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Crystal structure of histone demethylase LSD1 and tranylcypromine at 2.25 Å

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Abstract

Transcriptional activity and chromatin structure accessibility are correlated with the methylation of specific histone residues. Lysine-specific demethylase 1 (LSD1) is the first discovered histone demethylase, which demethylates Lys4 or Lys9 of histone H3, using FAD. Among the known monoamine oxidase inhibitors, tranylcypromine (Parnate) showed the most potent inhibitory effect on LSD1. Recently, the crystal structure of LSD1 and tranylcypromine was solved at 2.75 Å, revealing a five-membered ring fused to the flavin of LSD1. In this study, we refined the crystal structure of the LSD1–tranylcypromine complex to 2.25 Å. The five-membered ring model did not fit completely with the electron density, giving R_{work}/R_{free} values of 0.226/0.254. On the other hand, the N(5) adduct gave the lowest R_{work}/R_{free} values of 0.218/0.248, among the tested models. These results imply that the LSD1–tranylcypromine complex is not completely composed of the five-membered adduct, but partially contains an intermediate, such as the N(5) adduct.

Keywords: Chromatin; Histone demethylase; Nucleosome; SWIRM domain; Transcription

Histones are subjected to several types of covalent modifications, such as acetylation, phosphorylation, ubiquitination, and methylation. These modifications alter the structure of chromatin, and provide binding platforms for adaptor proteins that specifically recognize them. Consequently, they regulate various processes involving DNA, such as transcription, replication, repair, and heterochromatin formation. A "histone code" hypothesis was proposed, in which combinations of histone modifications dictate distinct downstream biological phenomena [1].

Lysine-specific demethylase 1 (LSD1), a nuclear homolog of the amine oxidases, was the first histone demethylase to be discovered [2]. Using FAD as a cofactor, LSD1 oxidatively demethylates mono- and di-methyl lysines on histone H3 [2,3] (Fig. 1A). Consistent with its proposed reaction mechanism, which requires protonated nitrogen, LSD1 cannot demethylate tri-methyl lysine [2]. LSD1 was initially identified as a component of corepressor complexes [4–6]. In non-neural cells, LSD1 removes the transcriptionally active mark of histone H3 Lys4 (H3K4) methyl groups, thereby repressing neuron-specific genes [2]. Recombinant LSD1 alone cannot demethylate nucleosomal H3K4, and CoREST, another component of the complex, is required for the nucleosome-dependent demethylation [7,8]. LSD1 can also act as a transcriptional activator. Androgen receptor and LSD1 form a complex in a ligand-dependent manner and remove the transcriptionally repressive H3K9 methyl groups, thereby

