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Non-peptidic propargylamines as inhibitors of lysine specific demethylase 1 (LSD1) with cellular activity

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Abstract

Lysine demethylases play an important role in epigenetic regulation and thus in the development of diseases like cancer or neurodegenerative disorders. As the lysine specific demethylase 1 (LSD1/KDM1) has been strongly connected to androgen and estrogen dependent gene expression, it serves as a promising target for the therapy of hormone dependent cancer. Here, we report on the discovery of new small molecule inhibitors of LSD1 containing a propargylamine warhead, starting out from lysine containing substrate analogues. Based on these substrate mimicking inhibitors we were able to increase potency by a combination of similarity-based virtual screening and subsequent synthetic optimization resulting in more druglike LSD1 inhibitors that lead to histone hypermethylation in breast cancer cells.

Keywords

LSD1; histone demethylase; propargylamine; *in-vitro* inhibition; small molecule inhibitors

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ASSOCIATED CONTENT

Supporting Information.

Experimental details and spectral data for compounds **5b–5r**, **6a**, **7a–d** and **9b–9k**. Figure for irreversibility of inhibition. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

INTRODUCTION

Transcriptional regulation in eukaryotic cells is shaped and maintained beyond cell division by the posttranslational modification of histones.¹² These modifications include the reversible attachment of small moieties like acetyl or methyl groups but also of polypeptides like ubiquitin.

The equilibrium of histone lysine methylation is maintained by lysine methyltransferases that transfer the methyl group to the histone tail and histone demethylases that remove the modification. It is not surprising that an imbalance of the methylation state leads to aberrant transcription and this has been linked to the development of diseases like cancer and neurodegenerative disorders. The lysine specific demethylase 1 (LSD1) for instance has been shown to have a crucial impact on androgen dependent gene expression and to be overexpressed in human prostate cancer cell lines and prostate cancers.³ Thus, this histone demethylase serves as a valuable target for drug development towards new therapies of hormone dependent cancers.

LSD1 is an amine oxidase and its activity depends on the co-factor flavine adenine dinucleotide (FAD).⁴ The native substrate of LSD1 is mono- and dimethylated lysine 4 in histone H3 (H3K4me1/me2) as depicted in Figure 1. In androgen dependent tissue, however, a shift in substrate specificity to H3K9me1/me2 is observed.³

After the LSD1 crystal structure was solved,⁵ it was shown that it shares close sequence homology to the FAD dependent monoamine oxidases MAO A and MAO B. Due to this homology, it was not surprising that MAO inhibitors like pargyline and deprenyl (see Chart 1A) also possess an inhibitory effect on LSD1 but their inhibitory activity is in the millimolar range.³⁶ An overview of these and other LSD1 inhibitors is given in reference⁷.

In search for optimized inhibitors of LSD1, the combination of the inhibitory propargylamine group known from MAO inhibitors like pargyline with the LSD1 substrate histone H3 led to the discovery of an oligopeptide that appears as a covalent modifier and thus irreversible inhibitor of LSD1 (see Chart 1B).⁸ But due to its peptidic nature, compounds like this are rather mechanistic tools in biochemical studies and unlikely will have potential for drug development. So far other small molecule inhibitors of LSD1 have rather focused on tranylcypromine and analogues⁹¹⁰¹¹¹² as well as polyamines and amidines.¹³¹⁴¹⁵ A reversible inhibitor is the chromone namoline¹⁶. In order to investigate the biological consequences of reversible vs. irreversible inhibition of LSD1 and to investigate differences among irreversible inhibitors with different warheads (cyclopropylamines vs. propargylamines), it would be very valuable to obtain more potent small molecule propargylamine inhibitors of LSD1 with cellular activity.

Hence, we set up a strategy for the design and synthesis of lysine-mimicking small molecules carrying the propargyl warhead known from MAO inhibitors but initially resembling more the natural substrate of LSD1. This led us first to inhibitors which were subsequently optimized by molecular modelling and refinement by synthesis, resulting in small molecule propargylamines with cellular inhibition of histone demethylation.

