Evaluation of phenylcyclopropylamine compounds by enzymatic assay of lysine-specific demethylase 2 in the presence of NPAC peptide

Taeko Kakizawa,^a Tamio Mizukami,^b Yukihiro Itoh,^c Makoto Hasegawa,^b Ryuzo Sasaki^b and Takayoshi Suzuki^{c,*}

^aDepartment of Chemistry and Biochemistry, School of Advanced Science and Engineering, Waseda University, Shinjuku, Tokyo 169-8555, Japan

^bGraduate School of Bio-Science, Nagahama Institute of Bio-Science Technology, 1226 Tamura-cho, Nagahama, Shiga 526-0829, Japan

^cGraduate School of Medical Science, Kyoto Prefectural University of Medicine, 1-5 Shimogamohangi-cho, Sakyo-ku, Kyoto 606-0823, Japan

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*Corresponding author. Tel and fax: +81-75-703-4937; e-mail: suzuki@koto.kpu-m.ac.jp

Abstract— Lysine-specific demethylase 2 (LSD2) demethylates mono- and dimethylated Lys-4 of histone H3 (H3K4me1 and H3K4me2). NPAC protein is known to interact with LSD2 and promote its H3K4 demethylase activity. In this study, we established a demethylation assay system that utilizes recombinant LSD2 in the presence of a synthetic NPAC peptide. Several phenylcyclopropylamine (PCPA)-based inhibitors were examined for their LSD2 inhibitory activity in the LSD2 enzymatic assay with the NPAC peptide. The assay results showed that the PCPA derivatives, including NCD41, selectively inhibited LSD1 in preference to LSD2.

Lysine-specific demethylase 1 (LSD1) and LSD2 are flavin adenine dinucleotide (FAD)-dependent LSD family proteins that demethylate mono- and dimethylated Lys-4 of histone H3 (H3K4me1 and H3K4me2).¹ As LSD1 is an important target in cancer therapy, many groups have pursued the development of LSD1 inhibitors.² A majority of the previously reported LSD1 inhibitors are the derivatives of phenylcyclopropylamine (PCPA) (Figure 1). PCPA per se is known to inhibit several flavin-dependent enzymes, such as LSD1 and monoamine oxidases (MAOs), by forming the FAD-PCPA adduct through the reaction with FAD in the active site of the enzyme (Figure 2).² We have identified PCPA-based LSD1 inhibitors, such as NCL1^{3,4} and the NCD series of compounds^{5,6} (Figure 1) that efficiently inhibit LSD1 in preference to MAOs. However, the LSD2 inhibitory activities of NCL1 and NCDs have not yet been tested due to the weak demethylase activity of recombinant LSD2. Thus, we

Figure 1 Chemical structures of PCPA, NCL1, NCD18, NCD25, NCD33, and NCD41.

Figure 2. Proposed mechanism of flavin-dependent enzyme inhibition by PCPA.

have been interested in the LSD2-inhibitory activity of the inhibitors. Recently, Yang *et al.* disclosed that LSD2 possesses unexpected E3 ubiquitin ligase activity and inhibits lung cancer cell growth by promoting the ubiquitylation and degradation of *O*-linked *N*-acetylglucosamine transferase (OGT), and indicated that the inhibition of LSD2 may disrupt these pathways.⁷ They also suggested that LSD1 inhibitors that do not affect LSD2 activity may be effective as anticancer agents. We report herein the establishment of an LSD2 assay system by using a peptide and the evaluation of the LSD2 inhibitory activity of PCPA-based inhibitors, which led to the identification of selective inhibitors for LSD1 over LSD2.

Initially, we tried to conduct a mass-spectrometry-based *in vitro* enzymatic assay using recombinant LSD2⁸ to evaluate the inhibitory activity of several LSD1 inhibitors. However, the demethylase activity of recombinant LSD2 was so weak that we could not accurately evaluate the LSD2 inhibitory activity of the PCPA derivatives. Thus, further investigations were needed to establish an LSD2 enzyme assay system that would enable us to accurately evaluate the LSD2 inhibitory activity of our inhibitors.

Fang *et al.* characterized NPAC protein (also known as GLYR1) as an LSD2-specific cofactor that facilitates LSD2-mediated H3K4me1 and H3K4me2 demethylation.⁹ They also determined the crystal structures of LSD2 alone and LSD2 in complex with the NPAC protein in the absence and presence of a histone H3 peptide. The structures revealed that the NPAC protein stabilizes the interaction between LSD2 and the histone H3 peptide, and thus enhances the enzymatic activity of LSD2.¹⁰ Based on these reports, we synthesized an NPAC peptide and attempted to perform a demethylation assay with recombinant LSD2 to confirm that the demethylation activity of LSD2 is enhanced in the presence of the NPAC peptide. First, the dodecapeptide of NPAC (residues 214-225) was produced as a wild-type (WT) sequence of H₂N-DPHFHHFLLSQT-OH (Figure 3) by standard solid-phase peptide synthesis using the Fmoc strategy.^{11,12} Its