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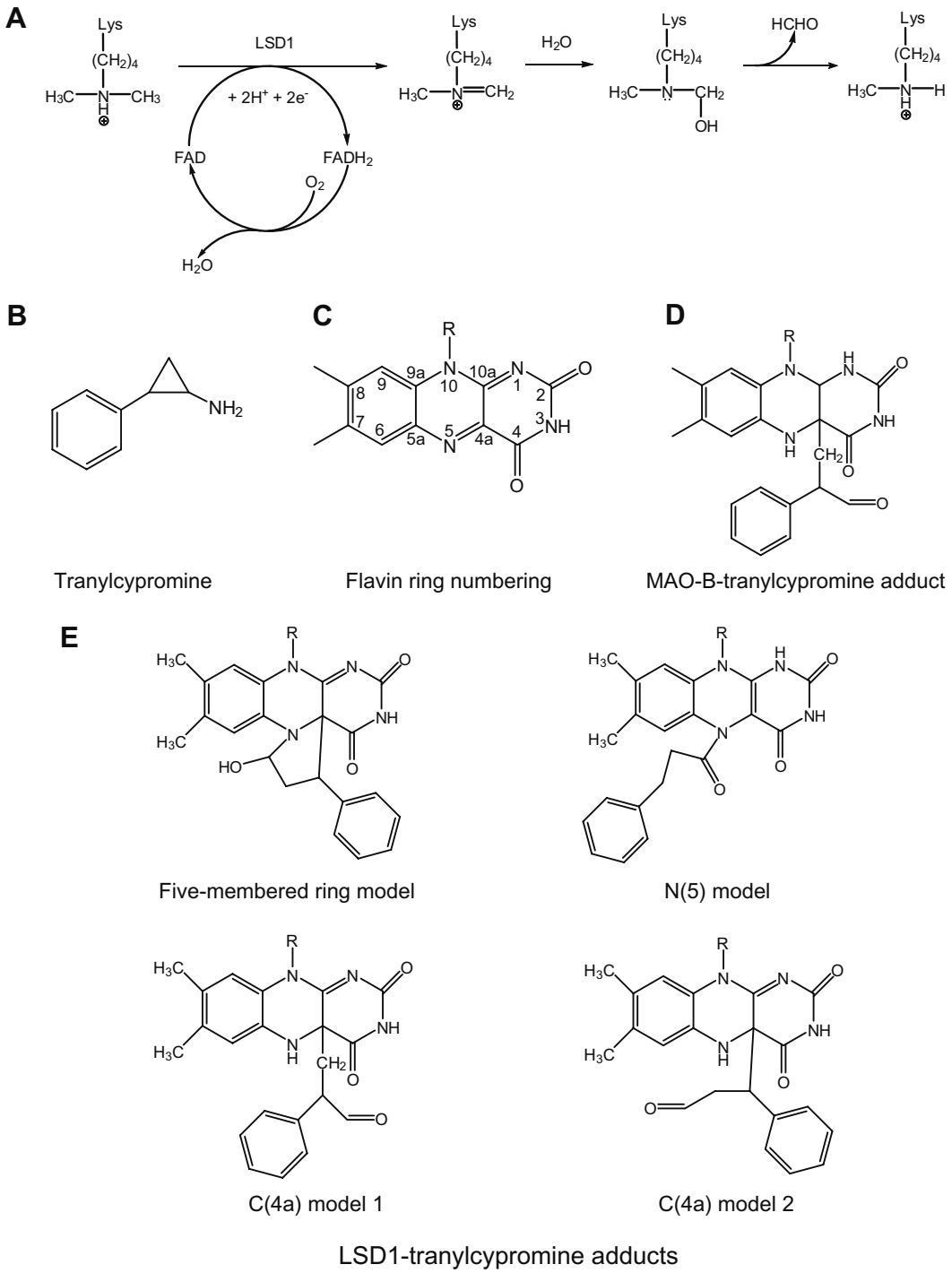

Fig. 1. Reaction chemistry of LSD1. (A) Proposed LSD1 reaction mechanism. (B) Chemical structure of tranylcypromine. (C) Atomic numbering of the flavin ring. (D) Chemical structure of the FAD–tranylcypromine adduct in the MAO-B complex. (E) Four chemical models of the FAD–tranylcypromine adduct in the LSD1 complex.

de-repressing androgen-receptor-target genes [9,10]. Thus, LSD1 can target different lysine residues and regulate transcription positively or negatively, depending on its binding partners. *In vitro* analyses showed that the demethylation activity is decreased by other modifications on the H3 tail, such as acetylation and phosphorylation, suggesting possible regulatory mechanisms [11,12]. A recent genome-wide study revealed an unexpectedly large number of LSD1-enriched promoters, suggesting its broad role in transcriptional regulation [13].

The crystal structures of LSD1 fragments containing most of the conserved regions have been determined in the free form and in complex with a CoREST fragment [14–16]. The histone-specific recognition of LSD1 has been analyzed through structural studies, which revealed three gamma turns of the H3 peptide inside the LSD1

reaction pocket [17,18]. In contrast to the closed, small, and hydrophobic active site of monoamine oxidase B(MAO-B), the active site cavity of LSD1 is open, large, and relatively hydrophilic. Specific inhibitors of LSD1 would provide a powerful tool for analyzing the biological significance of histone demethylation. Moreover, as LSD1 acts as a coactivator of androgen and estrogen-responsive gene expression [9,10,13], specific LSD1 inhibitors may also be useful in treating nuclear hormone-dependent cancer proliferation. In fact, the LSD1 expression level is correlated with the malignancy of prostate cancer [19].

