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Mechanistic Analysis of a Suicide Inactivator of Histone Demethylase LSD1[†]

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Abstract

The lysine-specific demethylase 1 (LSD1) is a transcriptional repressor and a flavin-dependent amine oxidase that is responsible for methyl removal from lysine-4 of histone H3. In this study, we characterize the mechanism and scope of LSD1 inhibition by a propargylamine derivatized histone H3 substrate (**1**). Unlike aziridiny and cyclopropylamine-derivatized histone H3 peptide substrate analogs, compound **1** appears to covalently modify and irreversibly inactivate LSD1 with high potency. Accompanying this inactivation is a spectroscopic change, which shifts the absorbance maximum to 392 nm. Spectral changes associated with the **1**-LSD1 complex and reactivity to decreased pH and sodium borohydride treatment were suggestive of a structure involving a flavin-linked inhibitor conjugate between the N⁵ of the flavin and the terminal carbon of the inhibitor. Using ¹³C-labeled inhibitor, NMR analysis of the **1**-flavin conjugate was consistent with this structural assignment. Kinetic analysis of the spectroscopic shift induced by **1** showed that the flavin adduct formed in a reaction with similar kinetic constants to that of the LSD1 inactivation process. Taken together, these data support a mechanism of LSD1 inactivation by **1** involving amine oxidation followed by Michael addition to the propargylic imine. We further examined the potential for a biotinylated analog of **1** (**1-Btn**) to be used as a tool in affinity pull-down experiments. Using **1-Btn**, it was feasible to selectively pull-down spiked and endogenous LSD1 from HeLa cell nuclear extracts, setting the stage for activity-based demethylase proteomics.

Chromatin remodeling has emerged as a major mechanism of epigenetic gene regulation (1-4). Within the framework of chromatin modulation, reversible, covalent modifications of histone proteins play key roles in accessibility of DNA to transcription, replication, and repair (1-4). Acetylation, phosphorylation, and methylation have long been known to site-specifically modify histone residues and the functions of these modifications are beginning to become better understood (1-4). Until relatively recently, histone methylation on lysine was viewed as a static modification but with the discovery of lysine-specific demethylase 1 (LSD1²) (5,6) and the JmjC domain (6-8) containing family of demethylases, there is now recognition that lysine methylation is a dynamic protein modification.

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LSD1 belongs to the amine oxidase superfamily, which are flavin enzymes that utilize oxygen and generate hydrogen peroxide (9). The LSD1-catalyzed reaction converts mono- and dimethyl-lysine 4 of histone H3 to demethylated products (5). In a complex with CoREST, LSD1 can efficiently demethylate histones in nucleosomes (10-12). LSD1 serves as a transcriptional repressor since methylation of histone H3 can activate gene expression. LSD1 is an ~100 kDa protein which contains 2 domains, SWIRM and amine oxidase (5,6). Recent crystal structures of the amine oxidase domain reveal that it shares a fold with other amine oxidases and suggest models for how substrate selectivity may be achieved (12-14). Inhibitors of LSD1 may be useful biological tools and have therapeutic properties in the treatment of diseases involving abnormal epigenetic regulation, such as cancer (15,16).

Previous approaches for development of amine oxidase inhibitors have exploited the potential that these enzymes have for suicide inactivation (17). Suicide inactivators are typically substrate analogs that can be processed by the targeted enzyme to generate highly reactive species that then covalently modify the enzyme and reduce its catalytic activity (18,19). Because they are relatively inert until acted upon by the targeted enzyme, they have the potential for high specificity. Furthermore, they typically show irreversible inhibition such that more enzyme has to be biosynthesized before a catalytic pathway can recover.

Based on the finding that pargyline is a suicide inactivator of monoamine oxidases (20-22), we previously designed and synthesized a peptide substrate analog in which the nitrogen atom of Lys-4 was derivatized with a propargyl group **1** (15). We showed that this compound displayed time-dependent inactivation of LSD1 and generated a covalent flavin adduct which was characterized by mass spectrometric analysis (15). In contrast, a peptide aziridine inhibitor appeared to behave as a standard reversible inhibitor (15). In this study, we investigate the kinetic and mechanistic basis of inhibition by compound **1** in greater detail. We have shown that compound **1** induces a spectroscopic change in the flavin cofactor consistent with an N⁵ adduct. In contrast, a novel cyclopropylamine derivative **2**, which behaves as a competitive inhibitor does not induce this spectroscopic change. Further analysis of the **1**-flavin adduct using NMR is consistent with the proposed structure. We have measured the optical spectroscopic change induced by **1** as a function of time and found that it proceeds with kinetic constants similar to the rate of inactivation. Finally, we show that a biotin-labeled analog of compound **1**, **1-Btn**, can be used to isolate endogenous LSD1 and CoREST from nuclear extracts, suggesting applications in proteomics.