mutant (MT) peptide H₂N-DPHAHHFLLSQT-OH (Figure 3) was also prepared as a negative control whose cofactor activity has been reported to be lower than that of WT NPAC.⁹ Next, to confirm the enhanced activity of LSD2, the histone demethylation assay was carried out in 20 μ L of 50 mM HEPES-NaOH (pH 8.5) containing 0.1% BSA buffered solution at 37 $^{\circ}$ C for 1 h with 10 μ M H3K4me2 substrate peptide that bears a dimethylated lysine at position 4 of a 21-amino-acid LSD1 substrate peptide, in the absence or presence of 0.5 μ g of recombinant LSD2 and 0.5 μ g of NPAC peptide.¹³ Demethylation efficiency was estimated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) on the basis of the peak intensities of the products (Figure 4). The analysis of the substrate and demethylated product peptides is summarized in Figure 5. Compared with the three controls (Figure 5, entries 1 to 3), in the LSD2-NPAC system, LSD2 efficiently removed a methyl group from its substrate H3K4me2 to give H3K4me1 as the major product (Figure 5, entry 4). Interestingly, the addition of MT NPAC peptide also moderately accelerated the removal of the methyl groups by LSD2, although the activity of the MT NPAC peptide was lower than that of the WT NPAC peptide (Figure 5, entry 4 vs entry 5). The moderate activity of the MT NPAC peptide is due to the decrease in interaction between the MT peptide and the H3K4 substrate peptide.⁹

We then evaluated PCPA for its LSD2 inhibitory activity using the same assay conditions as those in entry 4 of Figure 5. As shown in Figure 6, PCPA dose-dependently decreased the amounts of the demethylated H3K4 products (mono- and nonmethylated H3K4 peptides), confirming the LSD2 inhibitory activity of PCPA.

WT : H₂N-DPHFHHFLLSQT-OH

MT : H₂N-DPHAHHFLLSQT-OH

Figure 3. Wild-type (WT) and mutant (MT) NPAC dodecapeptide (residues 214-225).

Figure 4. MALDI-TOF MS analysis of H3K4 peptides: (A) 0 µg of LSD2 and 0 µg of NPAC peptide, (B) 0.5 µg of LSD2 and 0 µg of NPAC peptide, and (C) 0.5 µg of LSD2 and 0.5 µg of NPAC peptide.

Figure 5. H3K4 demethylation by recombinant LSD2 in the presence or absence of LSD2 and NPAC peptide. Percentages of H3K4me2 peptide (blue), H3K4me1 peptide (red), and nonmethylated H3K4 (green) were measured by MALDI-TOF MS analysis. The % peptide abundance values are means of two separate experiments. The differences in duplicate experiments are within 10%.

Figure 6. LSD2 inhibition by PCPA. Percentages of H3K4me2 peptide (blue), H3K4me1 peptide (red), and nonmethylated H3K4 (green) were measured by MALDI-TOF MS analysis. The % peptide abundance values are means of two separate experiments. The differences in duplicate experiments are within 10%.

Table 1. LSD2 and LSD1 inhibitory activities of PCPA, NCL1, NCD18, NCD25, NCD33 and NCD41.

compound	IC ₅₀ (µM) ^a		selectivity LSD2 IC ₅₀ /LSD1 IC ₅₀
	LSD2	LSD1 ^b	
PCPA	2300 ± 1400	31 ± 12 ^c	74
NCL1	26 ± 10	2.5 ± 0.5 ^d	10
NCD18	60 ± 18	0.30 ± 0.13 ^c	200
NCD25	230 ± 120	0.48 ± 0.28 ^c	480
NCD33	250 ± 92	0.41 ± 0.08 ^c	610
NCD41	>540	0.58 ± 0.10 ^c	>930

^aValues are means of at least three experiments. ^bLSD1 inhibitory activity was examined using peroxidase-coupled assay (ref 3 and 4). ^cTaken from the literature (ref 5). ^dTaken from the literature (ref 3).

Next, using the LSD2 assay system with the NPAC peptide, the LSD2 inhibitory activities of several LSD1 inhibitors, NCL1, NCD18, NCD25, NCD33, and NCD41 (Figure 1), were evaluated and the IC₅₀ values determined.¹⁴ As shown in Table 1, the IC₅₀ value of PCPA was 2300 ± 1400 µM in the LSD2 assay system. LSD1 inhibitors NCL1 and NCD18 inhibited the demethylase activity of LSD2 with IC₅₀ values of 26 ± 10 µM and 60 ± 18 µM, respectively. NCD25 (IC₅₀ = 230 ± 120 µM) and NCD33 (IC₅₀ = 250 ± 92 µM) moderately inhibited LSD2. The inhibitory effect of NCD41 (IC₅₀ > 540 µM) was obviously weaker than those of the other inhibitors. These assay results showed that the LSD2-inhibitory activity of NCL1 is greater than that of NCD series of compounds while NCL1 is less active LSD1 inhibitor than NCDs, which leads to high LSD2-selectivity of NCD compounds as compared with NCL1. In addition, the assay data highlighted the significance of the biphenyl group in LSD1-selectivity (Table 1, NCD18 vs NCD25, NCD33 and NCD41). As a result, NCD41 was found to be the most selective inhibitor for LSD1 rather than LSD2. As mentioned above, LSD2 has been reported to inhibit lung cancer cell growth,⁷ and it has been suggested that compounds that selectively inhibit LSD1 over LSD2 may be desirable as anticancer agents. Therefore, the NCD series of compounds, including NCD41, are expected to serve as lead compounds for anticancer agents.

