Experimental

Molecular Modeling

Docking and subsequent scoring were performed using Macromodel 8.1 software. Coordinates of LSD1 complexed with FAD-*N*-propargyl lysine peptide adduct were taken from the Brookhaven Protein Data Bank (PDB code 2UXN) and hydrogen atoms were added computationally at appropriate positions. The structure of NCL1 (**4**) bound to LSD1 was constructed by molecular mechanics (MM) energy minimization. The starting position of compound **4** was determined manually: the amino acid part was superimposed on the active site of the lysine peptide counterpart. The conformation of compound **4** in the active site was minimized by MM calculation based upon the OPLS_AA force field with parameters set as follows: method: LBFGS, max. no. iterations: 10000, converge on: gradient, convergence threshold: 0.05.

Synthesis of Compounds 5 and 6

General Methods. Reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, and Kanto Kagaku and used without purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 GF-254, and visualization on TLC was achieved by UV light or iodine indicator. Flash column chromatography was performed using Silica Gel 60 (particle size 0.046–0.063 mm) supplied by Merck. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JEOL JNM-LA500 spectrometer in solvent as indicated. Chemical shifts (δ) were reported in parts per million (ppm) relative to the internal standard, tetramethylsilane. Coupling constants are reported in Hertz (Hz). Elemental analysis was performed with a Yanaco CHN CORDER NT-5 analyzer, and all values were within ±0.4% of the calculated values. Fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMS-SX102A mass spectrometer. Melting points were determined with a Yanagimoto micro melting point apparatus or a Büchi 545 melting point apparatus and were left uncorrected.

Synthesis of compound 5 (Scheme 1).

***tert*-Butyl (2-(3-((*S*)-3-benzamido-4-(benzylamino)-4-oxobutoxy)phenyl)cyclopropyl)carbamate (9).** To a solution of **8** (200 mg, 0.640 mmol), **7** (230 mg, 0.923 mmol), and PPh_3 (504 mg, 1.920 mmol) in dry THF (3 mL) was added DIAD (1 mL, 1.987 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature for 5 h. Concentration in vacuo and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 1/1) gave 64 mg (18%) of **9** as a yellow solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.82 (2H, d, *J* = 8.5 Hz), 7.52 (1H, t, *J* = 8.5 Hz), 7.46 (2H, t, *J* = 8.5 Hz), 7.26–7.21 (5H,

m), 7.16 (1H, t, *J* = 8.0 Hz), 6.92 (1H, s), 6.75 (1H, d, *J* = 8.0 Hz), 6.63 (1H, d, *J* = 8.0 Hz), 6.60 (1H, broad s), 4.88 (1H, q, *J* = 6.0 Hz), 4.47 (2H, d, *J* = 6.0 Hz), 4.32–4.29 (1H, m), 4.13–4.10 (1H, m), 2.70–2.65 (1H, m), 2.43–2.37 (2H, m), 1.99–1.97 (1H, m), 1.45 (9H, s), 1.14–1.10 (2H, m).

***tert*-Butyl (2-(3-((*S*)-3-amino-4-(benzylamino)-4-oxobutoxy)phenyl)cyclopropyl)(2-(4-methylpiperazin-1-yl)-2-oxoethyl)carbamate (11).**

To a suspension of 60% NaH (7.36 mg, 0.18 mmol) in dehydrated DMF (1 mL) at 0 °C was added compound **9** (50 mg, 0.09 mmol) and the mixture was stirred for 10 min. Then 1-(chloroacetyl)-4-methylpiperazine hydrochloride (**10**, 30 mg, 0.14 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. The progress of the reaction was monitored by TLC and after completion, reaction mixture was diluted with ice cold water and extracted with ethyl acetate. The organic layers were washed with water and brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Purification by column chromatography using $CHCl_3$ and methanol (50:1 to 20:1) afforded the desired product **11** as a colourless solid (42 mg, 67%): mp 70-72 °C; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.81 (d, *J* = 8.5 Hz, 2H), 7.55 (dt, *J* = 7.5, 1.2 Hz,1H), 7.45 (dt, *J* = 7.5, 1.2 Hz, 3H), 7.25-7.20 (m, 5H), 7.14 (t, *J* = 7.9 Hz, 1H), 7.03-6.95 (bs, 1H), 6.73 (d, *J* = 7.9 Hz, 1H), 6.63 (d, *J* = 8.2 Hz, 1H), 6.57-6.51 (bs, 1H), 4.95-4.84 (m, 1H), 4.45 (d, *J* = 5.7 Hz, 2H), 4.33-4.35 (m, 1H), 4.15-4.05 (m, 1H), 4.00-3.92 (m, 1H), 3.65-3.55 (m, 3H), 3.50-3.50 (m, 3H), 3.02-2.95 (m, 1H), 2.45-2.35 (m, 5H), 2.31 (s, 3H), 2.18-2.12 (m, 1H), 1.39 (s, 9H), 1.18-1.05 (m, 2H); MS (FAB): *m/z* 684 [M^++1].

