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Structure-activity study for (bis)ureidopropyl- and (bis)thioureidopropyldiamine LSD1 inhibitors with 3-5-3 and 3-6-3 carbon backbone architectures

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Abstract

Methylation at specific histone lysine residues is a critical post-translational modification that alters chromatin architecture, and dysregulated lysine methylation/demethylation is associated with the silencing of tumor suppressor genes. The enzyme lysine-specific demethylase 1 (LSD1) complexed to specific transcription factors catalyzes the oxidative demethylation of mono- and dimethyllysine 4 of histone H3 (H3K4me and H3K4me2 respectively). We have previously reported potent (bis)urea and (bis)thiourea LSD1 inhibitors that increase cellular levels of H3K4me and H3K4me2, promote the re-expression of silenced tumor suppressor genes and suppress tumor growth *in vitro*. Here we report the design additional (bis)urea and (bis)thiourea LSD1 inhibitors that feature 3-5-3 or 3-6-3 carbon backbone architectures. Three of these compounds displayed single-digit IC₅₀ values in a recombinant LSD1 assay. In addition, compound **6d** exhibited an IC₅₀ of 4.2 μ M against the Calu-6 human lung adenocarcinoma line, and 4.8 μ M against the MCF7 breast tumor cell line, in an MTS cell viability assay. Following treatment with **6b–6d**, Calu-6 cells exhibited a significant increase in the mRNA expression for the silenced tumor suppressor genes *SFRP2*, *HCAD* and p16, and modest increases in GATA4

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Supplementary Material

Supplementary material for this manuscript includes Figure S1 (IC₅₀ value determinations for compounds **2**, **6b**, **6c**, **6d**, **8f** and **21** against LSD1); Figure S2 (IC₅₀ value determinations for compounds **1**, **2**, **6b**, **6c** and **6d** against monoamine oxidase A and B); Figure S3 (cell viability dose-response in Calu-6, MCF7 and MCF-10A cells for compounds **2**, **6b**, **6c**, **6d** and **20**), and is available on line.

message. The compounds described in this paper represent the most potent epigenetic modulators in this series, and have potential for use as antitumor agents.

Keywords

Epigenetics; Histone demethylase; Lysine-specific demethylase 1; Oligoamine; Antitumor agent

1. Introduction

DNA methylation at CpG islands in the promoter regions of DNA and the post-translational modification of histones play a crucial role in the regulation of chromatin architecture and gene expression.^{1–3} It is well established that CpG island hypermethylation at gene promoters alone or in combination with specific histone modifications is associated with silencing of tumor suppressor genes.⁴ Prior to 2004, it was thought that the dynamic ratio of methylated to unmethylated lysine residues on histone tails was mediated by histone methyltransferases and the subsequent replacement of methylated histones with non-methylated histones. The discovery of the enzyme lysine-specific demethylase 1 (LSD1) established that histone methylation was a dynamic, reversible process.⁵ LSD1 is a flavin-dependent amine oxidase that catalyzes the oxidative demethylation of both activating and deactivating chromatin marks such as histone 3 lysine 4 (H3K4) and histone 3 lysine 9 (H3K9) through an FAD-dependent oxidative reaction.⁶ It is now known that LSD1 is overexpressed in a variety of human cancers, and excessive demethylation of activating chromatin marks such as H3K4 leads to silencing of critical tumor suppressor genes.^{7, 8} For this reason, LSD1 and other chromatin remodeling proteins have become attractive targets for drug discovery.^{9, 10}

In light of the central role of LSD1 in demethylating chromatin marks, and given its validity as a chemotherapeutic target, we initiated a program to design, synthesize and evaluate small molecules that act as epigenetic modulators of gene expression through LSD1 inhibition. The structure and mechanism of LSD1 are similar to those of monoamine oxidase A and B, leading to the observation that tranlycypromine 1 (Figure 1) was a modest irreversible inhibitor of LSD1 (IC_{50} 242 μ M, k_{inact} 0.011 sec^{-1}).¹¹ A number of inhibitors based on the tranlycypromine scaffold have now been discovered, and in a few cases they have nanomolar IC_{50} values against recombinant LSD1.^{9, 12–17} In 2007, we described a series of (bis)guanidines and (bis)biguanides that act as potent LSD1 inhibitors, increase H3K4 methylation and promote the re-expression of aberrantly silenced tumor suppressor genes in vitro.⁸ The lead compound emerging from these studies, verlindamycin (2, Figure 1, aka 2d), is synergistic with the deoxynucleotide-N-methyltransferase (DNMT) inhibitor 5-azacytidine (5-AC) in limiting tumor growth in an HCT116 xenograft study in athymic mice,¹⁸ and has been shown to promote the re-expression of the silenced e-cadherin gene in acute myeloid leukemia cells in vitro.¹⁹ Subsequently, we reported a series of (bis)alkylureas and (bis)alkylthioureas that are isosteric to 2, and found that these analogues were more potent epigenetic modulators in vitro.²⁰ The IC_{50} values for the three most promising compounds from the (bis)thiourea series, 3–5, suggested that the ability of these analogues to promote epigenetic changes was related to the length of the central chain. Each

of these compounds featured (bis)-2,2-(diphenyl)ethyl substituents on the terminal nitrogens, and their relative inhibitory activity was in the order **4**>**3**>**5**.²⁰ Low micromolar concentrations of compounds **3** and **4** cause a significant increase in the global H3K4me2 methylation mark in Calu-6 lung carcinoma cells, accompanied by an increase in the mRNA levels of the secreted frizzled-related protein 2 (*SFRP2*) and the transcription factor *GATA4*.^{8, 20} In order to add to our library of LSD1 inhibitors that could serve as epigenetic modulators and potential antitumor agents, we designed and synthesized homologous (bis)ureas and (bis)thioureas **6** and **7**, which are previously unreported compounds featuring a 3-5-3 carbon/nitrogen backbone architecture, and compounds **8** and **9**, which possess a 3-6-3 backbone structure.²¹ The 3-6-3 compounds have been reported,²¹ but have not been characterized as epigenetic modulators (Figure 1). In the present manuscript, we describe the synthesis, biological evaluation and structure/activity correlations for these oligamines. We also describe molecular docking analysis of the most promising LSD1 inhibitor, compound **6b** (Table 1), which produces the most significant inhibition in the LSD1 in vitro assay. These in silico studies were conducted using the software tool GOLD,²² with the goal of characterizing the binding of **6b** and its homologues to the enzyme.