The amine oxidase domain of LSD1 shares moderate sequence similarity with monoamine oxidases (MAO), which oxidatively deaminate neurotransmitters. Several non-specific inhibitors of MAOs, which show an antidepressant effect, also inhibit LSD1. For example, pargyline, chlorgyline, and deprenyl impair the LSD1-dependent expression of androgen-responsive genes [9]. Tranylcypromine (Fig. 1B) and phenelzine also inhibit LSD1 *in vivo* and *in vitro*, where tranylcypromine shows the most potent inhibition [20,21]. Tranylcypromine is a mechanism-based suicide inhibitor of MAO-B and LSD1: it covalently binds to the protein–FAD complex, dependent on the enzymatic catalysis. For two decades, the site where MAO becomes modified by tranylcypromine remained unknown [22–26]. Several studies suggested that MAO forms an adduct at the N(5) position of FAD [25,26]. However, the crystal structure of MAO-B complexed with tranylcypromine revealed that the inhibitor forms a covalent adduct with the cofactor FAD, through the C(4a) atom of the flavin ring (Fig. 1B–D) [27]. The phenyl ring of the tranylcypromine is accommodated in the active site cavity of MAO-B, and thus is inaccessible from the outside [27].

Recently, the crystal structure of LSD1 inhibited by tranylcypromine was solved at 2.75 Å, revealing a five-membered ring adduct fused to the flavin ring of LSD1 (Fig. 1E) [28]. This five-membered ring model is derived from a previous 1.8 Å crystal structure of monomeric sarcosine oxidase (MSOX) reacted with *N*-(cyclopropyl)glycine (CPG) [29], and is supported by visual/UV spectra, mass-spectrometry and kinetic assays [28,29].

In the present study, we solved the structure of residues 172–833 of LSD1 complexed with tranylcypromine at an improved resolution of 2.25 Å. In this refined crystal structure, however, we were unable to fit the five-membered ring model completely into our electron density, suggesting the presence of other, different adducts. We thus attempted to build the models of the C(4a) and N(5) adducts protruding from LSD1-flavin, and validated the models with the electron density. We propose that the five-membered ring model is the major adduct formed in the LSD1–tranylcypromine reaction, based on the supporting data, but cannot exclude the presence of the N(5) adduct.

Materials and methods

Protein preparation. A cDNA clone encoding human LSD1 (KIAA0601) was obtained from Kazusa DNA Research Institute. The human LSD1 fragment, consisting of residues 172–833, was expressed in Escherichia coli as a GST-fusion protein, by using a modified pGEX-6P-1 vector (GE Healthcare Biosciences). The fusion protein was purified by Glutathione Sepharose chromatography, followed by GST removal and cation-exchange chromatography (UNO-S, Bio-Rad).

Crystallization, data collection, and structure determination. Purified LSD1 was mixed with a histone H3 peptide (residues 1–21) at a protein to peptide ratio of 1:5, in 20 mM MES–Na buffer (pH 6.0) containing 1 mM DTT, and was crystallized with 100 mM HEPES–Na buffer (pH 7.5) containing 5% 2-methyl-2,4-pentanediol and 3.5–4.0% (w/v) polyethylene glycol monomethyl ether 2000, with or without 5 mM tranylcypromine. The crystals were soaked briefly in a cryoprotectant containing 25% (w/v) 2-methyl-2,4-pentanediol and were flash-cooled in liquid nitrogen prior to the data collection. The diffraction data were collected on the BL41XU beamline at SPring-8 under cryogenic conditions. The data were processed and scaled with HKL2000 [30]. The inhibitor-free structure was solved by the single-wavelength anomalous dispersion method for selenomethionine (Table S1). The selenium sites were located by Shelx [31], and then the model was generated by phase calculation, density modification, and automatic model building/refinement with Resolve [32] and refmac5 [33]. The tranylcypromine-inhibited structure was solved with the same space group as the inhibitor-free model. Finally, the model was manually modified and refined by using Cuemol (http://cuemol.sourceforge.jp/en/) and CNS [34]. Structural figures were prepared using Cuemol and Pymol. The atomic coordinates and structure factors for the inhibitor-free model, the five-membered ring model, and the N(5) model have been deposited in the Protein Data Bank, with the accession codes 2DW4, 2Z5U, and 2Z3Y, respectively. Details of the mass-spectrometry are described in the Supplementary online material.