RESULTS

To mimic the native substrate, we initially synthesized several propargyl amines derived from *L*-lysine (see chart 2). To obtain these target compounds, different routes of synthesis were pursued (see Scheme 1). Both alkylation of lysine derivatives with propargyl bromides as well as alkylation of propargylamines with mesylates derived from hydroxynorleucines led to the desired compounds **1** and **2**. The latter route was especially valuable to realize an additional N-methylation (**2b**). Besides the propyne derivatives which have precedence in pargyline and the oligopeptide, we also synthesized a lysine derivative **1b**, bearing a butyne residue at the ϵ -amino group.

The initial substrate analogues **1** and **2** were evaluated for their inhibitory activity against LSD1 in a biochemical *in-vitro* assay that was previously described.¹⁷ The data is summarized in table 1. Only the benzoyl derivatives **1** showed considerable demethylase inhibition in the higher micromolar range, but we could show with this that in principle small molecules substrate analogues are able to inhibit LSD1. To further prove this principle and to obtain more drug-like inhibitors, the second part of our synthesis strategy included replacement of the amino acid core by an aromatic ring to limit conformational flexibility in this part of the molecule. To achieve this goal, two different synthesis pathways were followed starting from either methyl 3-hydroxybenzoate resp. 3-aminophenol (see schemes 2 and 3). This led to the synthesis of lysine-mimicking benzamide (**3**) and anilide (**4**) derivatives, all carrying a propargylamine group.

These compounds also were tested for *in-vitro* LSD1 inhibition. As shown in table 1, only the anilide derivatives possess a considerable inhibitory activity towards LSD1 in the micromolar range. With the synthesis of this small set of compounds, we could prove the hypothesis that lysine derived small molecules and analogues are able to inhibit the lysine demethylase LSD1 *in-vitro*.

To get an idea about the potential interaction of **4a** with LSD1 we carried out a docking study (details can be found in the Methods section 5.3). In the most favoured docking pose Tyr761 forms a hydrogen bond with the amine belonging to *N*-propargylamine warhead and the sidechain of Asp555 interacts, through a hydrogen bond, with the amide nitrogen atom of the benzamide moiety (Figure 3). The aromatic substituents are forming extensively hydrophobic and T-shaped aromatic interaction with Phe558, Phe560, Tyr807 and His812.

In order to identify related compounds in commercial databases, we then set out to perform a similarity based virtual screening starting from our initial active molecules (for details see Methods section 5.3). For this purpose we used the published X-ray structure of LSD1 complexed with inhibitors⁹ and docking programs. From this screen, two compounds were selected for *in-vitro* testing (Figure 4). The 3-aryloxy-2-hydroxy-propylamine **5a** with an *N*-propargyl group was identified as an LSD1-inhibitor with a potency of 44 μ M in a hydrogen peroxide dependent assay and 34 μ M in a formaldehyde dehydrogenase (FDH) assay (data of FDH assay not shown).

The docking showed that the *N*-propargylamine group of **5a** is located near the reactive N5-nitrogen of the cofactor, according to the covalent inhibitor hypothesis (Figure 5a). The hydroxyl group and the amine form hydrogen bonds with the backbone of Ala809 and the sidechain of Tyr761, respectively. The hydrophobic biaryl group interacts via T-shaped and edge to face alkyl-aryl interactions with Phe560 and Tyr807. The irreversible inhibitor hypothesis was confirmed in an *in-vitro* dilution assay experiment, where we could show that pre-incubation of compound **5a** with LSD1 and subsequent dilution leads to loss of enzyme activity, whereas dilution of a LSD1 preparation that was pre-incubated with namoline, a known reversible LSD1 inhibitor, results in recovery of the enzyme activity (see Figure S1).

We then set out to vary the structure of the screening hit by synthesis using our docking results. We first focused on varying the aryloxy substituent for a group of compounds (**5a–r**). As docking predicted that bulky lipophilic aryl groups would be beneficial for inhibition, we were mainly focusing on annellated aryl and biaryl structures (Figure 5b–d). For some of the phenols (**8a,b**), we had to synthesize the biphenylol by palladium catalyzed C-C coupling (see Scheme 4). The phenols were reacted with epichlorhydrin to aryloxymethyl oxiranes which were then opened with amine nucleophiles to obtain the final products **5a–r**. For some ring opening reactions, calcium trifluoromethanesulfonate was added as a catalyst that increased the yield. Again, we also investigated the effect of *N*-methylation on the enzyme inhibitory properties. The enzyme inhibitory properties are listed in Table 2.