In summary, we developed an LSD2 enzymatic assay system that uses recombinant LSD2 and a synthetic NPAC peptide. In the assay system, an enhancement of the demethylation reaction by LSD2 was observed with the addition of the NPAC peptide by mass-spectrometry-based analysis. Several LSD1 inhibitors were evaluated using the LSD2 assay system. The LSD1 inhibitors inhibited LSD2 with relatively high IC₅₀ values, showing their high selectivity for LSD1. Further exploratory studies of selective LSD inhibitors using this LSD2 assay with the NPAC peptide are under way.

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- Recombinant His-tagged human full-length LSD2 was obtained using the following protocol.¹⁵ The AOF1 gene encoding human LSD2 (amino acids 1–822) was amplified by PCR using cDNA from HeLa cells as template with forward primer 5'-CAGTAATCATATGGCAACTCCACGGGGGAGGACAAAG-3' and reverse primer 5'-TAGTCTCGAGTTAAATGCTGCAATCTTGCTTG-3'. Underlines show restriction enzyme sites for *Nde* I and *Xho* I that were introduced into the forward and reverse primers, respectively. The PCR product was ligated into pET28b (Novagen Inc., Madison, WI) and digested with *Nde* I and *Xho* I, and the resultant plasmid was designated pETLSD2. *Escherichia coli* BL21 (DE3) cells were transformed with pETLSD2 for the expression of full-length LSD2 bearing a hexahistidine tag at the N-terminus. Recombinant *E. coli* BL21 (DE3) cells carrying pETLSD2 were grown in 200 mL of NZCYM medium containing 50 µg/mL kanamycin at 37 °C with shaking at 200 rpm. When the absorbance at 600 nm reached 0.7, the temperature was lowered to 16 °C and isopropyl-β-D-thiogalactopyranoside was added to make a final concentration of 0.5 mM. The culture was continued for a further 19 h with shaking at 110 rpm, and the cells were harvested by centrifugation at 3,100×g for 10 min at 4 °C. LSD2 was purified by chromatography through a HisTrap column. The partially purified LSD2 was dissolved in 50 mM HEPES-NaOH (pH 8.0) containing 5% glycerol, 1 mM PMSF, 1.5 µg/mL pepstatin A, and 1.5 µg/mL leupeptin, and stored at –30 °C until use.
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- NPAC dodecapeptides (WT: H₂N-DPHFHHFLLSQT-OH, MT: H₂N-DPHAHHFLLSQT-OH) were prepared by conventional solid-phase peptide synthesis using Fmoc strategy. The synthetic peptides were purified by reverse-phase HPLC and lyophilized to give white powders. The peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using Bruker autoflex with α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. WT peptide, MALDI-TOF MS calcd. for C₆₉H₉₆N₁₉O₁₈ [M+H]⁺ 1479.6420, obsd. 1479.857. MT peptide, MALDI-TOF MS calcd. for C₆₃H₉₂N₁₉O₁₈ [M+H]⁺ 1403.5440, obsd. 1403.347, respectively.
- The histone demethylation reaction for assaying LSD2 activity was carried out for 1 h at 37 °C in 20 µL of 50 mM HEPES-NaOH (pH 8.5) containing 0.1% BSA and 10 µM H3K4me2 peptide (chemically synthesized peptide consisting of the first 21 amino acid residues of histone H3, incorporating dimethylated lysine at position 4; Sigma-Aldrich) in the absence or presence of 0.5 µg of His-tagged full-length LSD2 and 0.5 µg of NPAC peptides. The reaction was stopped by adding 20 µL of matrix solution (5 mg/mL CHCA, 37% acetonitrile, and 0.12% trifluoroacetic acid), and then sonicated for 30 sec. One µL of the reaction mixture was spotted on the sample plate, dried, and analyzed by MALDI-TOF MS using JMS-S3000 (JEOL).
- IC₅₀ evaluation assays of LSD2 were carried out in 20 µL of buffered solution incubated for 1 h at 37 °C with 10 µM H3K4me2 substrate peptide, 1 µg of LSD2, 1 µg of NPAC peptide (WT), and inhibitors at several concentrations. Mass-spectrometry-based analysis was carried out using the above-mentioned method to evaluate the demethylation activity of LSD2. IC₅₀ values are means ± S.D. of at least three experiments; PCPA = 2300 ± 1400 µM, NCL1 = 26 ± 10 µM, NCD18 = 60 ± 18 µM, NCD25 = 230 ± 120 µM, NCD33 = 250 ± 92 µM, NCD41 > 540 µM.
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