Results and discussion

Crystal structure of LSD1–tranylcypromine at 2.25 Å resolution

The crystal structure of LSD1 bound with tranylcypromine was analyzed at 2.25 Å. In order to determine the tranylcypromine-binding site of LSD1, an $F_o - F_c$ electron density map of inhibitor-free LSD1 including FAD in its reaction pocket was made (PDBID:2DW4). A significant peak protruding from LSD1-flavin was observed (Fig. 2A). No other significant peaks were observed in the structure, indicating that LSD1-flavin is the only place modified by this inhibition. The most likely tranylcypromine-binding site of LSD1 seems to be flavin N(5). Unfortunately, the electron density for the H3 peptide was not observed. The present LSD1 structure has almost the same unit cell parameters as those of the previously reported structures, which failed to show the H3 peptide [14–16]. The H3 peptide was observed in other reports, in which H3K4 was mutated to Met [18], and the H3 peptide was covalently attached to LSD1-flavin by suicide inactivation [17]. In the inhibitor-free LSD1 structure, a water molecule forms a hydrogen bond with the flavin N(5) atom and Lys661 (Fig. 2B). However, in our tranylcypromine adduct structure, this water is missing because of the orbital changes of the flavin atoms (Fig. 2C). Lys661 is highly conserved among LSD1 orthologues and

