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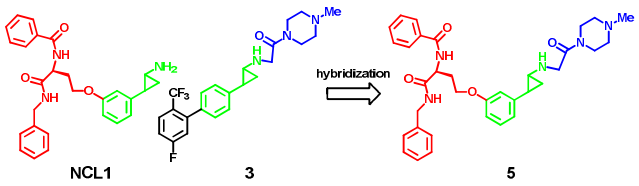

A hybridization of NCL1 and compound **5** led to the identification of a potent lysine-specific demethylase 1 inhibitor **5**.

Med. Chem. Commun.

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ARTICLE

Design, synthesis, and biological activity of *N*-alkylated analogue of NCL1, a selective inhibitor of lysine-specific demethylase 1

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2014,
Accepted ooth January 2014

DOI: 10.1039/xoxxoooooox

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Lysine-specific demethylase 1 (LSD1), the first histone demethylase to be identified, catalyzes specifically the demethylation of the mono- and dimethyl groups of histone H3 lysine 4, and its dysregulation is thought to contribute to the development of cancer. We have recently reported that NCL1 (**4**) is the first cell-active LSD1-selective inhibitor. To find LSD1 inhibitors that show higher potency than NCL1 (**4**), we designed and synthesized an *N*-alkylated analogue of NCL1 (**5**), and evaluated its biological activity. In enzyme assays, compound **5** was six times more potent than **4**, and compound **5** exhibited cell growth inhibition in cervical cancer HeLa cell line and neuroblastoma SH-SY5Y cell line. Compound **5** should be useful as a lead structure for anticancer drugs.

Introduction

Lysine-specific demethylase 1 (LSD1) catalyzes the oxidative demethylation of histone H3 methyllysine 4 (H3K4me1) and histone H3 dimethyllysine 4 (H3K4me2) through flavin adenine dinucleotide (FAD)-dependent enzymatic oxidation.[1] LSD1 also catalyzes the demethylation of H3K9me1/2 in prostate cell line[2] as well as the demethylation of non-histone proteins, such as p53,[3] DNA methyltransferase 1,[4] and E2F1,[5] and regulates their cellular functions. LSD1 is overexpressed in several cancers, including leukemia and solid tumors.[6,7] Therefore, LSD1 inhibitors are of interest not only as tools to elucidate the biological functions of the enzyme, but also as potential therapeutic agents.

Monoamine oxidase (MAO) inhibitors, such as *trans*-phenylcyclopropylamine (PCPA, **1**, Fig. 1) and pargyline (**2**, Fig. 1), have been reported to inhibit LSD1 irreversibly.[8] Among the MAO inhibitors, PCPA (**1**) is the most well studied LSD1 inhibitor, and biological studies of PCPA (**1**) have uncovered the important roles of LSD1 in several diseases.[6,9,10] PCPA (**1**) inhibits LSD1 through a single-electron transfer mechanism[11] (Fig. 2). In the active site of LSD1, FAD first abstracts one electron from the nitrogen of PCPA to yield a cation radical. Then, the cyclopropyl ring opening occurs, followed by covalent bond formation with FAD. Since the discovery that PCPA (**1**) is a potent mechanism-based inhibitor of LSD1, several research groups,[11-13] including ours,[14-17] have focused on the PCPA scaffold for the development of more potent and selective LSD1 inhibitors. For example, Oryzon Genomics reported *N*-alkylated PCPA analogues, including compound **3** (Fig. 1), as LSD1-selective inhibitors.[18] On the other hand, we reported a PCPA-lysine hybrid compound (NCL1, **4**, Fig. 1) as the first cell-active LSD1-selective inhibitor. The design of compound **4** was based on the X-ray crystal structures of the PCPA-FAD adduct and the FAD-*N*-propargyl