As mentioned above, the *p*-biphenyl moiety led to enzyme inhibition but shifting the second phenyl ring in the *ortho*-position decreased inhibition strongly. Also substitution of the *p*-phenyl substituent in the biphenyl group with different halogens eliminated activity (**5i–k**). The substitution of the second phenyl ring by more polar ester groups or amides (**5l–r**) led to inactive congeners as well. For the 2-naphthyl compound, *N*-methylation increased inhibition from 92 to 52 μ M, while in the *p*-biphenyl containing compounds *N*-methylation led to a decrease of activity. Shifting the alkoxy substituent in the naphthyl series from the 2- to the 1-position also decreased activity strongly. The most potent inhibitor **5f** (22 μ M) contains a 6-bromo-2-naphthyl-substituent and *N*-methylation. Replacing the oxygen bridging atom between the aryl group and the propylene chain by an amine led to inactive compounds (**6a** and **6b**, see Scheme 6, table 2). Using the cyclopropylamine warhead from tranlycypromine in compounds **7a–d** resulted in much weaker inhibitors (see Scheme 7, table 2).

We also analysed the inhibition of MAO-A and B for selected compounds, including pargyline. Most of our compounds are actually more potent on MAOs than on LSD1 but clearly we largely increased selectivity and potency as compared to pargyline which was the only small molecule propargylamine inhibitor available so far. E.g., **5d** shows only limited inhibition of MAO-B.

To examine whether the target actually is hit in a cellular setting, we selected two of the best inhibitors from the different series (**4b** and **5a**) which were soluble in cell culture media and incubated them with MCF7 cells for 24 hours. As shown in figure 2, increased levels of H3K4 dimethylation were observed in a western blot. To support this data two selected

compounds with good solubility in cell culture media, **4b** and **5a** also were tested in a proliferation assay with MCF7 cells. This experiment resulted in a GI₅₀ value of 90.5 μ M for **4b**, compound **5a** showed an inhibition of proliferation of 54% at a concentration of 100 μ M. This data shows that the inhibitors are affecting the growth of the cells in a concentration in which a significant *in-vitro* LSD1 inhibition is shown *in vitro* and *in vivo*.

DISCUSSION AND CONCLUSION

Starting out from lysine derivatives as substrate analogues, involving a cycle of synthetic variation, similarity based virtual screening and a second round of synthetic optimization we have demonstrated for the first time that small molecule inhibitors of LSD1 containing a propargyl amine structure with a potency in the lower micromolar region can be realized. *N*-methylation is usually beneficial for inhibitory potency. Selected inhibitors lead to elevated levels of H3K4 dimethylation in MCF7 cells, demonstrating that the compounds hit the desired target *in vivo*. Thus, while it is desirable further optimize the selectivity towards monoamine oxidases, this class of compounds can deliver interesting mechanistic probes for further analysis of the functional effects of irreversible LSD1 inhibition *in vivo*.

5. Experimental section

5.1 *In-vitro* assay system

Potency was measured in an established horseradish peroxidase (HRP) assay system based on the Amplex® Red protocol from Invitrogen™ (Life Technologies, Carlsbad, CA). The assay was performed in black 96-well microtiterplates (from Greiner Bio-one GmbH, Germany) with 4 μ l LSD1 enzyme (expressed in Sf9 cells as published elsewhere³, 5 μ l inhibitor (dissolved and diluted in DMSO) and 36 μ l demethylation buffer (contains 45 mM HEPES, 40 mM NaCl, pH 8.5). This solution was preincubated for 30 minutes, then demethylation was started by adding 5 μ l of a solution of 100 μ M H3K4(me2) peptide (from Peptide Specialty Laboratories Ltd., Heidelberg, Germany) in buffer. The mixture was incubated for 90 minutes at 30 °C, 50 μ l Amplex® Red mixture (containing 100 μ M Amplex® Red reagent and 2 U/ml HRP in demethylation buffer) was added and, after 5 minutes, detection performed on a BMG polarstar microplate reader (λ_{ex} : 550 nm, λ_{em} : 615 nm). For the testing of reversibility of enzyme inhibition, 192 μ l of LSD1 (about 50fold amount as compared to standard assays) were incubated with of either 176 μ M **5a**, 224 μ M of the known reversible inhibitor namoline or DMSO. After 10 minutes of incubation, 1 μ l aliquots from all incubation batches were diluted into the assay solution containing the peptide substrate and the coupling reagents to a final volume of 40 μ l.