2. Chemistry

To complete the synthesis of oligoamine compounds **6–9**, we employed a variation of a synthetic route previously described by our laboratory, as shown in Scheme 1.^{20, 21} The appropriate diamine **10** (*n* = 1 or 2) was (bis)cyanoethylated (acrylonitrile, EtOH, reflux) to afford the corresponding (bis)cyano intermediates **11**. The central nitrogens in **11** were then N-Boc protected ((Boc)₂O, CH₂Cl₂/aq. NaHCO₃)²³ to yield **12**, and the cyano groups were reduced (Raney Ni) to yield the desired diamines **13**.^{24, 25} Compounds **13** were then reacted with the appropriate isocyanates **17** or isothiocyanates **19**²⁶ (see Scheme 2) to produce the corresponding protected (bis)ureas or (bis)thioureas **14**, followed by acid removal of the N-Boc protection groups (HCl in EtOAc)²³ to afford the desired urea or thiourea products **6–9**. In cases where the requisite isocyanate or isothiocyanate was not commercially available, it could be synthesized using the route shown in Scheme 2. Thus the appropriate amine **15** was treated with trichloroacetyl anhydride **16** in toluene to directly afford the desired isocyanate **17**. Likewise, treatment of **15** with carbon disulfide and triethylamine in THF produced intermediate **18**, which was converted to the corresponding isothiocyanate **19** using tosyl chloride.

3. Biological Evaluation

To determine the inhibitory potency of **6–9** against human recombinant LSD1, we employed an assay protocol previously described by our laboratories.^{8, 20} A preliminary screen at a concentration of 10 μM was initially run to measure relative inhibitory activity, as summarized in Table 1 and Figure 2. Compound **1**, a non-selective inhibitor of flavin-dependent amine oxidases, and **2**, the LSD1 inhibitor verlindamycin,⁸ were used as positive controls.^{8, 20, 27} Among the new compounds tested, (bis)aralkyl compounds **6b**, **6c**, **6d**, **7c**, **7d**, **7e**, **8e**, **8f** and **8g** produced greater LSD1 inhibition than the related monoaralkyl derivatives (**6a**, **7a**, **7b**, **8a**, **8b**, **8c**, **8d**, **9a**, **9b**, **9c**, **9d**), an observation that is in agreement with our previous observations involving variations in the size of the terminal substituent.²⁰

Similarly, the (bis)aralkylthiourea analogs **3**, **4**, **5**, **6a**, **6b**, **6c**, **6d**, **8a**, **8b**, **8c**, **8d**, **8e**, **8f**, **8g** were consistently more potent LSD1 inhibitors than the corresponding (bis)aralkylurea analogs **7a**, **7b**, **7c**, **7d**, **7e**, **9a**, **9b**, **9c**, **9d**, a finding that also agrees with our previous studies.²⁰ It is notable that (bis)ureido analogues **7a**, **9a**, **9b** and **9d** caused an increase in the activity of LSD1 at the concentration tested. In reaction mixtures containing **7a**, **9a**, **9b** and **9d** and inactivated (boiled) LSD1, the compounds gave identical results to control, and thus the compounds have no intrinsic luminescence. Interestingly, these 4 analogues are all (bis)ureido compounds that lack a benzylic carbon on the terminal substituents. The most likely explanation for the increase in LSD1 activity is that these compounds produce an allosteric effect that increases the efficiency of the enzyme or stabilizes the epigenetic complex. Additional mechanistic studies beyond the scope of this manuscript are required to verify this hypothesis.

A survey of the LSD1 inhibitory activity of (bis)thioureas with identical terminal nitrogen substituents and central chain lengths between 3 and 7 carbons at 10 μM revealed that the 3-5-3 carbon backbone found in **6b**, **6c** and **6d** produced the greatest inhibitory activity (Figure 3, left column). These analogues showed inhibitory activity at 10 μM in the order $\text{C5} > \text{C6} > \text{C4} = \text{C7} > \text{C3}$, based on the number of carbons in the central chain. When the central chain was held constant at 5 carbons, compounds **6b**, **6c** and **6d** were essentially equipotent. These data suggest that a 5-carbon central chain is optimal for LSD1 inhibition in this series.

We next determined the enzymatic IC_{50} values for compound **2**, 3-5-3 compounds **6b** (bis-1,1-diphenylmethyl, IC_{50} 8 μM), **6c** (bis-2,2-diphenylethyl, IC_{50} 7 μM) and **6d** (bis-3,3-diphenylpropyl, IC_{50} 5 μM), and the corresponding compounds **8f** (C6 central chain, IC_{50} 10 μM) and **21** (C4 central chain, IC_{50} 14 μM). The results of these studies are shown in Figure S1. Based on IC_{50} values, **6b**, **6c** and **6d** were again essentially equipotent inhibitors of recombinant LSD1. Compound **6d** appeared to be slightly more active, albeit by a small margin. A Lineweaver-Burk analysis of compound **6d** (Figure 4) demonstrated that the compound is a competitive inhibitor with a K_i value of 2.4 μM . These results are significant, in that our previous studies showed that the (bis)-3,3-diphenylpropyl biguanide **2** acts as a non-competitive inhibitor of recombinant LSD1.⁸ Importantly, our attempts to isolate a co-crystal of **2** and LSD1/CoREST have not yet been successful, and as such we are unable to explain the non-competitive kinetics observed for inhibition of the enzyme by biguanides such as **2**. In addition, although our kinetic results suggest that **6d** and its homologues are competitive inhibitors of the recombinant enzyme, the cellular mechanism may be quite different. There is increasing evidence to support the hypothesis that analogues such as **6d** could inhibit the function of LSD1 indirectly by disrupting the complex formed with HDAC 1 and 2, REST and CoREST.^{28, 29} Along those lines, we are conducting pull-down experiments to determine which cellular factors are associated with the complex following application of these LSD1 inhibitors, and these results will be reported separately.

In order to determine the selectivity of **1**, **2** and **6b–d** for LSD1, these compounds were evaluated for their ability to inhibit MAO-A and MAO-B using a commercial assay kit (MAO-Glo®, Promega, Madison, WI). These results are shown in Table 2 and Figure S2. The known MAO inhibitor **1** was a poor inhibitor of LSD1, and exhibited an IC_{50} value of 242 μM against the recombinant enzyme. As expected, **1** was a potent inhibitor of MAO-A

(IC₅₀ 4 μ M) and MAO-B (IC₅₀ 6 μ M). Compound **2** was significantly more potent against recombinant LSD1 (IC₅₀ 13 μ M), but also inhibited MAO-A (IC₅₀ 37 μ M) and MAO-B (IC₅₀ 10 μ M). By contrast, **6b–6d** did not inhibit MAO-A at concentrations up to 100 μ M, and showed 4-fold selectivity for LSD1 over MAO-B.