***N*-((2*S*)-1-(benzylamino)-4-(3-(2-((2-(4-methylpiperazin-1-yl)-2-oxoethyl)amino)cyclopropyl)phenoxy)-1-oxobutan-2-yl)benzamide Hydrochloride (5·HCl).**

To a solution of **11** (50 mg, 0.07 mmol) in DCM (1 mL) was added 4N HCl in AcOEt (0.4 mL) at 0 °C and the mixture was stirred for 4 h at room temperature. The solvent was removed by evaporation, and the product was purified by recrystallization from $CHCl_3$ and MeOH to give compound **5** hydrochloride salt (35 mg, 75%) as colourless crystals: mp 153-155 °C; ^{1}H NMR (D_2O, 500 MHz, δ; ppm) 7.55 (d, *J* = 7.3 Hz, 2H), 7.46 (t, *J* = 7.6 Hz, 1H), 7.35 (t, *J* = 7.6 Hz, 2H), 7.18-7.10 (m, 4H), 7.07 (d, *J* = 7.0 Hz, 2H), 6.71 (dd, *J* = 8.2, 1.2 Hz, 1H), 6.65 (dd, *J* = 7.9, 4.5 Hz, 1H), 6.59-6.52 (m, 1H), 4.20-4.10 (m, 2H), 4.03 (bs, 2H), 3.80-3.70 (m, 1H), 3.45-3.30 (m, 5H), 3.05-2.84 (m, 4H), 2.82-2.76 (m, 2H), 2.75 (s, 3H), 2.40-2.31 (m, 1H), 2.30-2.29 (m, 1H), 2.20-2.10 (m, 1H), 1.41-1.32 (m, 1H), 1.20-1.10 (m, 1H); MS (FAB): *m/z* 585 [M^++2]; Anal. Calcd. For $C_{34}H_{43}Cl_2N_5O_4 \cdot 8/3H_2O$: C, 57.95; H, 6.91; N, 9.94. Found: C, 57.69; H, 6.96; N, 10.34.

Synthesis of compound 6 (Scheme 2).

***tert*-Butyl 2-(3-(methoxymethoxy)phenyl)cyclopropyl(2-(4-methylpiperazin-1-yl)-2-oxoethyl)carbamate (13).**

To a suspension of 60% NaH (14 mg, 0.068 mmol) in dry DMF (1 mL) at 0 ^{o}C was added compound **10** (100 mg, 0.34 mmol) and the mixture was stirred for 10 min. Then, 1-(chloroacetyl)-4-methylpiperazine hydrochloride (86 mg, 0.40 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. The progress of the reaction was monitored by TLC and after completion, reaction mixture was diluted with ice-cold water and extracted with ethyl acetate. The organic layers were washed with water and brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Purification by column chromatography using $CHCl_3$ and methanol (20:1) afforded the desired product **13** as colourless amorphas (115 mg, 78%). ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.15 (t, *J* = 8.0 Hz, 1H), 6.84 (ddd, *J* = 8.0, 2.5, 1.2 Hz, 1H), 6.72-6.69 (m, 2H), 5.15 (s, 2H), 4.21-4.15 (m, 1H), 4.01-3.95 (m, 1H), 3.68-3.60 (m, 2H), 3.46 (s, 3H), 3.45-3.40 (m, 2H), 3.03-2.98 (m, 1H), 2.45-2.35 (m, 4H), 2.30 (s, 3H), 2.20-2.12 (m, 1H), 1.40 (s, 9H), 1.15-1.05 (m, 1H), 0.90-0.80 (m, 1H); MS (FAB): *m/z* 434 [M^++1].

2-(2-(3-hydroxyphenyl)cyclopropylamino)-1-(4-methylpiperazin-1-yl)ethanone Hydrochloride (6·HCl).

