (MAO-B IC_{50})/(LSD1 IC_{50}) = 1.0 to >520]. In particular, the LSD1 selectivity of compounds **1** and **2** was 400 to >11000 times higher than that of PCPA while compounds **3** and **4** showed less selectivity, indicating the importance of the amino acid structure of the inhibitors for potency and selectivity toward LSD1.

To investigate the mechanism of LSD1 inhibition, we initially examined whether inhibition by compound **1** or **2** is time-dependent. The time course of product formation was monitored in the absence or presence of compound **1** or **2**. As shown in Figure S3, compounds **1** and **2** were found to be time-dependent inhibitors of LSD1, showing nonlinear progress curves and reaching a plateau value. These data suggest that compounds **1** and **2** are irreversible inhibitors. The values of k_{inact}, K_I, and k_{inact}/K_I for compounds **1**, **2**, and PCPA were obtained from kinetic assays using LSD1, MAO-A, and MAO-B (Table S2). The k_{inact}/K_I values toward LSD1 for compounds **1** and **2** are much larger than those toward MAO-A and MAO-B, while the k_{inact}/K_I value toward LSD1 for PCPA is smaller than those toward MAO-A and MAO-B, confirming that compounds **1** and **2** are highly selective for LSD1.

To gain further mechanistic insight, a mass spectroscopic analysis of an incubation mixture of LSD1 with compound **1**, the most potent inhibitor in this study, was performed. If compound **1** reacts with FAD as expected, an FAD−**1** conjugate should be generated. As depicted in Figure 3, while the peak for FAD was observed at *m*/*z* 783.9, significant peaks at *m*/*z* 1228.1 and 1210.1 were also observed. These peaks correspond to the predicted molecular weights of the FAD−**1** adduct. Furthermore, they were not detected in the absence of LSD1 (Figure S4). These results indicate that compound FAD−**1** adduct was generated as a result of LSD1-catalyzed reaction of compound **1** and FAD. The data from the kinetics and mass spectroscopic analyses support the idea that compound **1** inactivates LSD1 by mechanism-based enzyme inhibition in a manner similar to PCPA.

Unlike peptide inhibitors, compounds **1** and **2** are small molecules that might be active in cellular assays. We performed a cellular assay using Western blot analysis. Since LSD1 is known to catalyze the demethylation of H3K4me2, the methylation level of H3K4 in HEK293 cells was analyzed. As Figure S5 shows, the level of H3K4me2 was dose-dependently elevated in the presence of **1** or **2**. These results suggest that compounds **1** and **2** inactivate LSD1 in cells and can be used as tools for probing the biological role of LSD1.

Since it has been reported that RNAi-mediated knockdown of LSD1 suppresses the growth of tumor cells,[9] we carried out cell-growth inhibition assays of compounds **1** and **2**, the most selective and active compounds in this study, using HEK293 cells. Cell-growth suppression by the inhibitors was observed over the concentration range in which distinct H3K4 methylation was detected in the Western blot analysis (Figure S6). Thus, the demethylase function of LSD1 appears to be deeply involved in cell growth. Next, we evaluated growth inhibition by inhibitors **1** and **2** against various other human cancer cell lines (Table S3 and Figure S7). Compounds **1** and **2** exhibited growth inhibition with GI_{50} values ranging from 6.0 to 67 μM, suggesting that LSD1-selective inhibitors are anticancer-agent candidates.

Thus, we have identified the first cell-active LSD1-selective inhibitors **1** and **2**, which should be useful as lead structures in the development of more potent and selective LSD1 inhibitors through modification of the benzoyl and benzylamino groups. Such inhibitors are anticancer-agents candidate as well as tools for studying the biological roles of LSD1 in cells.

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Supporting Information Available: Experimental procedures, including spectral data for compounds **1**−**4** and biological methods, and supporting figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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