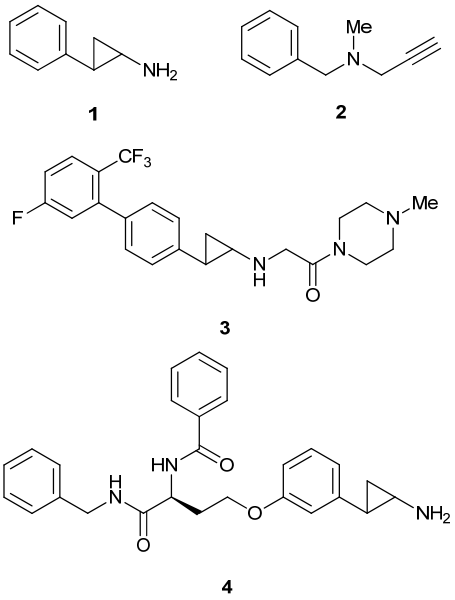


Fig. 1 Structures of phenylcyclopropylamine (PCPA, **1**), pargyline (**2**), *N*-alkylated PCPA analogue (**3**), and NCL1 (**4**).

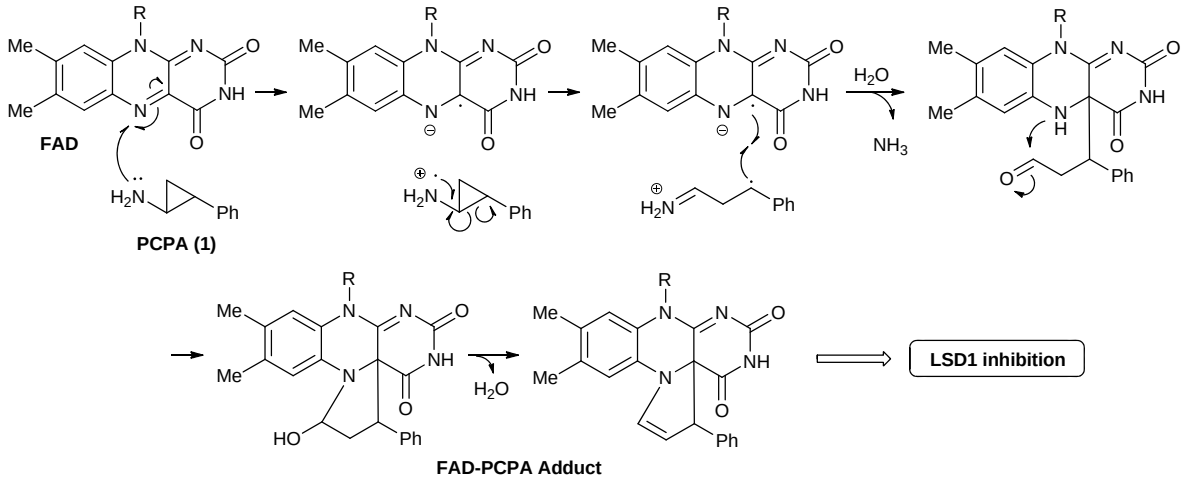


Fig. 2 Mechanism of LSD1 inhibition by PCPA (**1**).

lysine peptide adduct in the active site of LSD1.[14,15] Since then, we have been trying to identify LSD1 inhibitors that exhibit higher potency than NCL1 (**4**). In this report, we present the design, synthesis, LSD1 inhibitory activity, and cancer cell growth inhibitory activity of an *N*-alkylated NCL1 analogue.

Results and discussion

To identify LSD1 inhibitors that exhibit higher potency than NCL1 (**4**), we attempted to incorporate a piperazine acyl moiety into the structure of NCL1 (**4**) by *N*-alkylation of the cyclopropylamine functionality. We initially calculated the binding mode of NCL1 (**4**) complexed with LSD1, and found an additional space near the cyclopropylamine group of NCL1 (**4**) (Fig. 3). We hypothesized that the 1-acetyl-4-methylpiperazine moiety of compound **3** would fill the space, and the introduction of the 1-acetyl-4-methylpiperazine group on the nitrogen of NCL1 (**4**) would further enhance the interaction with the amino acid residues in the catalytic center (Fig. 4), thus leading to a potent LSD1 inhibitory effect. Hence, we developed a strategy for the design and synthesis of *N*-alkylated NCL1 analogue (**5**, Fig. 5) and its reference compound (**6**, Fig. 5), which lacks the side chain amino acid moiety.