To a solution of **13** (70 mg, 0.16 mmol) in DCM (1 mL) was added 4N HCl in AcOEt (0.4 mL) at 0 ^{o}C and the mixture was stirred for 4 h at room temperature. The solvent was removed by evaporation and the product was purified by recrystallization from $CHCl_3$ and MeOH to give compound **6** (35 mg, 75%) as colourless crystals: mp 145-147 ^{o}C; ^{1}H NMR (CD_3OD, 500 MHz, δ; ppm) 7.10 (t, *J* = 7.9 Hz, 1H), 6.67-6.61 (m, 2H), 6.58 (t, *J* = 2.1 Hz, 1H), 4.78-4.68 (m, 1H), 4.43-4.30 (m, 2H), 4.05-3.95 (m, 1H), 3.62-3.45 (m, 4H), 3.25-3.05 (m, 3H), 2.95-3.00 (m, 1H), 2.94 (s, 3H), 2.50-2.42 (m, 1H), 1.58-1.50 (m, 1H), 1.37-1.12 (m, 1H); MS (FAB): *m/z* 290 [M^++1]; HRMS (EI) *m/z*: calcd for $C_{16}H_{23}N_3O_2$ 289.1790, found $[M]^+$ 289.1777.

Purification of recombinant LSD1

The *AOF2* (amine-oxidase flavin-containing domain 2) gene encoding human LSD1 (1-852 aa) was amplified by PCR using the plasmid KIAA0601 obtained from Kazusa DNA Research Institute as a template. The PCR product was ligated into pET28b (Novagen) digested with *Nde* I and *Xho* I, and the resultant plasmid was designated pETAOF2. *Escherichia coli* BL21 (DE3) was

transformed with pETAOF2 for the expression of full-length LSD1 bearing a hexahistidine-tag at its N-terminus. Expression and purification of the His-tagged LSD1 were done as described previously[1] with some modifications. Recombinant *E. coli* BL21(DE3) cells containing pETAOF2 were grown in 200 mL of NZCYM medium, containing 50 μg/mL kanamycin at 37°C with shaking at 190 rpm. When the absorbance of 600 nm reached 0.9, the temperature was lowered to 25°C and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. The cultivation was continued for further 6 h with shaking at 180 rpm and the cells were harvested by centrifugation at 3,100 × *g* for 10 min at 4°C. Cells (4.1g obtained from 600 mL cultivation medium) were suspended in 80 mL of 50 mM sodium phosphate buffer, pH 7.5, containing 0.5 M NaCl, 20 mM imidazole, 5% glycerol, 0.01 mM FAD, 10 mM 2-mercaptoethanol, 1 mM PMSF, 3 μg/mL pepstatin A, and 3 μg/mL leupeptin, and sonically disrupted using a homogenizer (UD-201; Tomy). The suspension was centrifuged at 17,000×*g* for 1 h at 4°C. The supernatant was loaded on a HisTrap HP column (0.7×2.5 cm) (GE Healthcare) equilibrated with buffer A (20 mM sodium phosphate buffer, pH 7.5, containing 0.5 M NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol). Elution was done by linearly increasing the concentration of imidazole from 20 to 500 mM in buffer A at the flow rate of 0.8 mL/min. Fractions enriched in LSD1 were collected and concentrated. The enzyme solution was loaded on a HiLoad 16/60 Superdex 200 pg column (1.6×60 cm) (GE Healthcare) equilibrated with buffer B (50 mM sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl, 5% glycerol, 1 mM PMSF, 1 μg/mL pepstatin A, and 1μg/mL leupeptin) and fractionated with the same buffer at 1 mL/min. The purified LSD1 was dissolved in buffer C (buffer B without PMSF) and stored at –30°C until used. The protein concentration was measured by Lowry's method using BSA as a standard. The molar concentration of the recombinant LSD1 was calculated by using the molecular mass (95,084 Da) deduced from the amino acid sequence.

LSD1 inhibition assay

The LSD1 activity was assayed at 25°C by using the peroxidase-coupled method as described previously[2]. The chemically synthesized peptide consisted of the first 21 amino acid residues of histone H3, incorporating dimethylated lysine at position 4 (H3K4me2 peptide) (Sigma-Aldrich) was used as the substrate of LSD1. The reaction mixture contained 50 mM HEPES-NaOH, pH 7.5, 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 5.5 units/ml horseradish peroxidase, 20 μM H3K4me2 peptide, and appropriate amount of LSD1. To assess the

inhibitory effect of compounds **5** and **6** on the LSD1 activity in comparison with that of PCPA, the partially purified LSD1 obtained at the purification step of HisTrap HP chromatography was dissolved in buffer C and used. Compounds **5** and **6** were dissolved in DMSO. The final concentration of DMSO in the reaction mixture was adjusted to be 5%, and it was confirmed that 5% DMSO did not affect the LSD1 activity. Reaction without the inhibitors was also done as a control. Reaction mixtures (18 μL), containing all the materials except H3K4me2 peptide were first incubated for 5 min. Then reactions were started by adding 2 μl of 0.2 mM peptide solution into the assay mixtures. Absorbance at 515 nm was monitored for 30 min in 384-well plate (Nunc) by using a microplate reader (SpectraMax M2^e; Molecular Devices). Enzyme activity was determined from the linear part of the reaction curve. The ratio of the enzyme activity measured in the presence of inhibitor to the activity of the control was plotted against log[Inh].