The Calu-6 human anaplastic lung tumor cell line was chosen for subsequent experiments because these cells exhibit high levels of endogenous LSD1 activity.²⁰ In a standard MTS cell viability assay, the (bis)-3,3-diphenylpropyl-substituted 3-5-3 compound **6d** (IC₅₀ 4.2 μ M) displayed a lower IC₅₀ value than the homologous 3-5-3 compounds **6c** (IC₅₀ 6.5 μ M) and **6b** (IC₅₀ 9.7 μ M), **2** (IC₅₀ 12.5 μ M) and the previously described 3-4-3 analogue **20** (IC₅₀ 14.9 μ M)²⁰ (Figure S3, Panel A). The superior activity of **6d** in cells could be attributed to enhanced lipophilic character caused by the (bis)-3,3-diphenylpropylamine moieties. In our hands, compound **2** has only modest activity against the MCF7 breast tumor cell line in vitro. We thus examined the effects on cell viability of the same 5 compounds in the MCF7 cell line as well. Compounds **6b** (IC₅₀ 6.3 μ M), **6c** (IC₅₀ 6.1 μ M), **6d** (IC₅₀ 4.8 μ M) and **20** (IC₅₀ 6.8 μ M) exhibited IC₅₀ values that were similar to the values observed in the Calu-6 line, while the IC₅₀ value for **2** increased slightly to 17.4 μ M (Figure S3, Panel B). These results suggest that terminally substituted thioether compounds promote greater cytotoxicity in these two cell lines when compared to biguanides such as **2**. The human breast epithelial cell line MCF10A exhibits levels of LSD1 that are significantly lower than either the Calu-6 or MCF7 cell lines. As shown in Figure S3, Panel C, compounds **6a**, **6b**, **6d** and **20** were significantly less cytotoxic in this cell line, suggesting that the antitumor effect of these analogues is related to the LSD1 content in a given cell line. It should be noted that these structure-activity correlations are preliminary, and an expanded library of analogues is now being synthesized to further refine the SAR model.

It has been demonstrated that the inhibition of LSD1 by oligoamine analogues leads to the re-expression of several abnormally silenced tumor suppressor genes.^{8, 18, 30} Thus, we tested the ability of compounds **6b–6d** to promote re-expression of silenced tumor suppressor genes in the Calu-6 cell line. The secreted frizzled-related protein 2 (SFRP2), a modulator of the Wnt signaling pathway, H-cadherin (HCAD), a membrane bound mediator of calcium-dependent cell to cell adhesion, GATA4, a zinc finger transcription factor, and p16, a cell cycle-regulating protein were chosen as candidate genes for these expression studies because it has been hypothesized that their silencing promotes tumorigenesis^{8, 18, 30–32}. In the present study, Calu-6 cells were treated for 24 or 48h in the presence of 10 μ M of compound **6b**, **6c** or **6d** and the mRNA levels of *SFRP2*, *HCAD*, *GATA4*, and *p16* expression were subsequently determined by qRT-PCR analysis (Figure 5). Interestingly, the expression levels of both *HCAD* and *p16* mRNA were significantly up regulated at both 24 and 48h after treatment with all three compounds. However, *SFRP2* and *GATA4* mRNA exhibited very different expression profiles, with *SFRP2* mRNA displaying a significant increase in expression only after 48h of treatment with **6c** or **6d**. *GATA4* mRNA significantly increased at 24h after treatment with compound **6c**, and at 48h after treatment with compound **6d**. These data provide strong evidence that the inhibition of LSD1 by these three novel oligoamines containing 3-5-3 linkers induces the re-expression of several epigenetically silenced tumor suppressor genes. Compound **6d** appears to be the most promising of these

candidate compounds by displaying induction of all 4 tumor suppressor genes at 48h. These data provide a rationale for further study of these compounds in other *in vitro* and *in vivo* cancer models.

It has been previously shown that the inhibition of LSD1 can be accompanied by significant increases in global H3K4me2.²⁷ Therefore, the global levels of H3K4me2 in Calu-6 cells treated with 10 μ M of compound **6b**, **6c** or **6d** were compared to non-treated cells. When normalizing H3K4me2 to total histone H3 (Figure 6, Panel A), a trend towards higher global H3K4me2 protein levels was observed in all compound-treated Calu-6 cells at 48h; however, none of the increases of H3K4me2 were statistically significant. Additionally, H3K4me2 protein levels are reduced at 24h when treated with compounds **6b** and **6c** and only show a modest induction with compound **6d**. These results are similar to those observed in cells where LSD1 has been deleted through homologous recombination.³³ In those studies, although global levels of H3K4me2 do not change with the loss of LSD1, H3K4me2 at specific gene promoters is increased significantly, suggesting gene-specific effects resulting from loss of LSD1 activity.³³ Thus, the modest increases in global H3K4me2 observed in Calu-6 cells after treatment with **6b**, **6c** or **6d** could be entirely due to more significant increases at specific gene promoters. As an additional determinant of the ability of **6b**, **6c** and **6d** to increase H3K4me2 levels, we measured the levels of H3K4me2 in a bulk histone preparation, as previously described.¹³ The results of these studies, shown in Figure 6, Panel B, suggest that inhibition of LSD1 by **6b**, **6c** or **6d** results in increased H3K4me2 content in isolated histone proteins.

To determine the molecular basis for the observed biological activity of compound **6b**, we performed *in silico* molecular docking experiments using the GOLD software package, version 5.1. For our modeling purposes, we used the coordinates of X-ray crystal structure 3ZMT from the Protein Data Bank, because it featured the necessary components including the LSD1 binding pocket, a co-crystallized inhibitor, free FAD and the associated CoREST complex. The top ranked binding mode of **6b** in the LSD1 binding site is shown in Figure 7.

Interestingly, both **6b** and **6d** are predicted to have similar binding modes in the catalytic site of LSD1 (Figure 7, Panel A). Inspection of the binding mode of **6b** revealed three potential H-bonding interactions (Figure 7, Panel B): the backbone C=O of Ala 539 with both the thiourea nitrogens (2.5 Å and 2.8 Å, respectively), the side chain NH₂ of Asn 535 with the sulfur atom of the second thiourea (3.5 Å) and the C=O at the 4-position of the FAD with one of the amines of **6b** (3.0 Å). The significance of Ala 539 in LSD1 inhibitor binding has been documented previously thereby adding value to the computer-predicted pose.^{34, 35} In addition, the close contact observed between FAD and **6b** provides direction for further optimization studies by preserving the 3-5-3 linker. Binding of **6b** is also influenced by hydrophobic residues lining the LSD1 pocket including Val 333, Phe 382, Phe 538, Ala 539, Trp 552, Trp 695, Tyr 761, Val 764 and Pro 808. Thus, we reason that the binding of compound **6b** to LSD1 is favored by a combination of both H-bonding and hydrophobic interactions. Our future efforts will thus be aimed at constructing a focused library of compound **6b** analogues containing steric and electronic substituents designed to further explore the SAR of the 3-5-3 oligoamines.