Inhibition of MAO A and MAO B (purchased from Sigma-Aldrich) was determined in black 96-well microtiterplates (Greiner Bio-one GmbH, Germany) with 40 μ l of a 1:100 dilution of the respective oxidase, 5 μ l inhibitor (dissolved and diluted in DMSO) and 50 μ l PBS, pH 7.4 (containing 4.4 mM KH₂PO₄, 45 mM Na₂HPO₄, 100 mM NaCl). The assay reaction was started with the addition of 5 μ l of a 500 μ M DMSO solution of the substrate kynuramine (from Sigma-Aldrich). After 1 hour of incubation at 37 °C, the enzyme reaction was stopped by the addition of 15 μ l of aqueous sodium hydroxide solution (2 M), before

fluorescence intensity was measured with a BMG polarstar microplate reader (λ_{ex} : 330 nm, λ_{em} : 390 nm)

5.2 Docking Studies and Virtual Screening

The molecular structures of all compounds analyzed in the present study were generated using the MOE 2011.10 modeling software (Chemical Computing Group, Montreal, Canada). Initial ligand conformations resulted from an energy minimization using the MMFF94x force field as implemented in MOE. The available crystal structures of LSD1^{18, 19, 20, 21, 22, 23} were used to analyse the binding site and to design novel inhibitors. For the subsequent docking studies, all water and ligand molecules were removed and both structures were protonated and minimized using the Amber99 force field. LSD1 has an open cleft that hosts the histone H3 N-terminal tail residues through a network of very specific interactions. Docking studies were carried out to choose appropriate cap groups for lysine derivatives. Initially, the anilide derivatives (**4a, b**) were docked into the LSD1 substrate pocket. The docking protocol was set keeping the warhead moiety (*N*-propargylamine group) constrained to the N5 nitrogen atom of the tricyclic isoalloxazine ring of FAD, according to the hypothesis of a covalent inhibitory mechanism.^{24,25} We used the GOLD 4.1 docking program, GoldScore, and three LSD1 crystal structures (pdb code 2DW4²², 2UXN²³ and 2XAJ⁹, for the current docking study).

In order to identify related compounds for in vitro testing, a similarity based virtual screening was set up. We have chosen the Enamine database comprising more than 750 000 compounds, for this purpose. The *N*-propargylamine moiety was selected as a substructure search query. We identified two compounds which were docked using the setup described above into the LSD1 binding pocket. Both compounds were purchased from Enamine (Figure 5).

5.3 Synthesis and characterization of compounds

The starting materials (chemicals) were purchased by Acros Organics, Sigma-Aldrich, Alfa Aesar and Fluka and were used in synthesis without any further purification. With TLC Silica gel 60 F₂₅₄ plates from Merck, analytical thin-layer chromatography (TLC) was performed. All yields were not optimized. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Avance DRX Spectrometer (¹H-NMR: 400 Hz, ¹³C-NMR: 100 Hz) from Bruker. Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane, compounds were dissolved in CDCl₃ or DMSO-d₆. Proton coupling patterns were described as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), septet (s), multiplet (m), and broad singlet (bs). Aromatic ¹H-NMR proton signals were generally described as multiplets (m). Mass spectra with electric (EI) or chemical ionization (CI) were performed on a TSQ 700 from Thermo Fisher, with ammonia as reactant gas. Atmospheric Pressure Photo Ionization (APPI) was performed on a API 2000 from AB Sciex with toluene as a dopant. GC-MS was carried out with an HP 6890 series GC system with a HP 5973 mass selective detector (EI, 70 eV, column: FS-Supreme-5, 30 m $\times$ 250 μ m; T_{GC} (injector) = 250 °C, T_{MS} (ion source) = 200 °C, time program (oven): T_{0 min} = 60 °C, T_{3 min} = 60 °C, T_{14 min} = 280 °C (heating rate 20 °C·min⁻¹), T_{19 min} = 280 °C). Purity was determined for all tested compounds by HPLC and UV detection and/or GC analysis