Materials and Methods

¹³C- propargylamine hydrochloride

Diethylazodicarboxylate (DEAD, 767 μ L, 4.87 mmol) was added dropwise over 5 min to a solution of triply ¹³C labeled propargyl alcohol (Cambridge Isotope Lab) (250 mg, 4.23 mmol), triphenylphosphine (1.28 g, 4.87 mmol), and *N*-(tert-Butoxycarbonyl)phosphoramidic Acid Diethyl Ester (TCI) (1.07 g, 4.23 mmol) in 20 mL of anhydrous THF at 0 °C. After addition, the reaction was allowed to warm to room temperature while stirring for 4 hours. The reaction was concentrated *in vacuo* to a yellow oil. Without further purification, the oil was dissolved in 20 mL of anhydrous benzene and saturated with dry hydrogen chloride for 2 hours with stirring. The solution was allowed to stand 12 hours without stirring. The reaction was

²The abbreviations used are: LSD1, lysine-specific demethylase 1; H3-21, histone H3 residues 1-21; K4, lysine 4; **1**, propargylK4H3-21; **2**, cyclopropylK4H3-21; **1-Btn**, propargylK4H3-21-biotin; DEAD, diethylazodicarboxylate; RP-HPLC, reverse phase high pressure liquid chromatography; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption/ionization - time of flight; diMe, dimethyl; IPTG, isopropyl β -D-1-thiogalactopyranoside; BSA, bovine serum albumin; Gdn-HCl, guanidine hydrochloride; FAD, flavin adenine dinucleotide; HSQC, heteronuclear single quantum correlation; COSY, correlation spectroscopy; ROESY, rotational nuclear overhauser effect spectroscopy.

concentrated *in vacuo* and resuspended in dry diethyl ether and allowed to stand at -80 °C for 4 hours. The amine hydrochloride was pelleted by centrifugation and washed 3× with dry diethyl ether before being dissolved in H₂O and lyophilized to a white powder, yielding 255 mg. ¹H (CD₃OD, 400 MHz): δ 3.80 (dm, *J* = 148 Hz, 2H); δ 3.13 (dm, *J* = 306 Hz, 1H).

Fmoc-Hydroxynorleucine-OH

Fmoc-Lys-OH (5.2 g, 14.1 mmol) was added to 300 mL of ddH₂O and 50.0 mL of glacial acetic acid (15). The solution was gently heated until all solids dissolved. The solution was then cooled to 0 °C with an ice-water bath. Sodium nitrite (2.92 g, 42.3 mmol) in 65 mL H₂O was added dropwise to the stirring Fmoc-Lys-OH solution over 180 minutes. The reaction warmed to 25 °C while stirring over 20 hours. The reaction was concentrated *in vacuo* to a yellow oil, dissolved in H₂O and acidified with glacial acetic acid. The aqueous solution was extracted 3 × 75 mL with ethyl acetate. The pooled organics were washed 1 × 75 mL with brine. The organic phase was dried over MgSO₄, filtered, and concentrated *in vacuo* to a yellow solid. Crude product was purified by prep scale RP-HPLC, yielding 1.3 g. ¹H (CDCl₃, 400 MHz): δ 7.77 (d, *J* = 7.2 Hz, 2H); 7.60 (d, *J* = 7.2 Hz, 2H); 7.40 (t, *J* = 7.2 Hz, 2H); 7.32 (t, *J* = 7.2 Hz, 2H); 5.38 (d, *J* = 8.4 Hz, 1H); 4.43 (m, 3H); 4.23 (t, *J* = 6.4 Hz, 1H); 3.68 (t, *J* = 6.4 Hz, 2H); 1.93 (m, 1H); 1.78 (m, 1H); 1.61 (m, 2H); 1.49 (m, 2H). Electrospray ionization-mass spectrometry, PE Biosystems SCIEX API 150EX, *m/z* = 369.

MesylK4H3-21

Standard Fmoc solid phase peptide synthesis techniques were utilized to assemble the mesylK4H3-21 peptide. At the 4 position, Fmoc-hydroxynorleucine-OH was coupled; subsequent protection of the ε alcohol by acetic anhydride yielded the acetic ester prior to the coupling of Thr3. The fully protected H3-21 peptide on wang resin was treated with 125 mM hydroxylamine at pH 10 in 50:50 H₂O:dimethylformamide for 12 hours at room temperature. The primary alcohol, in position 4, was then treated with 20 eq. of mesyl chloride in the presence of 40 eq. of triethylamine in tetrahydrofuran for 20 hours at 25 °C. Universal deprotection and cleavage from the resin was accomplished with 95:5 trifluoroacetic acid (TFA): H₂O in the presence of phenol, ethanedithiol, and thioanisole for 5 hours at 25 °C. The stability of the mesylate to these conditions is ascribed to its location on a primary carbon (pH ≤ 1.0). Precipitation of the peptide with diethyl ether followed by lyophilization yielded crude peptide as an off-white solid that was purified by prep scale RP-HPLC. Analysis by MALDI-TOF, Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems), showed an expected/observed *m/z* = 2335.

PropargylK4H3-21 (1)

Lyophilized mesylK4-H3-21 (5.0 mg, 2.1 μmol) was dissolved in 500 μL of 1:1 H₂O:CH₃CN. Freshly distilled propargylamine (44 μL, 640 μmol) in 500 μL of 1:1 H₂O:CH₃CN was added and the solution rotated 65 hours at 25 °C. The crude reaction mixture was diluted to 3 mL with H₂O, acidified to pH 2 with TFA, and injected onto a prep scale column for RP-HPLC purification. Analysis by MALDI-TOF showed an expected/observed *m/z* = 2294. The pure peptide was lyophilized to a white solid and stored at -80° C.