In the present study, we have synthesized 9 unreported oligoamines containing a 3-5-3 backbone architecture, and evaluated these compounds, as well as 11 previously described but untested compounds with a 3-6-3 architecture, as inhibitors of recombinant LSD1. Of these compounds, 9 displayed more than 50% inhibition at a concentration of 10 μ M, and 3 compounds (**6b**, **6c** and **6d**) produced greater than 95% inhibition of LSD1 at 10 μ M in a commercial rLSD1 assay. Compound **6d** proved to have the lowest IC₅₀ value (4.8 μ M) for inhibition of the recombinant enzyme, and this inhibition was found to be competitive in nature. Compound **6d** also exhibited the lowest IC₅₀ value against both Calu-6 anaplastic lung tumor cells and MCF7 breast tumor cells in vitro as determined by an MTS cell viability assay. Structure/activity data suggest that (bis)aralkyl substitution with 1,1-diphenylmethyl in the 3-5-3 thiourea series produced the optimal effect on LSD1. Inhibition of LSD1 by compound **6b** and **6c** produced modest increases in H3K4 methylation, and significantly induced the re-expression of aberrantly silenced tumor suppressor genes such as *SFRP2*, *HCAD*, *GATA4* and *p16*. Computational modeling revealed H-bonding and hydrophobic interactions that govern the binding of the inhibitor to the LSD1 pocket. In light of these data, compounds **6b–d** represent the most effective LSD1 inhibitors of this type to date. The synthesis and evaluation of additional compounds in the thiourea series, and in vivo evaluation of compounds **6b–d** are ongoing concerns in our laboratories.

4. Experimental Section

All reagents and dry solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO), VWR (Radnor, PA) or Fisher Scientific (Chicago, IL) and were used without further purification except as noted below. Pyridine was dried by passing it through an aluminum oxide column and then stored over KOH. Triethylamine was distilled from potassium hydroxide and stored in a nitrogen atmosphere. Methanol was distilled from magnesium and iodine under a nitrogen atmosphere and stored over molecular sieves. Methylenechloride was distilled from phosphorus pentoxide and chloroform was distilled from calcium sulfate. Tetrahydrofuran was purified by distillation from sodium and benzophenone. Dimethyl formamide was dried by distillation from anhydrous calcium sulfate and was stored under nitrogen. Preparative scale chromatographic procedures were carried out using E. Merck silica gel 60, 230–440 mesh. Thin layer chromatography was conducted on Merck precoated silica gel 60 F-254. Ion exchange chromatography was conducted on Dowex 1 $\times$ 8–200 anion exchange resin. Compounds **8a–g** and **9a–d** were synthesized as previously described.²¹

All ¹H- and ¹³C-NMR spectra were recorded on a Varian Mercury 400 mHz spectrometer, and all chemical shifts are reported as δ values referenced to TMS or DSS. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak. In all cases, ¹H-NMR, ¹³C-NMR and MS spectra were consistent with assigned structures. Mass spectra were recorded by LC/MS on a Waters autopurification liquid chromatograph with a model 3100 mass spectrometer detector. Prior to biological testing procedures, compounds **14a–i**, **6a–d**, **7a–e**, **8a–g** and **9a–d** were determined to be >95% pure by UPLC chromatography (95% H₂O/5% acetonitrile to 20% H₂O/80% acetonitrile over 10 minutes) using a Waters Acquity H-series ultrahigh-performance liquid chromatograph fitted with a C18 reversed-phase column (Acquity UPLC BEH C18 1.7 μ M, 2.1 $\times$ 50 mm). Compounds

2, 3, 5, 8a–g, 9a–d and 11–13 were synthesized as previously described.^{8, 20, 25} Synthetic H3K4me2 peptides were purchased from Millipore (Billerica, MA). Calu-6 cells and MCF7 cells were maintained in RPMI medium, both supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) and grown at 37°C in 5% CO₂ atmosphere.

General procedure for the synthesis of N-Boc protected (Bis)ureas and (Bis)thioureas

The appropriate aryl isocyanate or aryl isothiocyanate dissolved in anhydrous dichloromethane was added by dropwise addition to a stirred solution of **13** (n=1) in anhydrous dichloromethane at 0°C. The reaction mixture was warmed to room temperature and allowed to stir for 24 h. During the reaction, the formation of product was monitored by TLC (75% EtOAc in hexanes). After 24 hours or when the reaction was complete, the solvent was removed in vacuo and the crude product was purified by chromatography on silica gel eluted with 75% EtOAc in hexanes to afford the final product in moderate to good yields.

1,13-bis-{3-[1-(benzyl)thioureido]}-4,10-[N-(tertbutyl)oxycarbonyl]-4,10-diazatridecane (14a)—Compound **14a** was prepared from **13** (270 mg, 0.648 mmol) and benzyl isothiocyanate (203 mg, 1.361 mmol) using the general procedure described above to afford 315 mg (68%) of pure **14a**. ¹H NMR (CDCl₃): δ 7.32–7.25 (m, 10H), 4.57(s, 4H), 3.90 (m, 4H), 4.1 (m, 4H), 1.71 (m, 4H), 1.56 - 1.47 (m, 4H), 1.40 (s, 18H), 1.23–1.20 (m, 2H). ¹³C NMR (CDCl₃): δ 181.01, 156.70, 136.97, 128.68, 127.64, 79.98, 47.51, 47.01, 43.25, 41.14, 28.38, 27.09, 24.18. MS calculated 687.37, found 687.61 ([M+1]⁺).

1,13-bis-{3-[1-(1,1-diphenylmethyl)thioureido]}-4,10-[N-(tertbutyl)oxycarbonyl]-4,10-diazatridecane (14b)—Compound **14b** was prepared from **13** (218 mg, 0.523 mmol) and 1,1-diphenylmethyl isothiocyanate (236 mg, 1.047 mmol) using the general procedure described above to afford 348 mg (71%) of pure **14b**. ¹H NMR (CDCl₃): δ 7.43 - 7.26 (m, 20H), 6.50 (s, 2H), 3.53 (m, 4H), 3.075 (m, 8H), 1.67 (m, 4H), 1.51 - 1.48 (m, 4H), 1.38 (s, 18H), 1.24 - 1.18 (m, 2H). ¹³C NMR (CDCl₃): δ 180.07, 156.64, 140.25, 128.75, 127.84, 127.58, 79.89, 61.42, 47, 42.93, 41.31, 28.32, 26.95, 24.17. MS calculated 867.47, found 867.26 ([M+1]⁺).