and were >95% except for MS56 (92%). HPLC analyses were performed using the following protocols: M1: analytical column: LiChroCART[®]₂₅₀₋₄, LiChrospher[®] 60 RP-select B, 5 μ m from Merck. Elution was performed at room temperature under gradient conditions. Eluent A was H₂O containing 0.05% TFA; eluent B was acetonitrile (ACN), also containing 0.05% TFA. Linear gradient conditions were as follows: 0–4 min, A=90%, B=10%; 4–29 min, linear increase to B=100%; 29–31 min, B=100%, 31–40 min A=10%, B=90%. A flow rate of 0.5 mL·min⁻¹ was maintained. UV detection at 210 nm was applied. Protocol M2–M5: analytical column: Synergi 4 μ m MAX-RP 80 Å, 150 - 4.6 mm. Elution was performed at room temperature under isocratic conditions with ACN in Water plus 0.05% TFA (M2: 45% ACN; M3: 25% ACN; M4: 35% ACN; M5: 10% ACN).

General procedure for the synthesis of propargylamine derivatives (5a–5r)—

The oxirane and N-methyl propargylamine or propargylamine (1–15 equiv) and 5 mol% zinc oxide were mixed in a pear shaped flask with a reflux (open). Alternatively, instead of zinc oxide, calcium trifluoromethanesulfonate (0.5 equiv) was used in a microwave tube with seal (closed). The mixtures were irradiated in a microwave reactor for 10 minutes (100 W, 100 °C) with the sealed tube or for 20 minutes (100 W, 83 °C) in the open system under reflux. After TLC control, the irradiation was continued as described for each compound or the reaction mixture was purified by flash chromatography.

3-(Prop-2-ynylamino)-2-hydroxy-1-(biphenyl-4-yloxy)propane (5a)—9a and 3

equiv of propargylamine were used in the open system. Yield: 26% of a white solid; ¹H-NMR (CDCl₃, δ [ppm]): 7.60-7.51 (m, 4H_{ar}, 3',5',2'',6''), 7.47-7.40 (m, 2H_{ar}, 3'',5''), 7.36-7.30 (m, 1H_{ar}, 4''), 7.05-6.99 (m, 2H_{ar}, 2',6'), 4.18-4.03 (m, 3H, Ar-OCH₂-CHOH-), 3.52 (d, 2H, N-CH₂-C $\equiv$ CH, ⁴J = 2.3 Hz), 3.03 (dd, 1H, CHOH-CH₂-N, ²J = 12.2 Hz, ³J = 3.9 Hz), 2.90 (dd, 1H, CHOH-CH₂-N, ²J = 12.2 Hz, ³J = 7.6 Hz), 2.28 (t, 1H, N-CH₂-C $\equiv$ CH, ⁴J = 2.3 Hz), 2.22 (bs, 1H, NH); ¹³C-14 NMR (CDCl₃, δ [ppm]): 158.12 (C-1'), 140.65 (C-1''), 134.18 (C-4'), 128.70 (C-3',-5'/-3'',-5''), 128.15 (C-3',-5'/-3'',-5''), 126.71 (C-4'',-2'',-6''), 114.82 (C-2',-6'), 81.75 (N-CH₂-C $\equiv$ CH), 71.71 (N-CH₂-C $\equiv$ CH), 70.45 (Ar-O-CH₂-CH), 68.50 (CH₂-CHOH-CH₂), 50.65 (CHO-CH₂-N), 38.19 (N-CH₂-C $\equiv$ CH); APPI-MS (Toluol) [m/z] = 282.2 (M-H⁺, 100%); Purity: >98% (4.26 min, M2)

Synthesis of 1-(N-Methyl-4-phenylanilino)-3-(prop-2-ynylamino)propan-2-ol (6b)

- A. **tert-Butyl N-(4-phenyl)carbamate (11):** Biphenyl aniline and di-*tert*-butyl dicarbonate (1.3 equiv) were dissolved in 20 ml THF and 10 ml of water with 10% NaHCO₃ (m/m) was added. After 5 minutes irradiation in a microwave reactor (150 W, 100 °C), the THF was removed by evaporation and the water phase extracted with dichloromethane to yield 92% of a white solid. ¹H-NMR (DMSO-d₆, δ [ppm]): 9.45 (bs, 1H, NH), 7.64-7.52 (m, 6H_{ar}, 3',5',3'',5'',2'',6''), 7.46-7.40 (m, 2H_{ar}, 2',6'), 7.34-7.27 (m, 1H_{ar}, 4''), 1.50 (s, 9H, 3 $\times$ CH₃)
- B. **N-Methyl-4-phenyl aniline (12):** **11** (1 mmol) and 3 ml iodomethane were mixed and irradiated in a microwave reactor for 5 minutes (150 °C, 300 W). After quenching with water and extraction with ethyl acetate, flash chromatography was performed. Yield: 60% of a white solid; ¹H-NMR (DMSO-d₆, δ [ppm]): 7.75-7.53