¹³C-propargylK4H3-21 (1*)

Lyophilized mesylK4H3-21 (5.0 mg, 2.1 μmol) was dissolved in 1000 μL of 1:1 H₂O:CH₃CN. ¹³C-propargylamine hydrochloride (57 mg, 600 μmol) in 100 μL of 1:1 H₂O:CH₃CN was added to the solution followed by triethylamine (112 μL, 800 μmol). The reaction rotated 65 hours at 25 °C. The crude reaction mixture was diluted to 25 mL with H₂O, acidified to pH 2 with TFA, and lyophilized to an oil. The oil was again diluted to 25 mL with H₂O, acidified, and lyophilized to a solid/oil that was diluted to 3 mL with H₂O and

injected onto a prep scale column for RP-HPLC purification. Analysis by MALDI-TOF showed an expected/observed $m/z = 2297$. The pure peptide was lyophilized to a white solid and stored at -80°C .

MesylK4H3-21-Biotin

This peptide was made in an analogous way to the non-biotinylated mesyl peptide with the following variations. Fmoc-Gly-Wang resin was used and in the 22 position Fmoc-Lys(biotin)-OH (Novabiochem) was coupled, resulting in a 23 amino acid peptide. Residues 1 through 21 being histone H3 while 22 and 23 are a biotinylated lysine followed by a glycine. Analysis by MALDI-TOF showed an expected/observed $m/z = 2746$.

PropargylK4H3-21-Biotin (1-Btn)

This peptide was made in an analogous way to the non-biotinylated peptide. Analysis by MALDI-TOF showed an expected/observed $m/z = 2705$.

CyclopropylK4H3-21 (2)

Lyophilized mesylK4H3-21 (5.0 mg, 2.1 μmoles) was dissolved in 500 μL of 1:1 $\text{H}_2\text{O}:\text{CH}_3\text{CN}$. Freshly distilled cyclopropylamine (Fluka) (55 μL , 800 μmol) in 500 μL of 1:1 $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ was added and the solution rotated 65 hours at 25°C . The crude reaction mixture was diluted to 3 mL with H_2O , acidified to pH 2 with TFA, and injected onto a prep scale column for RP-HPLC purification. Analysis by MALDI-TOF showed an expected/observed $m/z = 2296$.

diMeK4H3-21

Standard Fmoc solid phase peptide synthesis technique was utilized to assemble the diMeK4H3-21 peptide. The diMeK4 residue was coupled as commercially available Fmoc-diMeLys-OH (Novabiochem). Universal deprotection and cleavage of the peptide from the wang resin was accomplished with 95:5 TFA: H_2O in the presence of phenol, ethanedithiol, and thioanisole for 5 hours at 25°C . Precipitation of the peptide with diethyl ether followed by lyophilization yielded crude peptide as an off-white solid that was purified by prep scale RP-HPLC. Analysis by MALDI-TOF showed an expected/observed $m/z = 2284$.

LSD1 expression and purification

LSD1 (171-852) subcloned into the pGEX-6P-1 vector (12) (GE Healthcare) was overexpressed in *E. coli* BL21-CodonPlus[®](DE3)-RIPL cells (Stratagene). Cells were grown to an $\text{OD}_{600\text{ nm}}$ of 1.8 in CircleGrow[®] Media (Q-Biogene) at 37°C then induced with 1 mM final IPTG and grown for 20 hrs at 16°C . Cell pellets were harvested by centrifugation at $5000 \times g$ for 15 min and resuspended in ice cold lysis buffer (280 mM NaCl, 5.4 mM KCl, 20 mM Na_2HPO_4 , 3.6 mM KH_2PO_4 , 1 mM EDTA, 10 mM DTT, 10% glycerol, pH 7.4). The cells were then lysed via double pass on a French press (16,000-18,000 psi), and the lysates clarified by centrifugation at $25,000 \times g$ for 30 min. The clarified lysate from 1 L of culture was double loaded (0.5 mL/min) onto a 5 mL glutathione sepharose 4 fast flow column (GE Healthcare) that was pre-equilibrated with lysis buffer. The column was then washed with 75 mL of lysis buffer and eluted with $5 \times 5\text{ mL}$ fractions of lysis buffer containing 50 mM reduced glutathione (Sigma). GST-LSD1 containing fractions were pooled and dialyzed against $3 \times 1\text{ L}$ changes of lysis buffer containing 1 mM β -mercaptoethanol instead of 10 mM DTT. The dialyzed protein was concentrated to 1-2 mL and further purified by size exclusion chromatography using sephacryl S100 high resolution media (GE Healthcare, $1.5 \times 90\text{ cm}$ column). The protein was eluted with lysis buffer containing 1 mM β -mercaptoethanol instead of 10 mM DTT at a flow rate of 0.25 mL/min. GST-LSD1 containing fractions were pooled, concentrated, aliquoted, and stored at -80°C . Final protein concentration was determined by Bradford assay

using BSA as the standard. Purification of GST-LSD1 by this procedure yielded approximately 1 mg of protein (>90% pure) per 1 L of culture.

Demethylase Assays

Initial velocity measurements were performed using a peroxidase-coupled assay, which monitors hydrogen peroxide production as previously described (23). The time courses of the reaction were measured under aerobic conditions using a Beckman Instruments DU series 600 spectrophotometer equipped with thermostated cell holder ($T = 25^\circ\text{C}$). The 150 μL reactions were initiated by adding 50 μL of buffered substrate (diMeK4H3-21, final concentration = 60 μM) solution to reaction mixtures (100 μL) consisting of 50 mM HEPES buffer (pH 7.5), 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 0.76 μM horseradish peroxidase (Worthington Biochemical Corporation), and 120-360 nM LSD1. Reaction mixtures were equilibrated at 25°C for 2 min prior to activity measurement. Absorbance changes were monitored at 515 nm, and an extinction coefficient of $26,000\text{ M}^{-1}\text{ cm}^{-1}$ was used to calculate product formation. Under these conditions, our GST-LSD1 displayed at k_{cat} of $3.1 \pm 0.1\text{ min}^{-1}$ and a K_{m} for diMeK4H3-21 of $50 \pm 5\text{ }\mu\text{M}$.