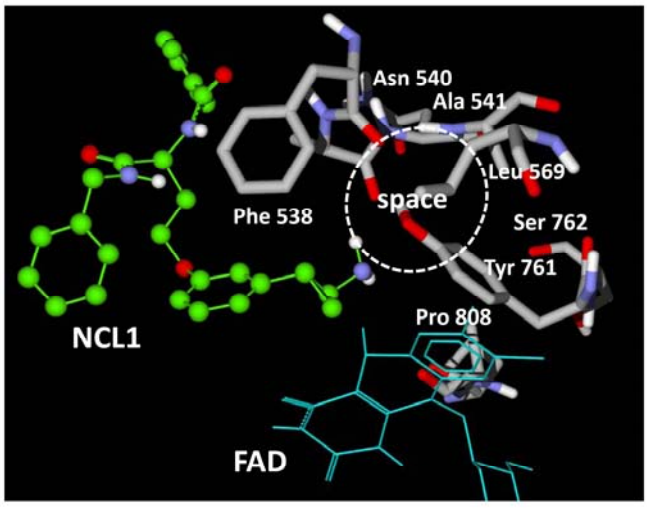


Fig. 3 View of conformation of NCL1 (**4**) (ball-and-stick) docked in the LSD1 catalytic core. Residues within 5 Å from the PCPA moiety of NCL1 (**4**) are displayed in the tube graphic, and FAD is displayed in the wire graphic.

The synthesis of compound **5** is illustrated in Scheme 1. Compound **9** was synthesized by carrying out the Mitsunobu reaction between compounds **7** and **8**, which were prepared by methods reported in our previous study.[14] Chloro compound **10** was coupled with **9** in the presence of NaH and DMF to afford **11**. Deprotection of the Boc group of **11** under the acidic condition furnished desired compound **5**.

The synthesis of compound **6** is shown in Scheme 2. Starting material **12** was prepared by employing our previously reported method.[14] Compound **12** was further coupled with **10** using NaH and DMF to provide compound **13**. Deprotection of the Boc group of **13** under the acidic condition afforded **6**.

The LSD1 inhibitory activities of compounds **5** and **6** were evaluated by using a previously described peroxide-coupled assay[14] that was conducted in the presence of a fixed concentration of substrate containing a dimethyllysine residue, and the results are summarized in Table 1. As we expected, *N*-alkylated NCL1 analogue (**5**) exhibited six times more potent LSD1 inhibitory activity than NCL1 (**4**) and analogue **6**. In addition, compound **5** did not inhibit MAO A or MAO B (IC_{50} > 100 μM) (data not shown) as is the case in NCL1 (**4**),[14] showing high selectivity for LSD1 over MAO A and MAO B. We also examined the cell growth inhibitory activity of those compounds in cervical cancer HeLa cell line and neuroblastoma SH-SY5Y cell line. As shown in Table 1, whereas compound **6,** which lacks a hydrophobic amino acid moiety, did not exhibit any growth