1,13-bis-{3-[1-(2,2-diphenylethyl)thioureido]}-4,10-[N-(tertbutyl)oxycarbonyl]-4,10-diazatridecane (14c)—Compound **14c** was prepared from **13** (200 mg, 0.48 mmol) and 2,2-diphenylethyl isothiocyanate (230 mg, 0.96 mmol) using the general procedure described above to afford 292 mg (68%) of pure **14c**. ¹H NMR (CDCl₃): δ 7.33–7.21 (m, 20H), 5.9 (s, 2H), 4.14–4.05 (m, 4H), 3.55(m, 4H), 3.26 (m, 4H), 3.12 (t, 4H), 1.71 (m, 4H), 1.54 (m, 4H), 1.29 (s, 18H), 1.27 (t, 4H, J = 6.8 Hz). ¹³C NMR (CDCl₃): δ 181.22, 156.78, 141.55, 128.78, 128.08, 126.94, 80.08, 49.85, 47, 43.29, 40.99, 28.41, 26.96, 24.22. MS calculated 895.50, found 895.36 ([M+1]⁺).

1,13-bis-{3-[1-(3,3-diphenylpropyl)thioureido]}-4,10-[N-(tertbutyl)oxycarbonyl]-4,10-diazatridecane (14d)—Compound **14d** was prepared from **13** (200 mg, 0.48 mmol) and 3,3-diphenylpropyl isothiocyanate (255 mg, 1.008 mmol) using the general procedure described above to afford 328 mg (74%) of pure **14d**. ¹H NMR

(CDCl₃): δ 7.31–7.17 (m, 20H), 4.05 (t, 2H, J = 8.32 Hz), 3.55 (m, 4H), 3.3–3.25 (m, 8H), 3.12 (m, 4H), 2.41–2.36 (m, 4H), 1.71 (m, 4H), 1.56–1.53 (m, 4H), 1.43 (s, 18H), 1.29–1.26 (m, 2H). ¹³C NMR (CDCl₃): δ 171.19, 156.77, 143.95, 128.63, 127.75, 126.46, 80.01, 48.69, 46.99, 43.22, 40.94, 34.48, 31.59, 27.05, 24.22, 22.66. MS calculated 923.53, found 923.42 ([M+1]⁺).

1,13-bis-{3-[1-(phenyl)ureido]}-4, 10-[N-(tertbutyl)oxycarbonyl]}-4,10-diazatridecane (14e)—Compound **14e** was prepared from **13** (268 mg, 0.643 mmol) and phenyl isocyanate (153 mg, 1.287 mmol) using the general procedure described above to afford 282 mg (67%) of pure **14e**. ¹H NMR (CD₃OD): δ 7.34–7.32 (m, 8H), 7.23 (t, 2H, J = 7.6 Hz), 6.99 (t, 2H, J = 7.24 Hz), 3.19–3.10 (m, 12 H), 1.65 (m, 4H), 1.44 (m, 22H), 1.19 (m, 2H). MS calculated 655.42, found 655.28 ([M+1]⁺).

1,13-bis-{3-[1-(benzyl)ureido]}-4, 10-[N-(tertbutyl)oxycarbonyl]}-4,10-diazatridecane (14f)—Compound **14f** was prepared from **13** and benzyl isocyanate (189 mg, 1.422 mmol) using the general procedure described above to afford 324 mg (70%) of pure **14f**. ¹H NMR (CDCl₃): δ 7.28 (m, 10 H), 4.32 (s, 4H), 3.21–3.11 (m, 12 H), 1.61 (m, 4H), 1.52–1.47 (m, 22H), 1.24–1.20 (m, 2H). ¹³C NMR (CDCl₃): δ 158.59, 156.27, 139.53, 128.49, 127.37, 127.08, 79.56, 46.99, 44.32, 43.6, 36.8, 28.44, 24.11. MS calculated 683.45, found 683.37 ([M+1]⁺).

1,13-bis-{3-[1-(1,1-diphenylmethyl)ureido]}-4,10-[N-(tertbutyl)oxycarbonyl]}-4,10-diazatridecane (14g)—Compound **14g** was prepared from **13** (230 mg, 0.552 mmol) and 1,1-diphenylmethyl isothiocyanate (243 mg, 1.159 mmol) using the general procedure described above to afford 332 mg (72%) of pure **14g**. ¹H NMR (CDCl₃): δ 7.40–7.1 (m, 20H), 5.99 (d, 2H), 3.3–2.9 (m, 12 H), 1.54 (m, 4 H), 1.43 (m, 22H), 1.2 (m, 2H). ¹³C NMR (CDCl₃): δ 157.78, 156.25, 142.62, 128.51, 127.36, 127.14, 58.13, 47.01, 45.09, 43.67, 36.73, 28.46, 25.30, 24.15. MS calculated 835.51, found 835.33 ([M+1]⁺).

1,13-bis-{3-[1-(2,2-diphenylethyl)ureido]}-4,10-[N-(tertbutyl)oxycarbonyl]}-4,10-diazatridecane (14h)—Compound **14h** was prepared from **13** (200 mg, 0.48 mmol) and 2,2-diphenylethyl isothiocyanate (225 mg, 1.008 mmol) using the general procedure described above to afford 290 mg (70%) of pure **14h**. ¹H NMR (CDCl₃): δ 7.31–7.19 (m, 20H), 4.19 (t, 2H, J = 7.8 Hz), 3.81 (t, 2H, J = 7.1 Hz), 3.22 (m, 4H), 3.10 (m, 8H), 1.61 (m, 4H), 1.51 (m, 4H), 1.43 (s, 18H), 1.23 (m, 2H). ¹³C NMR (CDCl₃): δ 158.16, 142.22, 128.63, 128.16, 126.66, 79.61, 51.21, 46.91, 44.98, 44.46, 36.5, 28.45, 28.24. MS calculated 863.55, found 863.37 ([M+1]⁺).

1,13-bis-{3-[1-(3,3-diphenylpropyl)ureido]}-4,10-[N-(tertbutyl)oxycarbonyl]}-4,10-diazatridecane (14i)—Compound **14i** was prepared from **13** (217 mg, 0.521 mmol) and 3,3-diphenylpropyl isothiocyanate (260 mg, 1.094 mmol) using the general procedure described above to afford 348 mg (75%) of pure **14i**. ¹H NMR (CDCl₃): δ 7.30–7.16 (m, 20H), 3.99 (t, 2H, J = 7.5 Hz), 3.30–3.26 (m, 4H), 3.15–3.10 (m, 12H), 2.30–2.24 (m, 4H), 1.67 (m, 4H), 1.54–1.51 (m, 4H), 1.46 (s, 18H), 1.25–

1.22 (m, 2H). ^{13}C NMR (CDCl_3): δ 158.48, 156.5, 144.41, 128.54, 127.81, 126.29, 79.59, 48.8, 46.94, 39.21, 36.01, 28.47. MS calculated 891.58, found 891.42 ($[\text{M}+1]^+$).