LSD1 inhibitors were tested by using the peroxidase-coupled assay described above. In these experiments, assays were initiated by addition of buffered substrate and inhibitor (either **1** or **2**) simultaneously. Final substrate concentrations were either 240 μM ($> 4 \times K_{\text{m}}$ when examining **1**) or 60 μM ($\sim K_{\text{m}}$ when examining **2**). Progress curves obtained in the presence of **1** were fit to the following single exponential for slow-binding inhibitors which assumes a steady-state velocity of zero (24):

$$\text{Product} = v_0(1 - e^{-kt})/k + \text{offset} \quad (\text{Eq. 1})$$

The k_{obs} values were then analyzed by the method of Kitz and Wilson to yield k_{inact} and $K_{\text{i(inact)}}$. The following equation was used to extract kinetic constants from the Kitz-Wilson analysis (25):

$$k_{\text{obs}} = (k_{\text{inact}} * [\text{I}]) / (K_{\text{i(inact)}} + [\text{I}]) \quad (\text{Eq. 2})$$

$K_{\text{i(inact)}}$ was extrapolated to zero substrate by:

$$K_{\text{i(inact)app}} = K_{\text{i(inact)}} * (1 + S/K_{\text{m}}) \quad (\text{Eq. 3})$$

The $t_{1/2}$ for inactivation at saturation was obtained from equation 4:

$$t_{1/2} = (\ln 2) / k_{\text{inact}} \quad (\text{Eq. 4})$$

Compound **2** did not display time-dependent inhibition. Initial velocities at increasing concentrations of **2** were obtained by linear regression to reaction progress curves. These velocities were used to determine the K_{i} of **2** by Dixon analysis (assuming a competitive mode of inhibition).

Absorbance spectroscopy

LSD1 (10 μM) was incubated with **1** (50 μM), **2** (60 μM), or no inhibitor in 50 mM HEPES (pH 7.5) at 25°C . After 1 hr, the samples were clarified by centrifugation at $14,000 \times g$ for 10 min (25°C) and the flavin absorbance spectra was recorded versus a buffer blank (350-550 nm). The flavin adduct that formed upon incubation with **1** was then acidified with HCl (300 mM final, pH < 2) or reduced with NaBH_4 (1 mM final) at 25°C (10 min) and the flavin

absorbance spectra was recorded again. Difference spectra were generated by subtracting the native (untreated) LSD1 spectrum from the inhibitor treated spectra.

Time dependence of 1-flavin (3) formation

Difference spectroscopy indicated a maxima at 404 nm for LSD1 treated with **1**. The time- and concentration-dependent formation of this species could be monitored by absorbance spectroscopy. LSD1 was clarified by centrifugation at $14,000 \times g$ for 10 min (4 °C) prior to analysis. The reactions contained 0.75 μM LSD1 in 50 mM HEPES (pH 7.5) and were initiated with buffered inactivator (3, 6, 12, 24, 48, and 96 μM). Absorbance at 404 nm was recorded every 1.2 sec for 10 min at 25 °C. The k_{obs} values for the formation of **3** were extracted using Eq. 1 and replotted versus the concentration of **1**. Since these experiments directly followed formation of **3** and not enzyme activity, the following equation was used to extract kinetic constants, where k_{lim} is the limiting rate of adduct formation and $K_{\text{D(app)}}$ is the concentration of **1** necessary to yield $\frac{1}{2} k_{\text{lim}}$:

$$k_{\text{obs}} = (k_{\text{lim}} * [1]) / (K_{\text{D(app)}} + [1]) \quad (\text{Eq. 5})$$

1*-flavin adduct formation and isolation (3)

LSD1 (2 mg, purified by glutathione sepharose only) was incubated with 50 μM **1*** at 25 °C for 16 hours in $2 \times \text{PBS}$ (pH 7.4). The final concentration of LSD1 in the reaction was 10 μM and the final volume of the reaction was 750 μL . The inactivation reaction was quenched by the addition of 1 volume of 8 M guanidine hydrochloride (Gdn-HCl) with 0.2% TFA. This solution was injected onto a semi-prep C₄ RP-HPLC column and eluted in a water:acetonitrile gradient containing 0.05% TFA. The **1***-flavin adduct peak was collected and lyophilized to dryness. The crude adduct was further purified by size exclusion chromatography on P-10 resin (Bio-Rad) to remove remaining protein. The elution buffer was 10% acetonitrile + 0.025% TFA. Fractions containing the **1***-flavin adduct were pooled and lyophilized to dryness. Analysis by MALDI-TOF showed an expected/observed $m/z = 3082$. This process was repeated a total of 10 times to achieve enough product for analysis by NMR spectroscopy.