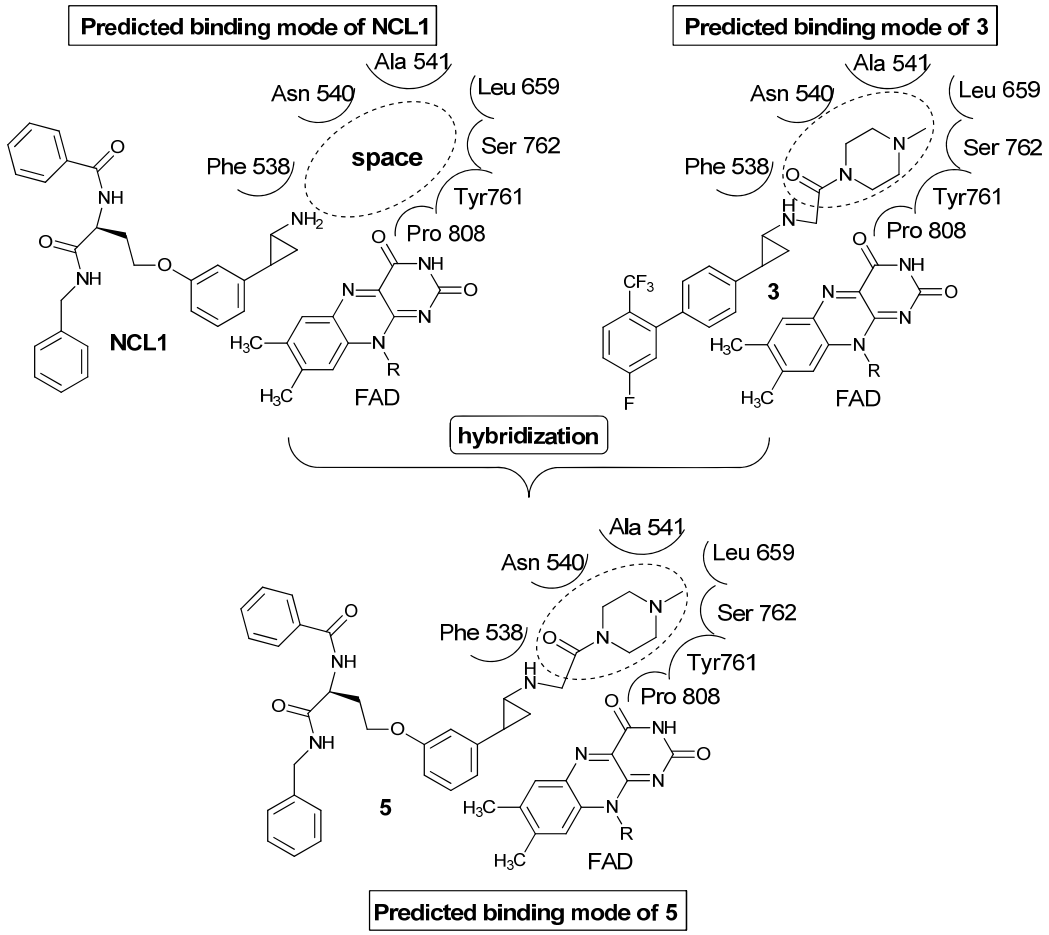


Fig. 4 Design of *N*-alkylated NCL1 analogue (**5**).

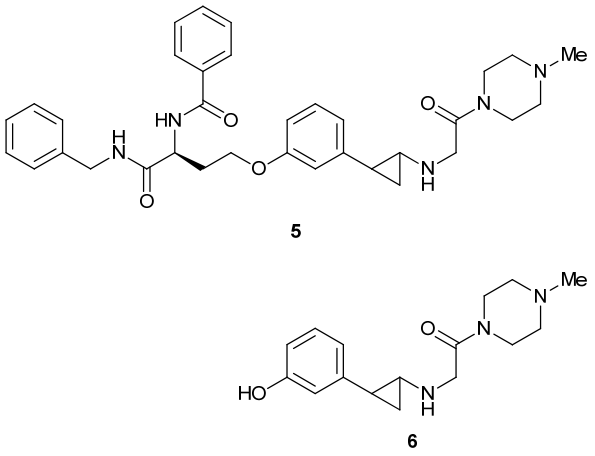


Fig. 5 Structures of compounds **5** and **6**.

inhibitory effect, compound **5** displayed comparable growth inhibitory effect to NCL1 (**4**) against neuroblastoma SH-SY5Y cell line in which LSD1 is overexpressed.[19] On the other hand, compound **5** was found to be 8-fold less potent than NCL1 (**4**) in HeLa cells as an antiproliferative agent. The reasons for the differences in results between *in vitro* LSD1 inhibition assays and cell-based assays and between HeLa cell line and SH-SY5Y cell line are not clear. Nevertheless, we surmise that the differences may be due to the sensitivity to LSD1 that complexed with proteins in the cells. LSD1 has been reported to bind to such proteins as HDAC1, CoREST, and TLX.[20] The activity in cell-based assays of LSD1 inhibitors may depend on which protein LSD1 binds to in cells. The binding of LSD1 to the proteins may alter the structure of the active site and affect the binding of inhibitors to LSD1. Regarding the weak antiproliferative activity of compound **6**, it is reasonable to assume that it was due to poor membrane permeability resulting from the highly polar character of this compound.