General procedure for the acid catalyzed removal of N-Boc protecting groups

The appropriate (bis)-N-Boc-protected intermediate **14** was dissolved in HPLC-grade ethyl acetate under a nitrogen atmosphere was added 6 molar equivalents of 1M HCl in ethyl acetate was added with stirring. The reaction mixture was allowed to stir at room temperature for 12 hours, during which time the formation of product was monitored by TLC. The product precipitated as the crystalline solid dihydrochloride salt during the course of the reaction. The solvent was subsequently removed in vacuo, fresh ethyl acetate was added and stirred for 15 minutes, and the liquid was decanted. The solid so obtained was vacuum dried to afford the final product in moderate to good yield.

1,13-bis-{3-[1-(benzyl)thioureido]}-4,10-diazatridecane dihydrochloride (6a)—Pure compound **6a** was synthesized from **14a** (100 mg, 0.153 mmol) using the procedure described above in 85% yield (68.5 mg). ^1H NMR (CD_3OD): δ 7.39 (d, 4H, $J = 7.92$ Hz), 7.28 (t, 4H, $J = 7.6$ Hz), 7.02 (t, 2H, 7.28 Hz), 3.36 (t, 4H, $J = 6.2$), 3.10–3.03 (m, 8H), 1.96–1.91 (m, 4H), 1.83–1.76 (m, 4H), 1.60–1.56 (m, 2H). ^{13}C NMR (CD_3OD): 157.76, 139.20, 128.51, 122.46, 119.25, 119.12, 47.13, 44.91, 35.83, 26.99, 25.3, 23.06. MS calculated 515.30, found 515.09 ($[\text{M}+1]^+$); mp 195–198°C, HPLC retention time 1.98 min.

1,13-bis-{3-[1-(1,1-diphenylmethyl)thioureido]}-4,10-diazatridecane dihydrochloride (6b)—Compound **6b** was synthesized from **14b** (100 mg, 0.146 mmol) using the procedure described above in 90% yield (73.2 mg). ^1H NMR (CD_3OD): δ 7.47–7.28 (m, 10 H), 4.7 (s, 4H), 3.75 (b, 4H), 3.04–3.01 (m, 8 H), 1.99–1.96 (m, 4H), 1.81–1.75 (m, 4H), 1.55 (m, 2H). ^{13}C NMR (CD_3OD): δ 160.47, 139.87, 128.48, 128.16, 126.69, 47.09, 44.71, 43.42, 35.72, 27.12, 25.32, 22.99. MS calculated 666.35, found 666.20 ($[\text{M}+1]^+$); mp 154–56°C, HPLC retention time 2.44 min.

1,13-bis-{3-[1-(2,2-diphenylethyl)thioureido]}-4,10-diazatridecane dihydrochloride (6c)—Compound **6c** was synthesized from **14c** (100 mg, 0.140 mmol) using the procedure described above in 85% yield (69.9 mg). ^1H NMR (CD_3OD): δ 7.4–7.26 (m, 10 H), 4.34 (s, 4H), 3.31 (t, 4H, $J = 6.2$ Hz), 3.01–2.98 (m, 4H), 2.91 (m, 4H), 1.88–1.85 (m, 4H), 1.72–1.68 (m, 4H), 1.48–1.46 (m, 2H). ^{13}C NMR (CD_3OD): δ 128.21, 127.09, 126.99, 47.06, 44.51, 40.20, 26.39, 25.31, 25.28, 23.15; MS calculated 694.39, found 694.25 ($[\text{M}+1]^+$); mp 125–28°C, HPLC retention time 2.48 min.

1,13-bis-{3-[1-(3,3-diphenylpropyl)thioureido]}-4,10-diazatridecane dihydrochloride (6d)—Compound **6d** was synthesized from **14d** (100 mg, 0.12 mmol) using the procedure described above in 88% yield (74.6 mg). ^1H NMR (CD_3OD): δ 7.37–7.25 (m, 20 H), 5.98 (s, 2H), 3.3 (m, 4H), 2.94 (m, 4H), 2.76 (m, 4H), 1.87 (m, 4H), 1.59 (m, 4H), 1.34 (m, 2H). ^{13}C NMR (CD_3OD): δ 159.72, 142.67, 129.63, 128.27, 126.93, 58.07, 47.12, 44.63, 35.52, 27.11, 25.27, 22.94. MS calculated 722.42, found 722.30 ($[\text{M}+1]^+$); mp 160–63°C, HPLC retention time 2.57 min.

1,13-bis-{3-[1-(phenyl)ureido]}-4,10-diazatridecane dihydrochloride e (7a)—

Compound **7a** was synthesized from **14e** (100 mg, 0.116 mmol) using the procedure described above in 89% yield (76 mg). ¹H NMR (CD₃OD): δ 7.33–7.21 (m, 20 H), 4.2 (t, 2H, J = 8 Hz), 3.81 (d, 4H), 3.23 (t, 4H, 5.8 Hz), 2.98 (t, 4H, J = 7.1 Hz), 2.92 (t, 4H, J = 6.3 Hz), 1.8 (m, 8H), 1.57 (m, 2H). ¹³C NMR (CD₃OD): δ 160.3, 142.54, 128.25, 127.82, 126.29, 51.37, 44.66, 44.33, 35.59, 27.12, 25.43, 23.09. MS calculated 455.32, found 455.47 ([M+1]⁺); mp 215–216°C, HPLC retention time 2.31 min.

1,13-bis-{3-[1-(benzyl)ureido]}-4,10-diazatridecane dihydrochloride (7b)—

Compound **7b** was synthesized from **14f** (100 mg, 0.1 mmol) as per the procedure described above in 82% yield (57 mg). ¹H NMR (CD₃OD): δ 7.3–7.17 (m, 20H), 4.02 (t, 2H, J = 7.8 Hz), 3.27 (t, 4H, J = 6.08 Hz), 3.09 (t, 4H, 7 Hz), 3.02–2.94 (m, 8H), 2.28–2.26 (m, 4H), 1.88–1.85 (m, 4H), 1.76–1.72 (m, 4H), 1.50–1.48 (m, 2H). ¹³C NMR (CD₃OD): δ 160.51, 144.59, 128.16, 127.47, 125.93, 48.48, 47.04, 44.71, 38.52, 35.69, 35.46, 27.15, 25.34, 23.0. MS calculated 482.35, found 482.52 ([M+1]⁺); mp 210–12°C, HPLC retention time 1.80 min.