1-Btn in protein analysis

HeLa cell nuclear extracts (135 μg , Biomol) either with or without 1% (1.35 μg) recombinant LSD1 (178-831) were incubated with 20 μM **1-Btn** for 15 min at 25 °C in 25 mM HEPES (pH 7.5). When a non-biotinylated competitor was used, the lysates were preincubated for 15 min with 200 μM **1** prior to the addition of **1-Btn**. After the incubation, the reaction was cooled to 4 °C and **1-Btn** was removed by repeated buffer exchange into 20 mM HEPES (pH 7.5), 1 mM EDTA, and 50 mM NaCl (Amicon® ultra centrifugal filter devices, 10K MWCO). In the final buffer exchange, the buffer was adjusted to contain 500 mM NaCl and 0.1% Tween-20. The lysate was applied to 50 μL of pre-equilibrated streptavidin-agarose resin (Sigma) at 4 °C for 60 min while rotating. The beads were washed 4 times with the high salt/detergent containing buffer, pelleted at $3000 \times g$, and eluted by denaturation (boiling) in 50 μL SDS loading buffer. Proteins were resolved on a 4-15% gradient gel (Bio-Rad) and visualized by either silver staining (GE Healthcare) or western blotting. For western blots, the primary antibodies were rabbit polyclonal α -LSD1 (Abcam) and rabbit polyclonal α -CoREST (Upstate) used at 2 $\mu\text{g}/\text{mL}$ and 0.5 $\mu\text{g}/\text{mL}$, respectively. The secondary antibody in each case was α -rabbit-HRP (1:10,000 dilution), and the proteins were visualized with SuperSignal® West Pico Chemiluminescent Substrate (Pierce).

NMR spectroscopy

NMR data was acquired on a 600 Mhz (¹H) Bruker Avance spectrometer equipped with a cryogenic triple-resonance probe. The triply ¹³C-labeled inactivator-flavin conjugate (**3**) was

present at an estimated 200 μM in D_2O . Data was collected at 25 $^\circ\text{C}$ in a D_2O using matched shigemi microtubes (Sigma-Aldrich) and processed with nmrPipe.

Results

Cyclopropylamine Synthesis

Cyclopropylamine analogs can be potent inhibitors for amine oxidases (26,27) so we prepared the corresponding substrate analog **2** for LSD1. The synthetic approach to **2** was similar to that used previously for **1** in that a hydroxy analog of lysine was used to generate a mesylated peptide (Scheme 1). The mesylated peptide was reacted with cyclopropylamine to produce the desired analog **2** which could be purified by RP-HPLC and showed the correct molecular weight by mass spectrometry.

LSD1 inhibition studies

In prior studies (15) we used baculovirus expressed recombinant LSD1 whereas a more convenient *E. coli* overproducing strain which generated GST-LSD1 was used here. Catalytic parameters for the bacterially expressed LSD1 with histone H3-21 peptide substrate were modestly different from the insect cell-derived protein as shown in Table 1 with $k_{\text{cat}}/K_{\text{m}}$ being about 9-fold higher for the former. The basis for these observed differences is uncertain may be due to slightly different construct sizes, post-translational modifications in insect cells, and/or the presence of the GST tag on the bacterially produced LSD1. Compound **1** proved to be a somewhat more potent time-dependent inactivator with the bacterially expressed LSD1 enzyme with a $k_{\text{inact}}/K_{\text{i(inact)}}$ increase of 13-fold over the insect cell derived enzyme (Table 1, Figure 1), consistent with the fact that this enzyme also showed higher k_{cat} and lower K_{m} . In contrast, the cyclopropylamine analog **2** did not exhibit time-dependent inactivation of LSD1 but instead appeared to show classical reversible inhibition (Figure 1). In addition, **2** was able to compete with **1** and decrease k_{obs} for inactivation by about 30% when held in a 5-fold excess (data not shown). Titration of **2** versus LSD1 and Dixon analysis revealed a K_{i} of 2.8 ± 1.1 μM (assuming a standard competitive model of inhibition).

Optical measurements with **1** and **2**

Prior studies with compound **1** and LSD1 showed that it generated a flavin adduct with molecular weight equal to the sum of the peptide and FAD (15). In principle, the covalent adduct induced by compound **1** could have several structures (**3-5**) as shown in Figure 2. Prior studies on monoamine oxidase inactivation by propargylic amines have employed absorbance spectroscopy to characterize the nature of analogous inhibitor-flavin adducts (21,27-32). In this light, we showed that compound **1** induced a major shift in the flavin absorbance spectrum. The ground state spectrum shows two maxima in the 350-550 nm region which are attributed to the oxidized and one electron reduced species (31). Upon addition of **1** to LSD1, these two peaks collapsed and a maximum at 392 nm appeared, consistent with flavin modification (28,33) (Figure 3). In contrast, the cyclopropylamine analog did not induce a significant absorbance shift, suggesting that the changes with **1** reflect suicide inactivation. To examine this further, we treated the **1**-LSD1 complex with sodium borohydride (32) which would have the potential to reduce the iminium functionality as well as the conjugated carbon-carbon double bonds present in **3** and **4**. This treatment led to another dramatic spectroscopic change with a near disappearance of the peak at 392 nm. This change was unlikely to have been related to reduction of the linker double bonds in compound **4** which are not in conjugation with the ring, whereas such changes would be entirely consistent with structure **3**. In a separate experiment, acidic treatment ($\text{pH} < 2$) of the **1**-LSD1 adduct led to a hypsochromic shift (20 nm) to 372 nm, presumably via protonation at the N^1 position and thus elimination of the zwitterionic character present in the ground state of the adduct (28) (Figure 3). Based on related studies with monoamine oxidase as well as model adducts (28,33), this behavior is also

consistent with a structural assignment in which the flavin N⁵ is linked to the vinyl imine as shown in **3** (Figure 2A) where the flavin ring nitrogen is protonated.