MTT assays

The cells were plated at initial densities of 5,000 cells/well (50 μL/well) in 96-well plates in RPMI 1640 with 10% fetal bovine serum and allowed to attach overnight. The cells were exposed to inhibitors for 48 h at 37 °C in 5% CO_2 incubator. A solution (5mg/mL) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added (10 μL/well) and incubated with the cells for 3 h before solubilization buffer (0.04 mol/l HCl-isopropanol) was added (100 μL/well) onto the cultured cells. The solubilized dye was quantified by colorimetric reading at 560 nm using a reference wavelength of 750 nm. Absorbance values of control wells (C) and test wells (T) were measured. Moreover, absorbance of the test wells (T_0) was also measured at time 0 (addition of compounds). Using these measurements, cell growth inhibition (percentage of growth) by a test inhibitor at each concentration used was calculated as: % growth = 100 x $[(T - T_0)/(C - T_0)]$, when $T > T_0$ and % growth = 100 x $[(T - T_0)/T]$, when $T < T_0$. Computer analysis of the % growth values afforded the 50% growth inhibition parameter (GI_{50}). The GI_{50} was calculated as 100 x $[(T - T_0)/(C - T_0)] = 50$.

Western Blot Analysis.

The SH-SY5Y cells (500,000 cells) were treated for 24 h with inhibitors at the indicated concentrations, in 10% FBS-supplemented in cell culture medium, then collected and extracted with SDS buffer. The each lysate were resolved in 10–20% SDS-polyacrylamide gels and

transferred onto PVDF membranes. After blocking with TBS containing 5% skim milk, the transblotted membranes were probed with the transblotted membranes were probed with rabbit monoclonal H3K4me2 antibody (Abcam, #ab32356) (1:10000 dilution) and rabbit polyclonal H3 antibody (Abcam, #ab1791) (1:200000 dilution) in TBS-T. The membrane was probed with the primary antibody, then washed with TBS-T, incubated with ECL rabbit IgG, HRP-linked whole Anti-body (GE Healthcare Life Sciences, #NA934) (1:2500 dilution), and again washed with TBS-T. The immunoblots were visualized by enhanced chemiluminescence with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, #P90718).

Kinetic Analysis

To determine rates of inactivation of LSD1 by compound **5**, reactions were initiated by addition of enzyme to assay solutions containing substrate and varying concentrations of inhibitor in a manner similar to that used in the enzyme inhibition assays described above. Progress curves obtained in the presence of inhibitor were fitted to a single exponential for slow-binding inhibitors, assuming a steady-state velocity of 0[3]:

product = $v_0(1 - e^{-kt})/k$ + offset

The k_{obs} values were then analyzed by the method of Kitz and Wilson to obtain k_{inact} and K_I. The following equation was used to extract kinetic constants from the Kitz–Wilson analysis[4]:

$k_{obs} = (k_{inact}[I])/(K_I + [I])$

MALDI-TOF/MS Analysis

LSD1 (12 μM) was incubated with 100 μM compound **5** for 22 h at room temperature in 50 mM sodium phosphate buffer (pH 7.5), 4% glycerol and 1% DMSO. The control was run with FAD instead of the enzyme. The reaction mixtures (10 μL) were then denatured with 6 M guanidine hydrochloride (10 μL) and acidified with 1% TFA (5 μL). An aliquot of the reaction mixture (10 μL) was desalted and concentrated using ZipTip μ-C18 (Millipore) (eluted with 5 μL of 50% MeCN containing 0.1% TFA). The eluate was analyzed on a 5800 MALDI-TOF/TOF mass spectrometer (AB SCIEX). MALDI mass spectra were acquired between m/z 800 and 4000 with negative ion mode. α-Cyano-4-hydroxycinnamic acid (CHCA, LaserBio) was used as the MALDI matrix without further purification. CHCA was dissolved in 50% MeCN containing 0.1% TFA at a concentration of 5 mg/mL. Samples for MALDI-TOF MS analysis were prepared by mixing the

desalted reaction mixture (2 μL) with the matrix solution (2 μL). The applied samples (1 μL) were allowed to dry at room temperature.

References

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