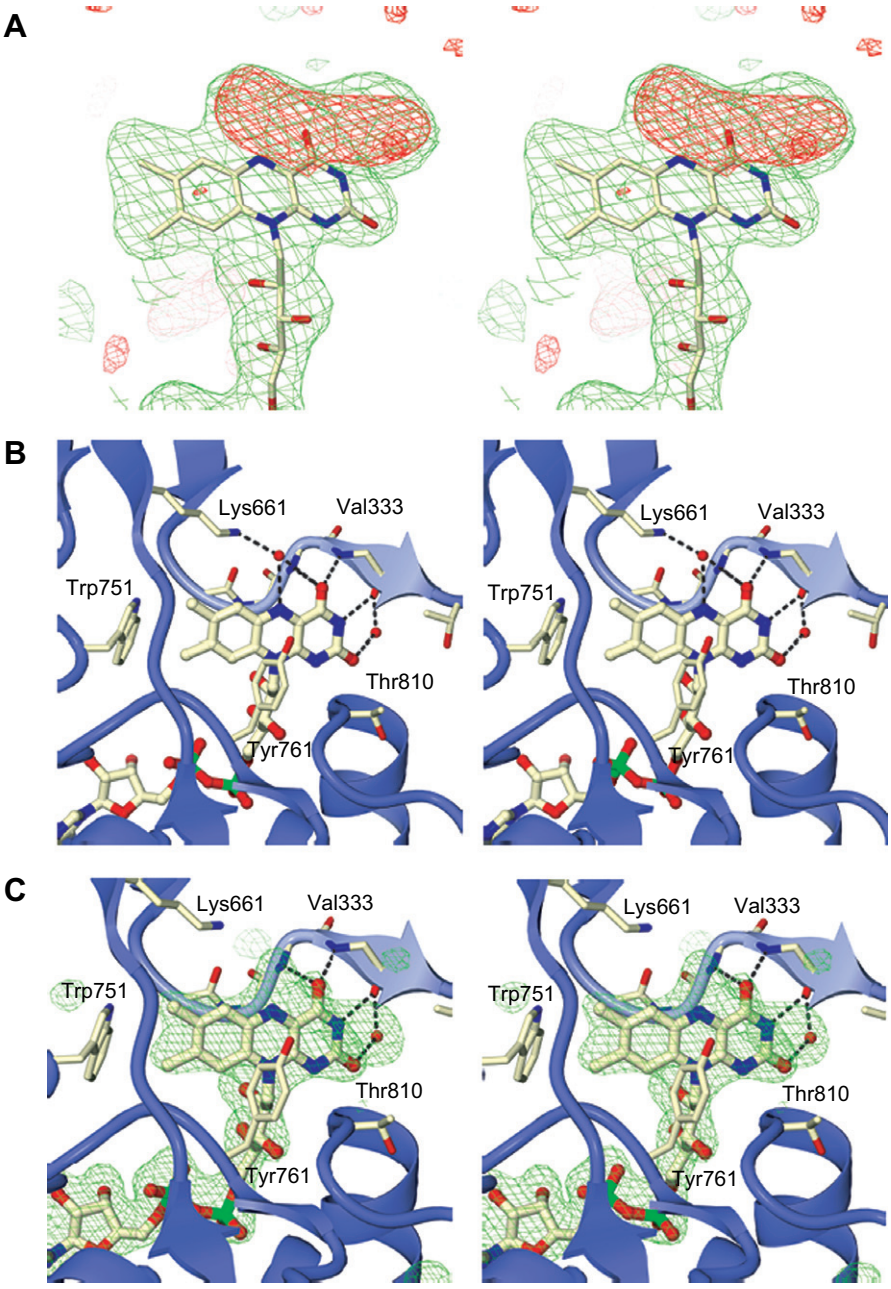

Fig. 2. Active site cavity. (A) $F_o - F_c$ maps of the tranylcypromine adduct contoured at 3 sigma (stereoview). The $F_o - F_c$ maps were calculated before the tranylcypromine adduct was built into the model and either with (red) or without (green) the inclusion of FAD. (B) Stereoview of the active site cavity in the inhibitor-free LSD1. (C) Stereoview of the active site cavity in the tranylcypromine-bound LSD1, with an $F_o - F_c$ map of the tranylcypromine adduct contoured at 3 sigma (green). Note that the water molecule above the flavin N(5) atom is present in the inhibitor-free form, but cannot be detected in the LSD1–tranylcypromine electron density map.

other FAD-dependent amino oxidases, and is essential for LSD1 activity [15]. This water would be important for the transport of electrons and protons, and is considered to play a vital role in the reaction scheme.

Mass-spectrometry of LSD1–tranylcypromine

In order to validate the molecular weight of this adduct, the FAD-adduct was examined by MALDI mass-spectrometry. In the absence of tranylcypromine, major peaks were detected at $m/z = 785.4$–786.4, which correspond to the masses of protonated FAD molecules (Fig. S1A). No other significant peak was observed, except for the peak at $m/z = 808.4$ (Fig. S1A). The mass difference of 22–23 Da between these peaks implies the addition of a sodium ion. In the presence of tranylcypromine, significant peaks were detected at $m/z = 919.7$–920.7 in addition to the FAD peak (Fig. S1B). The peaks at $m/z = 919.7$–920.7 were considered to be the FAD adduct, based on the mass increase of 133–135 Da from FAD. From the structural

model by Yang and colleagues, the difference in the masses between the FAD adduct and FAD was expected to be 133 Da, with a probable composition of C_9H_9O atoms [28]. A recent report also validates the molecular weight of the adduct [21].

Five-membered ring adduct model

There have been sound biochemical and chemical analyses of the LSD1–tranylcypromine reaction, which showed the five-membered ring adduct fusing C(4a) and N(5) of flavin (Fig. 1E) [28,29]. The structure is consistent with the supporting data, such as visible/UV spectra, mass spectra and kinetic assays [28,29]. The absorbance spectra of LSD1–tranylcypromine [28] and MSOX–CPG [29] show similar patterns, suggesting the structural similarity of the two adducts. A recent report has suggested that the tranylcypromine cyclopropane ring opens upon adduct formation with LSD1 [21]. An absorbance spectrum of this reaction and a comparison with the MAO-B tranylcypromine reaction suggested that the flavin ring changes its conformation and forms an adduct at flavin C(4a) [21]. Furthermore, two mechanisms of the tranylcypromine cyclopropane opening were proposed [21]. A mass-spectrometry analysis also revealed the molecular composition of the adduct [21].

We modeled the five-membered ring model proposed by Yang et al. to verify the established model with the electron density (Fig. 1E). The $F_o - F_c$ electron density map revealed that the five-membered ring model was not sufficient (Fig. 3, top panels). The phenyl ring of the model failed to fit completely with the $F_o - F_c$ map contoured at 3 sigma (Figs. 3 and S2). Although the five-membered ring model is supported by the absorbance spectrum analysis, in which the LSD1–tranylcypromine spectrum shows similarity to that of MSOX–CPG, the inadequate fit implies the presence of other intermediates in the structure.