NMR analysis of the 1*-flavin adduct **3**

In order to further characterize the **1**-flavin adduct, we elected to pursue NMR studies on the isolated compound. Gartner and colleagues (28) have previously analyzed the ¹H NMR spectrum of a chemically synthesized flavin adduct with a small molecule that would be analogous to **3**. To simplify the challenge of analyzing the rather complex predicted adduct by NMR, we prepared an analog of **1** in which the propargyl group was substituted with ¹³C at each site. We generated the ¹³C-labeled propargylamine from the commercially available triply labeled propargyl alcohol and then used this fragment in generating ¹³C-labeled **1** (**1***). **1*** showed the anticipated mass spectrometric and ¹³C-NMR properties. We then used **1*** in several large scale experiments with LSD1 and isolated **3** by RP-HPLC and size-exclusion chromatography. Mass spectrometry showed the correct molecular weight for the predicted **1***-flavin adduct (data not shown). Although there was some contamination observed in this mass spectrum with unreacted and dealkylated **1***, the latter being unlabeled should be invisible in the characterization of the **1***-flavin adduct. We used a combination of HSQC (34) and HCCH-COSY (35,36) experiments to identify the labeled three carbon fragment predicted for **3** along with the protons on these carbons. As shown in Figure 4 (A, B), the alpha and gamma carbons cluster in the expected chemical shift range of 160-170 ppm. The corresponding protons on these carbons also show the expected chemical shifts of 7.5-8 ppm (28). These experiments allow the C^β carbon and its attached hydrogen to be assigned with high confidence; moreover, the ¹³C and ¹H chemical shifts are also in excellent agreement with prediction at ca. 95 ppm and 5.3 ppm (28), respectively. The additional ¹³C-edited ROESY (37) experiment shown in Figure 4 C indicates the close proximity of the C^γ hydrogen with a downfield proton that is very likely the flavin H⁶ atom. It should be noted that only a subset of the peaks assigned to alpha/gamma protons show this NOE effect consistent with the prediction that only the gamma (rather than the alpha) proton is close enough to the flavin proton to give a significant effect. Taken together, we believe these NMR experiments provide compelling evidence for the flavin adduct being best represented by the covalent bond structure of **3**, with the recognition that the conformation and linker double bond stereochemistry cannot be stated with certainty and may well be heterogeneous.

Kinetic analysis of **3** formation

Based on the structural assignment of **3** using mass spectrometry, optical spectroscopy, and NMR spectroscopy, we elected to examine the kinetics of its formation by developing a spectroscopic continuous assay. In these experiments, an excess of inhibitor to enzyme was used and we could readily monitor the formation of **3** in real time. Reaction progress curves could be fit to a pseudo-first order process and the apparent rate-constants obtained at a range of concentrations. As can be seen, the rates of optical change show an apparent saturating rate constant of $0.50 \pm 0.01 \text{ min}^{-1}$ and an apparent K_D of $1.95 \pm 0.24 \text{ } \mu\text{M}$ (Figure 5). These values are quite similar to the k_{inact} and $K_{\text{i(inact)}}$ obtained from the plots of time-dependent demethylase inhibition (Table 1, Figure 1). Therefore, it is reasonable to conclude that the formation of **3** by LSD1 and **1** is the principal process responsible for enzyme inactivation.

Compound **1** as an affinity tag for proteomics analysis

We generated a biotinylated version of **1** (**1-Btn**) to assess whether it would be possible to use this chemical tool to specifically isolate LSD1 from cell extracts. Since the natural abundance of LSD1 in HeLa cell nuclear extracts is unknown, we initially used a spiked mixture containing 1% LSD1 by weight. In these affinity isolation experiments, extracts were mixed with $20 \text{ } \mu\text{M}$ **1-Btn** and then applied to a streptavidin-agarose resin. After washing, proteins were eluted by

denaturation and analyzed by silver-stained SDS-PAGE. As shown in Figure 6, we were able to identify a highly enriched band corresponding to recombinant LSD1 based on a side-by-side comparison. In additional experiments, we demonstrated that this spiked LSD1 could be competed if the non-biotinylated inactivator **1** was used in excess (Figure 6). Furthermore, at least two other bands were visualized that were competed by the addition of **1** (Figure 6). These bands were observed both in the LSD1 spiked and non-spiked extracts and had molecular weights that corresponded to endogenous LSD1 (~100 kDa) and its binding partner CoREST (~65 kDa). To examine the possibility that the identity of these 100 kDa and 65 kDa proteins might well be as predicted, we employed western blot analysis using antibodies to these known proteins. These western blots provide further evidence that these silver-stained bands are in fact CoREST and LSD1. Thus, strong bands appear in the **1-Btn** only lanes, but are effectively competed in lanes containing the non-biotinylated inactivator **1** (Figure 6). Taken together, it appears that **1-Btn** can be used to identify endogenous targets and their binding partners in cell extracts.