1,13-bis-{3-[1-(1,1-diphenylmethyl)ureido]}-4,10-diazatridecane

dihydrochloride (7c)—Compound **7c** was synthesized from **14g** (100 mg, 0.115 mmol) using the procedure described above in 85% yield (72.5 mg). ¹H NMR (CD₃OD): δ 7.45–7.27 (m, 20H), 6.67 (s, 2H), 3.78 (t, 4H, J = 5.76 Hz), 3.00–2.94 (m, 8H), 1.72–1.69 (m, 4H), 1.55 (b, 4H), 1.48 - 1.46 (m, 2H). ¹³C NMR (CD₃OD): δ 141.72, 132.48, 129.62, 128.23, 127.27, 127.07, 61.38, 47.04, 44.50, 40.12, 26.43, 25.83, 25.22, 23.10. MS calculated 635.41, found 635.20 ([M+1]⁺); mp 178–181°C, HPLC retention time 2.65 min.

1,13-bis-{3-[1-(2,2-diphenylethyl)ureido]}-4,10-diazatridecane dihydrochloride

(7d)—Compound **7d** was synthesized from **14h** (100 mg, 0.112 mmol) using the procedure described above in 90% yield (77 mg). ¹H NMR (CD₃OD): δ 7.32–7.22 (m, 20 H), 4.45 (s, 2H), 4.13 (t, 4H, J = 7.08 Hz), 3.67 (m, 4H), 3.04–2.97 (m, 8H), 1.92–1.89 (m, 4H), 1.80–1.77 (m, 4 H), 1.56–1.53 (m, 2H). ¹³C NMR (CD₃OD): δ 142.22, 128.29, 127.86, 126.39, 50.22, 47.09, 44.04, 39.87, 26.41, 25.31, 23.17. MS calculated 663.40, found 663.28 ([M+1]⁺); mp 160–163°C, HPLC retention time 0.39 min.

1,13-bis-{3-[1-(3,3-diphenylpropyl)ureido]}-4,10-diazatridecane

dihydrochloride (7e)—Compound **7e** was synthesized from **14i** (100 mg, 0.108 mmol) using the procedure described above in 87% yield (75 mg). ¹H NMR (CD₃OD): δ 7.30–7.16 (m, 20 H), 4.05 (t, 2H, J = 7.6 Hz), 3.70 (b, 4H), 3.41 (b, 4H), 3.04–2.99 (m, 8 H), 2.4–2.34 (m, 4H), 1.99–1.92 (m, 4H), 1.8–1.72 (m, 4H), 1.54–1.51 (m, 2H). ¹³C NMR (CD₃OD): δ 144.43, 128.17, 127.50, 125.97, 48.59, 44.48, 40.04, 34.41, 26.46, 25.26, 23.12. MS calculated 691.47, found 691.22 ([M+1]⁺); mp 141–144°C, HPLC retention time 2.25 min.

Recombinant LSD1 inhibition assay—The ability of the synthetic oligamine analogues **6–9** was determined using a commercially available LSD1 assay kit (BPS Bioscience, San Diego, CA, kit 50106). The substrate and all compounds were incubated in assay buffer from 30 min up to 4 hr at 37°C as described in the commercial protocol. The volume of each reaction well was 50 µl, containing 5 µl of a 200 µM solution of substrate

peptide and 20 μ l of a 15ng/ μ l enzyme solution. All compounds were diluted in 1% DMSO with assay buffer to a final volume of 50 μ M. Fluorescence was measured at the recommended wavelengths of λ_{ex} =530 nm, λ_{em} =590 nm. IC₅₀ determinations were performed using serial dilutions at 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 μ M).

Cell culture—Calu-6 and MCF7 cells were cultured in RPMI-1640 medium (Cellgro, Manassas, VA) supplemented with 10% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin streptomycin (Cellgro). Stock flasks were incubated at 37°C in a humidified atmosphere of 95%air/5%CO₂. Passages 17–35 were used for all of the experiments. For each experiment, cells were seeded at a starting density of 400,000 cells per T25 flask.

Determination of cell viability—For the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) reduction assay, 4000 cells/well were seeded in 100 μ l medium in a 96-well plate and the cells were allowed to attach at 37°C in 5% CO₂ for one day. The medium was aspirated and cells were treated with 100 μ l of fresh medium containing appropriate concentrations of each test compound. The cells were incubated for 4 days at 37°C in 5% CO₂. After 4 days 20 μ L of the MTS reagent solution (Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay) was added to the medium. The cells were incubated for another 2 hours at 37°C under 5% CO₂ environment. Absorbance was measured at 490 nm on a microplate reader equipped with SOFTmax PRO 4.0 software to determine the cell viability.

RNA isolation and qRT-PCR—RNA was extracted using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using Superscript III reverse transcriptase and oligo(dT)₂₀ primers (Invitrogen). qRT-PCR was conducted using the MyiQ single-color real-time PCR detection system (BioRad Laboratories, Hercules, CA). and the following primers: SFRP2 sense, 5'-AAGCCTGCAAAAATAAAAATGATG-3'; SFRP2 antisense, 5'-TGTAATGGTCTTGCTCTTGGTCT-3' (annealing at 57.4°C); GATA4 sense, 5'-GGCCGCCCGACACCCAATCT-3'; GATA4 antisense, 5'-ATAGTGACCCGTCCCATCTCG-3' (annealing at 64°C); HCAD sense, 5'-GGACCGAGAGACTCTGGAAAATC-3'; HCAD antisense 5'-GGGTCATCCTTATCTTCAACTGTC-3' (annealing at 64°C); p16 sense, 5'-CGGAGGCCGATCCAGGTCATG-3'; p16 antisense, 5'-CAATCGGGGATGTCTGAGGGAC-3' (annealing at 67.3°C). GAPDH was utilized as an internal control. The GAPDH primers were: 5'-GAAGGTCGGAGTCAACGGATTT-3' (sense) and 5'-ATGGGTGGAATCATATTGGAAC-3' (antisense). To quantify relative gene expression, the comparative cycle threshold (C_t) method was utilized and the C_t values for the gene of interest were normalized to the C_t values of GAPDH and were presented in relation to untreated control cells.