C(4a) and N(5) adduct models

To assess the possibility of other intermediates in the LSD1–tranylcypromine structure, two putative C(4a) models from the tranylcypromine reaction scheme were made and were superimposed on the $F_o - F_c$ map. (Figs. 1E, 3 and S2). These two models (Fig. 3, second and third panels) were also found to be unsuitable, since the adduct oxygen fails to fit with the electron density. Therefore, another LSD1–tranylcypromine model was made, in which the intermediate protrudes from flavin N(5) (Figs. 1E, 3 and S2). This model (Fig. 3, bottom panels), although slightly biased on one side of the electron density, fits satisfactorily with the $F_o - F_c$ map contoured at 3 sigma. The N(5) model also has the lowest R_{work}/R_{free} of 0.218/0.248, in comparison to the values determined for the five-membered ring model (0.226/0.254) and the C(4a) models (0.221/0.251 for C(4a) model 1 and 0.222/0.255 for C(4a) model 2; Table S2). The N(5) model seems to fit best with the 6 sigma contoured map as well as the 3 sigma contoured map, thus suggesting that the possible tranylcypromine-binding site of LSD1 is N(5) flavin. Therefore, the five-membered ring model alone is insufficient to explain the calculated electron density, but a conglomerate of both the five-membered and N(5) models would compensate for the bias of the models, and thus explain the electron density conundrum. From the electron density, it appears that the five-membered ring and the N(5) model occupancy occur roughly in a 1 to 1 ratio, although this is only an approximate estimate by observation. The five-membered ring model will still be the major inhibitory complex, because of the visible/UV spectral similarity of the FAD moieties between the LSD1–tranylcypromine [28] and the MSOX–CPG [29] complexes. Together, these results suggest that the LSD1–tranylcypromine complex is not completely composed of the five-membered ring adduct, and imply the presence of other intermediates, among which the N(5) adduct is the most likely.

The N(5) adduct is distinct from the C(4a) adduct of the MAO-B–tranylcypromine complex (PDBID:1OJB), which has branch-chained carbons (Fig. 1D) [27]. Previous studies revealed that irreversible MAO-B inhibitors can covalently bind to either the N(5) or C(4a) atom of FAD [27]. The amino group of tranylcypromine is substituted with a carbonyl, as a result of the oxidation reaction catalyzed by LSD1. We think that its cyclopropane ring, which is inherently reactive, split and formed a new covalent bond with the N(5) atom of FAD during this reaction.

The five-membered ring and C(4a) adduct reaction schemes were proposed by Yang et al. to occur by the single electron transfer mechanism (Figs. S3A, S3B, and S3C) [28]. Based on the adduct structure and the chemical properties of tranylcypromine, we propose an inhibitory reaction scheme for the N(5) adduct LSD1–tranylcypromine (Fig. S3D). The reaction involves the addition of tranylcypromine to the FAD heterocyclic system, which is enabled by the structural distortion of the tranylcypromine cyclopropane ring and the stability of the benzylic cation of tranylcypromine. Subsequently, the distorted cyclopropane ring causes a 1,3 hydride shift followed by hydrolysis of the iminium cation, resulting in our tranylcypromine adduct: 1-hydro-5-(3-phenylpropanoyl)-flavin-adenine dinucleotide (1-hydro-5-(3-phenylpropanoyl-riboflavin adenosine diphosphate)).

We compared the structures of the five-membered ring and N(5) LSD1 adducts with that of tranylcypromine-reacted MAO-B, to examine their structural differences in more detail (Fig. S4) [27]. Some of the residues around the LSD1 active site cavity have smaller side chains in comparison to those of MAO-B, such as Thr810 of LSD1, corresponding to Tyr435 of MAO-B. These small residues contribute to the creation of a larger active site cavity in LSD1. When the active sites of MAO-B (Fig. S4A) and tranylcypromine-inhibited LSD1 (Figs. S4B and S4C) are superimposed based on their flavin rings, Tyr435 of MAO-B overlaps with the aromatic ring of the LSD1