Discussion

Here we have shown that compound **1**, a propargylamine containing histone H3 tail peptide but not the corresponding cyclopropylamine analog, is a mechanism-based inactivator of LSD1. The differential reactivity of these two functional groups is somewhat unexpected based on results with monoamine oxidase A and B inactivation (33) and points to subtle differences in structure-function relationships among these enzymes. Interestingly, tranlylcypromine, a cyclopropylamine containing small molecule LSD1 inhibitor (16), does appear to show time-dependent inactivation of LSD1 with flavin modification (38, unpublished data from our labs). However, the cyclopropyl moiety in this compound is benzylic and oriented somewhat differently from that in the cyclopropylamine peptide analog **2**. In future work, it may be interesting to prepare an analog with the cyclopropyl functionality inserted between the delta and epsilon carbons of the lysine to more closely mimic the orientation in tranlylcypromine.

A combination of mass, optical, and NMR spectroscopy have allowed us to confidently assign the structure of the covalent adduct **3** formed upon LSD1 exposure to propargylamine compound **1**. One theoretical alternative to the proposed structure is the adduct **4** formed from reaction between the flavin C^{4a} and the propargylamine function (Figure 2B). However, this structure would be expected to be much less stable, given its loss of conjugation between the imine and the flavin. In fact, we found that **3** is very stable, showing minimal decomposition by NMR after standing in solution at room temperature for more than 4 weeks in a weakly acidic solution. Moreover, the optical spectra and the NMR chemical shifts for the alpha and gamma carbons would be expected to be quite different. Another theoretical adduct would involve a cyclized species **5** (Figure 2B). However, the NMR data is also inconsistent with such a compound which would not be expected to have three highly deshielded carbons and attached protons.

In sum, our data suggest an inactivation mechanism as indicated in Scheme 2. In this mechanism, oxidation of the amine (by single or two electron transfer reaction) yields the propargylic iminium ion which undergoes Michael addition with the N⁵ of the flavin. Although there is a formal possibility of a two-step mechanism whereby C^{4a} attacks the propargyl iminium species followed by rearrangement to an N⁵ adduct, we prefer the simpler model described. The optical time course shown in Figure 5 is in agreement with our one-step mechanism as there is no evidence of an intermediate buildup, and the data fits nicely to a single exponential. A wide range of conformational, tautomeric, and stereochemical isomers are possible for compound **3**. For example, each of the linker double bonds could exist in *cis* or *trans* configurations and the linker single bonds could occupy at least two rotamers, affording

at least 16 possible structural isomers of **3**. It is very likely that there is an ensemble of possibilities and these data do not allow deconvolution at this stage.

The optical spectroscopy change in Figure 3 was conveniently used for kinetic analysis of the reaction as shown in Figure 5. The clear similarity between the kinetics of spectroscopic change and the demethylase inactivation further validate the importance of the adduct in the inactivation process. Since this inactivation rate is not much lower than the demethylase rate, it is reasonable to speculate that oxidation of the inhibitor by the flavin may be at least a partially rate-determining step in inactivation and substrate turnover. It has recently been shown that the re-oxidation of flavin by molecular oxygen is quite fast (330 min^{-1}) excluding this as a rate-determining step (39).

The use of affinity labels and mechanism-based inactivators in proteomics analysis has undergone a renaissance with the advent of modern mass spectrometric and chromatographic techniques (40–43). Excellent probes have been developed for proteases, phosphatases, and protein arginine deiminases based on electrophilic reactivity. The advantage of a propargylamine probe is its lack of intrinsic reactivity in biological milieus. This has made it especially attractive for click chemistry (44,45). However, enzymatic oxidation of the propargylic amine creates an electrophilic target for protein active sites in addition to flavin moieties. The use of **1-Btn** for isolation of LSD1 and its binding partner CoREST offers a new opportunity for studying demethylases in complex systems. In particular, the possibility of identifying novel demethylases using such probes appears to represent an exciting opportunity. In this way, one can place the warhead (propargylamine moiety) on a peptide site thought to be targeted by a demethylase and extract it out. In a more sophisticated iteration, one can even imagine incorporating such a propargyl grouping by protein semisynthesis (46) or unnatural amino acid mutagenesis (46) for mimicking full-length folded methylated proteins of interest.

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Figure 1.

Inhibition of GST-LSD1 by **1** and **2**. (A) Steady-state progress curves obtained for the inactivation of LSD1 by 0 (●), 0.6 (○), 1.2 (▼), 2.4 (▽), and 4.8 (■) μM **1** at 240 μM diMeK4H3-21 ($> 4 \times K_m$) using 120 nM LSD1 in the peroxidase coupled assay system ($n=2$). Additional curves were obtained at 7.5 μM **1** using 360 nM LSD1 to increase signal to noise. (B) Rate constants (k_{obs}) for the time-dependent inactivation of LSD1 by **1** were extracted from steady-state data by single-exponential fits (Eq. 1) and analyzed by the method of Kitz and Wilson to yield k_{inact} and $K_{\text{i(inact)}}$. $K_{\text{i(inact)}}$ was then extrapolated to zero substrate using Eq. 3. (C) Steady-state progress curves obtained for the inhibition of LSD1 by 0 (●), 2.5 (○), 5 (▼), 10 (▽), 20 (■), and 40 (□) μM **2** at 60 μM diMeK4H3-21 ($\sim K_m$) using 120 nM LSD1 in the peroxidase coupled assay system ($n=2$). (D) Initial velocities for the inhibition of LSD1 by **2** were obtained by linear regression and replotted in a Dixon analysis to yield the K_i (assuming a competitive mode of inhibition).