Western blot analysis—Nuclear fractions were prepared for Western blot analysis using the NE-PER Nuclear Protein Extraction kit (Pierce, Rockford, IL). The primary antibody against H3K4me2 was from Millipore (Millipore, Billerica, MA). Histone H3 antibody was

obtained from Abcam (Cambridge, MA). Dye-conjugated secondary antibodies were used for Western blot quantification using the Odyssey Infrared Detection system and software (LI-COR Biosciences, Lincoln, NE). The effects of LSD1 inhibitors on H3K4 methylation of bulk histones was analyzed as we have previously reported.⁸

Molecular modeling—All molecular docking studies were performed using the GOLD software package, version 5.1 (Cambridge Crystallographic Data Centre, Cambridge, UK).²² The X-ray coordinates of LSD1 (PDB code 3ZMT) were downloaded from the Protein Data Bank,³⁶ and the active site was defined as a sphere enclosing residues within 9 Å around the substrate-like peptide inhibitor. The 3D structure of **6b** and **6d** was built using MOE software (version 2012.10) and was energy minimized using MM94x field and a convergence value of 0.001 kcal/mol/Å as the termination criterion.³⁷ The energy minimized compound **6b** and **6d** was docked in the binding site of LSD1 and scored using ChemPLP. All poses generated by the program were visualized; however, the pose with the highest fitness score was used for elucidating the binding characteristics of **6b** and **6d** in the LSD1 active site. Interaction diagram of compound **6b** in the LSD1 binding pocket was generated using Molecular Operations Environment (MOE) software, version 2010.12. The numbering sequence of amino acid residues in 3ZMT is preserved throughout this paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures of trans-1-aminobicyclo[1.1.0]butane **1**, verindamycin **2**, (bis)thioureas **3–5**, **6a–d** and **8a–g**, and (bis)ureas **7a–e** and **9a–d** (see Table 1).

Figure 2. Inhibition of recombinant human LSD1 by compounds **1**, **2** and **6–9** at a concentration of 10 μ M. Percent activity remaining was determined following treatment with each test compound as determined by the luminol-dependent chemiluminescence method. Each data point is the average of three determinations $\pm$ standard error of the mean. 1% DMSO was used as the negative control.

Figure 3. Structure/activity correlations for (bis)aralkylthiourea oligoamines **3**, **5**, **6b**, **6c**, **6d**, **8f** and **20**. Each data point is the average of 3 determinations $\pm$ standard error of the mean. Data for **3**, **5** and **20** were previously reported.²⁰

Figure 4.

Lineweaver-Burk analysis of the inhibition of recombinant LSD1 by compound **6d**. Each data point is the average of three determinations that in each case differed by 5% or less. The K_i value of 2.4 μM was calculated by the graphing software (KaleidaGraph).

Figure 5.

LSD1 inhibition through the compounds **6b**, **6c**, and **6d** causes the re-expression of aberrantly silenced genes. Calu-6 cells were seeded at a density of 400,000 cells per T25 flask. Upon 60% confluency, the cells were treated with 10 μ M of either compound **6b**, **6c**, or **6d** for 24h or 48h. RNA was harvested and qRT-PCR was conducted as described in the Materials and Methods. The data from 4 experiments were compiled (n=12). Data are shown as the fold mean $\pm$ S.E. of treated cells when compared to non-treated cells (NT) for each time point. Student's t tests were conducted comparing the treated to NT groups for each time point. *p<0.1 and **p<0.05.

Figure 6.

Panel A. Induction of global H3K4me2 following treatment with a 10 μ M concentration of compounds **6b**, **6c** or **6d**. Calu-6 cells were seeded at a density of 400,000 per T25 flask. Upon 60% confluency, the cells were treated with 10 μ M of **6b**, **6c**, or **6d** for 24h or 48h. 30 μ g of nuclear extract was used for Western blot analysis. Non-treated cells are denoted as NT. Gel bands were quantitated using the Odyssey software, and H3K4me2 bands were normalized to total histone H3 bands. The graphical data are the means $\pm$ S.E. from four experiments. Student's t tests were used to determine statistical significance. * $p < 0.1$ and ** $p < 0.05$. Panel B. 5 μ g of full-length LSD1 purified protein was incubated with 5 μ g of bulk histones in the presence or absence of 5, 10, or 20 μ M LSD1 inhibitor. The reaction was incubated at 37°C for 3h. Western blot analysis was performed and H3K4me2 levels were determined using the Odyssey Software program. All of the data were normalized to the histone only control and are shown as a percent of H3K4me2 in relation to the histone only condition. Data points in Panel B represent quantitation of single Western blots from a representative experiment that was repeated 4 times with similar results.

Figure 7. Molecular modeling studies of LSD1 inhibitors. Panel A: Computer-predicted binding mode of compound **6b** and **6d** in the LSD1 binding site shown as a cartoon. Panel B: Molecular interactions between LSD1 and **6b**. Hydrogen bonding interactions of **6b** with Ala 539, FAD and Asn 535 are highlighted using arrows. Both the picture were generated using MOE 2012.10

Scheme 1.

Scheme 2.

Table 1

Inhibition of purified recombinant LSD1 by tranlylcyppromine **1**, verlindamycin **2**, (bis)thioureas **3–6** and **8**, and (bis)ureas **7** and **9**. (Bis)ureas **7a**, **9a**, **9b** and **9d** stimulated LSD1 activity to 163, 119, 119 and 110% of control, respectively.

No.	Chemical structure	% LSD1 inhibition at 10 μ M
1		18 $\pm$ 4.3
2		79 $\pm$ 1.9
3		81 $\pm$ 1.7
4		83 $\pm$ 2.6
5		75 $\pm$ 3.7
6a		40 $\pm$ 2.5
6b		100 $\pm$ 1.2
6c		98 $\pm$ 0.9
6d		96 $\pm$ 3.4
7a		*
7b		29 $\pm$ 2.3
7c		56 $\pm$ 1.9

No.	Chemical structure	% LSD1 inhibition at 10 μ M
7d	2HCl	56 ± 3.4
7e	2HCl	77 ± 2.0
8a	2 HCl	34 ± 4.2
8b	2 HCl	42 ± 4.5
8c	2 HCl	5 ± 2.1
8d	2 HCl	17 ± 3.8
8e	2 HCl	91 ± 3.3
8f	2 HCl	80 ± 1.6
8g	2 HCl	77 ± 4.1
9a	2 HCl	*
9b	2 HCl	*
9c	2 HCl	9 ± 2.7
9d	2 HCl	*

Table 2

IC₅₀ values for the inhibition of recombinant LSD1, monoamine oxidase A and B by **1**, **2** and (bis)aralkylthioureas **6b**, **6c** and **6d**. IC₅₀ values were derived from dose-response curves shown in Figures 4a and S1. SI MAO-A = (IC₅₀ MAO-A ÷ IC₅₀ LSD1); SI MAO-B = (IC₅₀ MAO-B ÷ IC₅₀ LSD1).

Cmpd	LSD1 IC ₅₀ value, μ M	MAO-A IC ₅₀ value, μ M	* SI MAO-A	MAO-B IC ₅₀ value, μ M	* SI MAO-B
1	242	4	0.02	6	0.02
2	13	37	2.85	10	0.76
6b	8	>100	>12.5	36	4.46
6c	7	>100	>14.3	27	3.68
6d	5	>100	>20	19	4.04

* SI=Selectivity Index