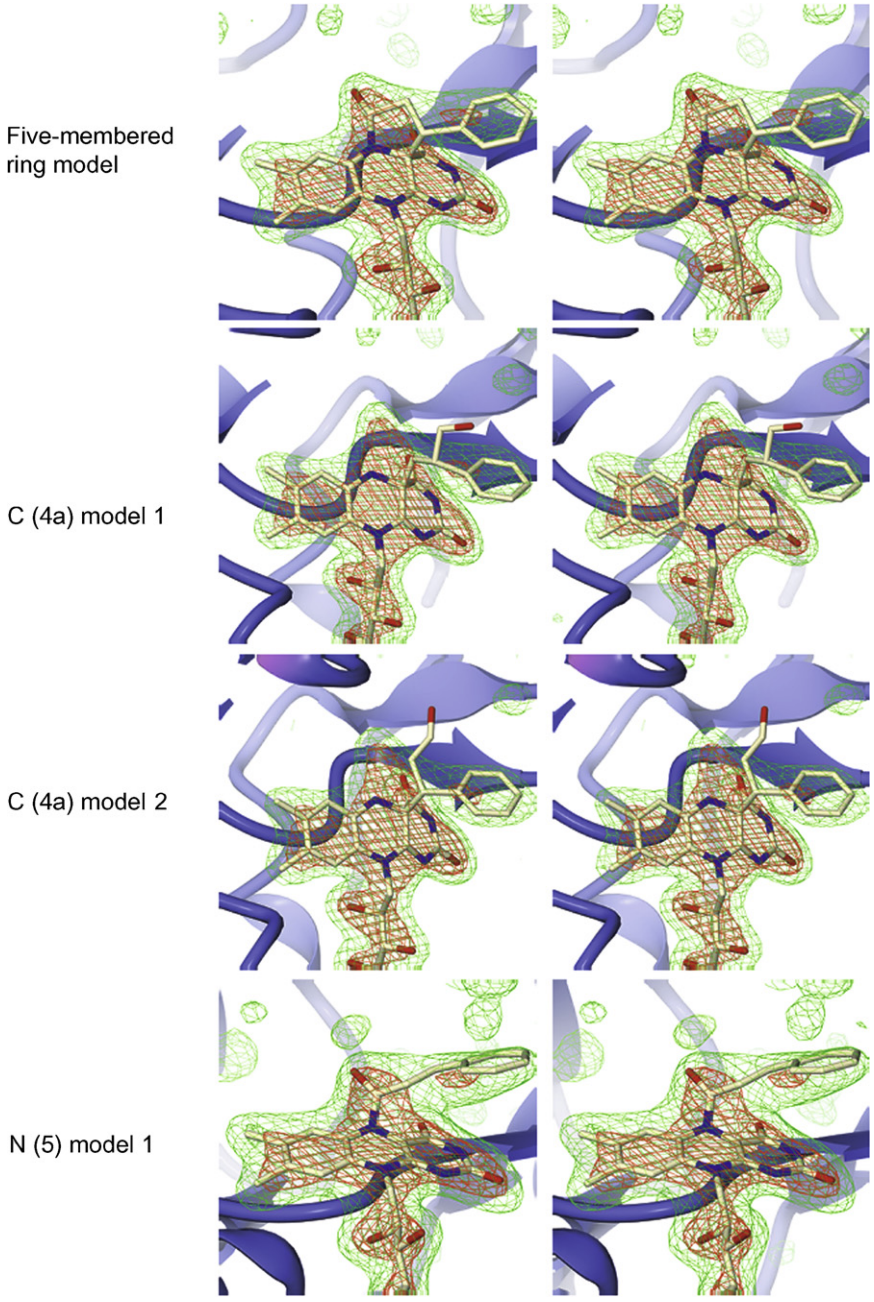

Fig. 3. Tranylcypromine adduct $F_o - F_c$ maps contoured at 3 sigma (green) and 6 sigma (red) (stereoview). The four models used for the refinements are indicated on the left. All $F_o - F_c$ maps were calculated before the tranylcypromine adducts were built into the model.

adduct, in both the five-membered ring (Fig. S4B) and N(5) (Fig. S4C) models, thus preventing the LSD1-type adduct from forming in MAO-B. On the other hand, when the MAO-B-type adduct was placed, no steric clash was observed in the active site of LSD1 (data not shown). It is interesting that LSD1 forms fewer interactions with tranylcypromine than MAO-B, since tranylcypromine inhibits LSD1 with an IC_{50} of 2 μM, which is ten times lower than that of MAO-B [20].

In summary, we have solved the crystal structure of LSD1 in complex with tranylcypromine at an improved resolution of 2.25 Å, and suggested that the five-membered ring model was not completely appropriate, with R_{work}/R_{free} values of 0.226/0.254. The N(5) model fit best with the electron density among the three models tested and generated the lowest R_{work}/R_{free} values of 0.218/0.248. The N(5) model is also oriented such that it compensates for the poorer fit of the five-membered ring model. These data, along with the spectral data, suggest that the LSD1–tranylcypromine complex is not composed entirely of the five-membered ring adduct, but contains other intermediates, such as the N(5) adduct. The reaction scheme of LSD1 and tranylcypromine forming an N(5) adduct requires the cyclopropane ring of tranylcypromine

to split and attach to the FAD heterocyclic system. These results provide valuable information, not only for understanding the inhibition mechanism of histone demethylases but also for the development of anti-cancer agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.066.

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