Figure 2. Proposed and theoretical structures of propargyl based inactivator – flavin conjugates. (A) Proposed structure of the adduct involving a trimethine linkage between the epsilon nitrogen of the lysine-4 inactivator and the N⁵ of the flavin (**3**). (B) Theoretical structure for an adduct involving a linkage between the epsilon nitrogen of the lysine-4 inactivator and the C^{4a} of the flavin (**4**) and a subsequent cyclization leading to a dihydro-pyrrole-amine (**5**).

Figure 3.

Spectral analysis of **1** and **2** treated GST-LSD1. (A) UV/Vis spectra of 10 μ M native LSD1 (pink), LSD1 treated with 50 μ M **1** for 1 hr (blue), LSD1 treated with 50 μ M **1** (1 hr) then acidified with 0.3 M HCl (orange), and LSD1 treated with 50 μ M **1** (1 hr) then reduced with 1 mM NaBH₄ (green). The samples were clarified by centrifugation at $14,000 \times g$ for 10 min prior to recording the absorbance spectrum from 350-550 nm. (B) UV/Vis spectra of 10 μ M native LSD1 (pink) and LSD1 treated with 60 μ M **2** for 1 hr (blue). The samples were clarified by centrifugation at $14,000 \times g$ for 10 min prior to recording the absorbance spectrum from 350-550 nm.

Figure 4.

NMR analysis of **1***-flavin conjugate **3**. (A) Constant-time ^1H , ^{13}C HSQC spectrum of **3**, showing regions consisting of $\text{H}^{\alpha/\gamma}:\text{C}^{\alpha/\gamma}$ (black) and $\text{H}^{\beta}:\text{C}^{\beta}$ (red) cross peaks, which are of opposite phase owing to the difference in the number of one-bond ^{13}C coupling partners (one for $\text{C}^{\alpha/\gamma}$, two for C^{β}). Insets *a* and *b* show the fine structures of representative peaks belonging to the $\text{H}^{\alpha/\gamma}:\text{C}^{\alpha/\gamma}$ and $\text{H}^{\beta}:\text{C}^{\beta}$ clusters respectively. These were obtained from a separate, high-resolution ^1H , ^{13}C HSQC spectrum. (B) Region of an "out-and-back" HCCH-COSY spectrum of **3**, showing cross-peaks between the C^{β} and $\text{C}^{\alpha/\gamma}$ clusters. The cross peaks are mediated through one-bond ^{13}C - ^{13}C coupling constants. (C) Region of ^{13}C -edited ^1H , ^{13}C HSQC-ROESY spectrum of **3**, showing cross-peaks between putative H^{γ} peaks and H^6 resonances of the flavin moiety. The ROESY mixing time was 250 ms.

Figure 5.

Time dependent formation of **1**-FAD adduct (**3**). (A) Difference spectrum generated by subtracting the absorbance spectrum of native LSD1 from the absorbance spectrum of LSD1 treated with 50 μM **1** (1 hr). The plot indicates that maximum change occurs at 404 nm. (B) LSD1 (0.75 μM) was incubated with **3** (●), 6 (○), 12 (▼), 24 (▽), 48 (■), and 96 (□) μM **1** and the increase in absorbance at 404 nm was monitored for 10 min (n=2). The k_{obs} values were extracted by fitting the data to Eq. 1. (C) The k_{obs} values from B were replotted versus the concentration of **1** and fit to Eq. 5 to yield the k_{lim} for formation of **3** and the apparent K_D of **1**. These values are similar to those obtained from steady-state demethylase assays.

Figure 6.

1-Btn as an affinity tag for proteomic analysis of HeLa cell nuclear lysates. (A) HeLa cell nuclear lysates were spiked with 1% recombinant LSD1 (178-831) and successfully pulled-down with **1-Btn** as compared to standard on a silver stained 4-15% gel (lanes 1-3). The addition of **1** as a competitor of **1-Btn** resulted in the loss of the recombinant LSD1 signal displaying the selectivity for LSD1 (lanes 4-5). Further analysis showed that at least two more signals (*a* and *b*) displayed competition when treated with **1-Btn** alone versus both **1-Btn** and **1** (lanes 6-7). (B) The identity of the competed bands (*a* and *b*) was confirmed as LSD1 and CoREST by western blot analysis using α-LSD1 (lanes 8-9) and α-CoREST (lanes 10-11) antibodies.

Scheme 1.
Synthetic procedure for the preparation of LSD1 inhibitors.

Scheme 2.

Proposed mechanism for the activation of **1** and subsequent inactivation of the LSD1 by flavin modification.

Table 1Kinetic constants for baculovirus produced and *E. coli* produced LSD1.

	Baculovirus LSD1 (178-831)	<i>E. coli</i> GST-LSD1 (171-852)
k_{cat}	$1.4 \pm 0.1 \text{ min}^{-1}$	$3.1 \pm 0.1 \text{ min}^{-1}$
K_{m}	$200 \pm 30 \text{ }\mu\text{M}$	$50 \pm 5 \text{ }\mu\text{M}$
$k_{\text{inact}} \text{ (1)}$	$0.26 \pm 0.03 \text{ min}^{-1}$	$0.29 \pm 0.02 \text{ min}^{-1}$
$K_{\text{i(inact)}} \text{ (1)}$	$8.3 \pm 1.5 \text{ }\mu\text{M}$	$0.69 \pm 0.10 \text{ }\mu\text{M}$