To examine LSD1 inhibitory activity in SH-SY5Y cells of compound **5**, we performed a Western blot analysis. As LSD1 is a demethylase of H3K4me2,[1] LSD1 inhibition was assessed by evaluating the accumulation of H3K4me2 in the cells. As shown in Figure 6, compound **5** induced an increase of H3K4me2 both at 10 μM and 30 μM, suggesting strong LSD1 inhibition in the cells.

Next, we investigated the LSD1 inhibitory mechanism to confirm that compound **5** is a mechanism-based LSD1 inhibitor. As shown in Fig. 7, compound **5** was expected to inhibit LSD1 in a similar manner to PCPA (**1**) (Fig. 2), i.e., through single-electron transfer, cyclopropane ring opening, and FAD-NCL1 adduct formation to release the 1-aminoacetyl-4-methylpiperazine moiety. We initially examined whether compound **5** inhibits LSD1 in a time-dependent

Table 1 LSD1 and cancer cell growth inhibitory activities of NCL1 (**4**), and compounds **5** and **6**.

	LSD1 inhibition	GI_{50} (μM)	
Compd	IC_{50} (μM)	HeLa	SH-SY5Y
5	0.38	109	42
6	2.4	>240	>240
NCL1 (4)	2.5	13	27

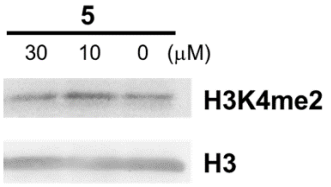
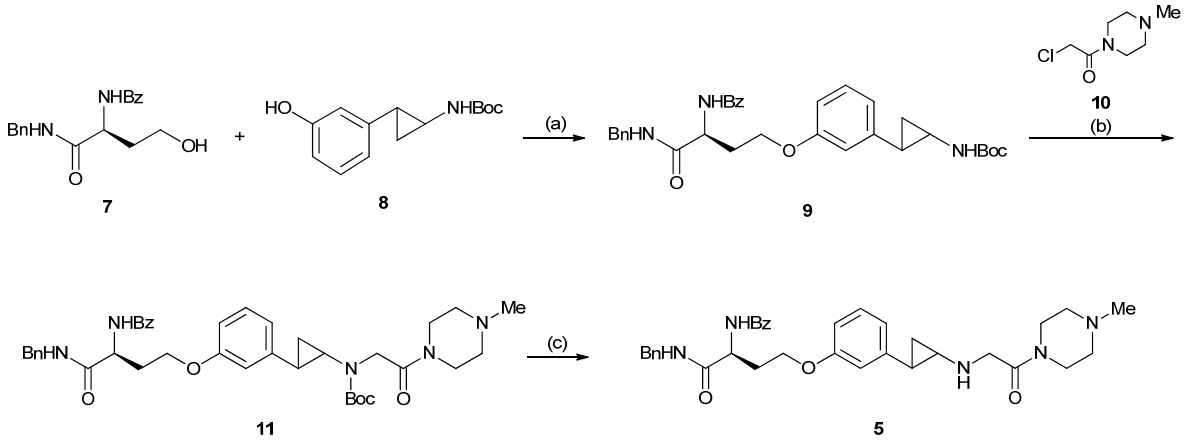
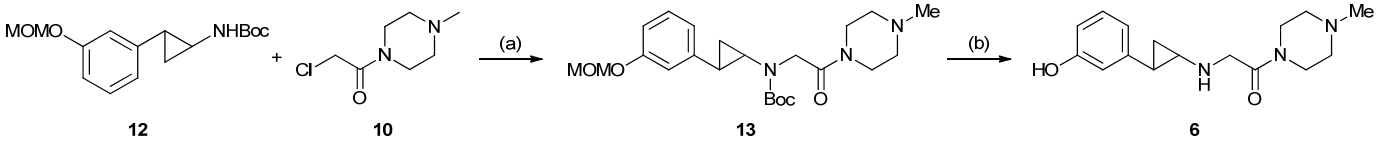


Fig. 6 Western blot analysis of H3K4me2 levels in SH-SY5Y cells after 24 h treatment with compound **5**.



Scheme 1 Synthesis of *N*-alkylated NCL1 analogue **5**. Reagents and conditions: (a) diisopropylazodicarboxylate, triphenylphosphine, THF, 0 °C, 1 h, rt, 5 h, 18%. (b) NaH, DMF, 0 °C to rt, 12 h, 67%. (c) 4 N HCl in EtOAc, DCM, 0 °C to rt, 4 h, 75%.



Scheme 2 Synthesis of reference compound **6**. Reagents and conditions: (a) NaH, DMF, 0 °C to rt, 12 h, 78%. (b) 4 N HCl in EtOAc, DCM, 0 °C to rt, 4 h, 75%.

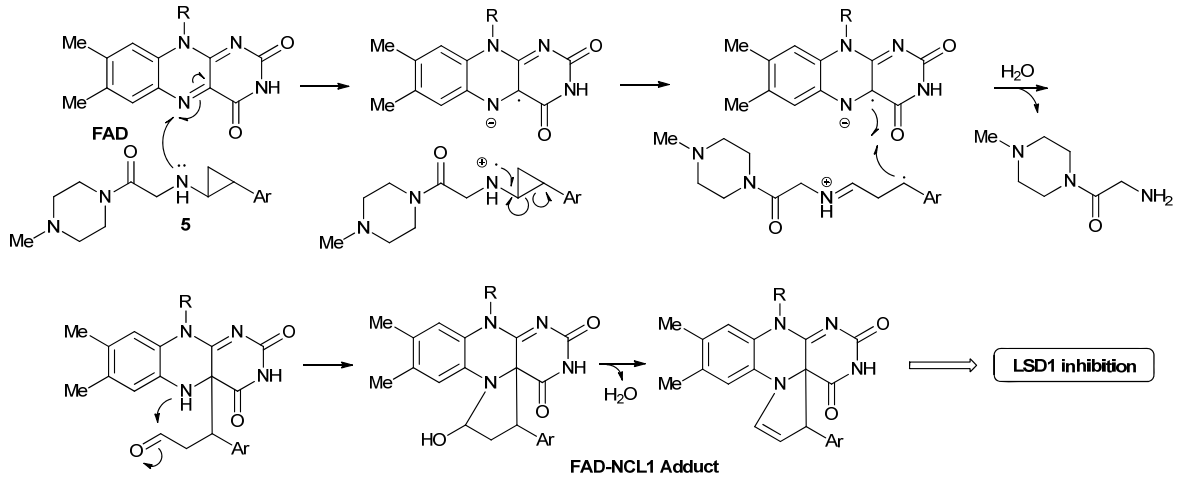


Fig. 7 Putative mechanism of LSD1 inhibition by compound **5**.

manner or not. As shown in Fig. 8, compound **5** was a time-dependent LSD1 inhibitor with K_i = 2.4 μM and k_{inact} = 0.0035 s^{-1}. The time-dependent LSD1 inhibition by compound **5** is in accordance with the irreversible mechanism we proposed (Fig. 7).[21] Next, to gain further insight into the mechanism of LSD1 inhibition by compound **5**, mass spectrometric analysis of a mixture of LSD1 incubated with compound **5** was performed. We expected that the inhibition of LSD1 by compound **5** would yield the FAD-NCL1 adduct (Fig. 7). As shown in Fig. 9, peaks with m/z 1228 and 1210, which correspond to the FAD-NCL1 adduct and the dehydrated one, respectively, were observed in the mass spectrum of the mixture of LSD1 and compound **5**. Such peaks were not observed in the absence of LSD1 (data not

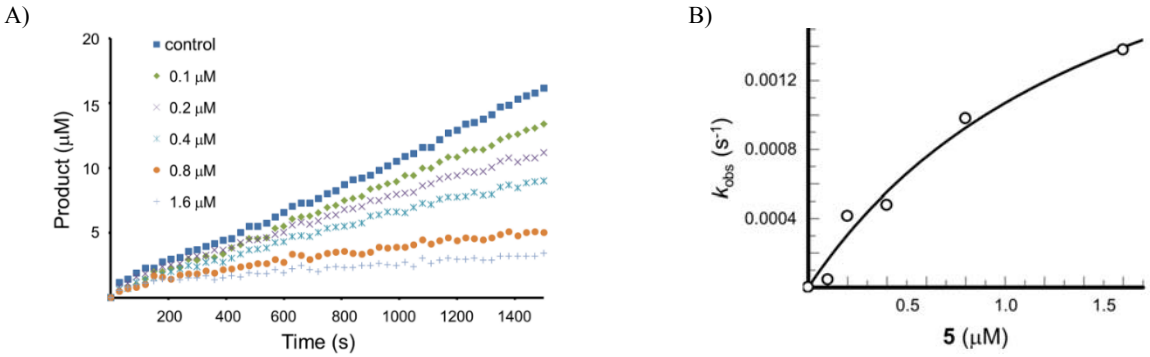


Fig. 8 Time- and concentration-dependent inhibition of LSD1 by **5**. A) Steady-state progress curves obtained for the inhibition of LSD1 by **5** at various concentrations. B) Rate constants (k_{obs}) for the time-dependent inactivation of LSD1 by **5** were extracted from the steady-state progress curves in (A) by single exponential fitting.

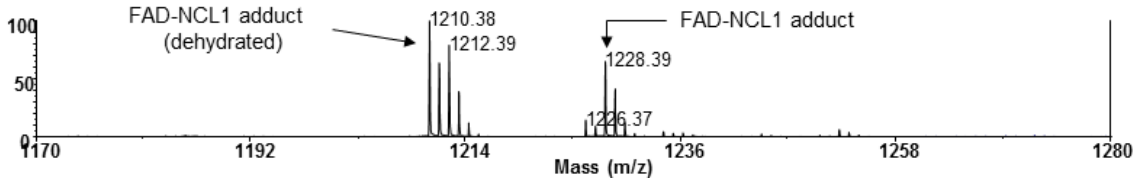


Fig. 9 Mass spectrum of FAD-NCL1 adduct.

shown). The data from the kinetic study and the mass spectrometric analysis strongly supported the LSD1 inhibitory mechanism by compound **5**, as shown in Fig. 7.

Conclusions

We have designed and synthesized an *N*-alkylated analogue of NCL1 (**5**), and evaluated its inhibitory effect on LSD1 and its growth inhibitory activities against human cancer cell lines. *N*-alkylated NCL1 analogue **5** exhibited six times more potent LSD1 inhibitory activity than reference compound **6**, which lacks an amino acid moiety, and NCL1 (**4**). Compound **5** also showed more potent antiproliferative activity than compound **6**. In addition, kinetic study and mass spectrometric analysis indicated that compound **5** is a mechanism-based LSD1 inhibitor. Additional studies on compound **5** are under way to gain more insight into its binding mode. Efforts are ongoing to find highly potent and selective inhibitors of LSD1.

Acknowledgements

We thank Ms. Mie Tsuchiya for technical support. The work described in this paper was partially supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (M.N.A., Y.I., T.S.), JST PRESTO program (T.S.), Takeda Science Foundation (T.S.), Naito Foundation (T.S.), and Terumo Life Science Foundation (T.S.).

Notes and references

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†Electronic Supplementary Information (ESI) available: Syntheses and analytical data of the test compounds; procedures for biological evaluation. See DOI: 10.1039/b000000x/

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