(m, 2H_{ar}, 3',5'), 7.45-7.34 (m, 4H_{ar}, 3',5',2'',6''), 7.25-7.18 (m, 1H_{ar}, 4''), 6.65-6.69 (m, 2H_{ar}, 2',6'), 5.85 (bs, 1H, NH), 2.71 (s, 3H, CH₃); ¹³C-NMR (DMSO-d₆, δ [ppm]): 149.83 (C-1'), 141.05 (C-1''), 129.13 (C-2'',-6''), 127.66 (C-4'), 127.56 (C-3',-5'/-3'',-5''), 126.06 (C-4''), 125.77 (C-3',-5'/-3'',-5''), 112.45 (C-2',-6'), 30.13 (NCH₃)

- C. N-Methyl-N-(oxiran-2-ylmethyl)-4-phenyl aniline (10b):** 12, epichlorohydrin (2 equiv) and calcium trifluoromethanesulfonate (0.5 equiv) were placed in a microwave tube and irradiated for 15 minutes (150 W, 90 °C). After cooling, the mixture was transferred into a round bottom flask, 5 ml water with KOH (2 equiv) and tetrabutylammonium bromide (0.1 equiv) was added and the mixture stirred for 2.5 h. After liquid extraction with dichloromethane and evaporation under reduced pressure, the product was purified by flash chromatography. Yield: 55% of a white solid; ¹H-NMR (DMSO-d₆, δ [ppm]): 7.61-7.57 (m, 2H_{ar}, 3'',5''), 7.56-7.52 (m, 2H_{ar}, 3',5'), 7.46-7.40 (m, 2H_{ar}, 2'',6''), 7.32-7.27 (m, 1H_{ar}, 4''), 6.89-6.84 (m, 2H_{ar}, 2',6'), 3.73 (dd, 1H, N-CH₂, ²J = 15.7 Hz, ³J = 3.1 Hz), 3.47 (dd, 1H, N-CH₂, ²J = 15.7 Hz, ³J = 4.9 Hz), 3.25-3.19 (m, 1H, CH₂-CHO-CH₂), 3.08 (s, 3H, NCH₃) 2.84 (dd, 1H, CH-CH₂-O, ²J = 4.9 Hz, ³J = 4.0 Hz), 2.62 (dd, 1H, CH-CH₂-O, ²J = 4.9 Hz, ³J = 2.7 Hz)
- D. 1-(N-Methyl-4-phenylanilino)-3-(prop-2-ynylamino)propan-2-ol (6b):** 10b, calcium trifluoromethanesulfonate (0.5 equiv) and 2 equiv of propargylamine were suspended in acetonitrile and irradiated (15 min, 50 W, 90 °C) in the closed system. Yield: 52% of a white solid; ¹H-NMR (CDCl₃, δ [ppm]): 7.60-7.55 (m, 2H_{ar}, 3'',5''), 7.55-7.50 (m, 2H_{ar}, 3',5'), 7.45-7.39 (m, 2H_{ar}, 2'',6''), 7.31-7.26 (m, 1H_{ar}, 4''), 6.91-6.85 (m, 2H_{ar}, 2',6'), 4.10-3.03 (m, 1H, CHOH), 3.50 (d, 2H, N-CH₂-C≡CH, ⁴J = 2.4 Hz), 3.47 (dd, 1H, Ar-N-CH₂, ²J = 15.0 Hz, ³J = 7.4 Hz), 3.40 (dd, 1H, Ar-N-CH₂, ²J = 15.0 Hz, ³J = 4.9 Hz), 3.06 (s, 3H, CH₃), 2.96 (dd, 1H, CHOH-CH₂-N, ²J = 12.0 Hz, ³J = 3.3 Hz), 2.70 (dd, 1H, CHOH-CH₂-N, ²J = 12.0 Hz, ³J = 8.5 Hz), 2.29 (bs, 1H, NH), 2.27 (t, 1H, N-CH₂-C≡CH, ⁴J = 2.4 Hz); ¹³C-NMR (CDCl₃, δ [ppm]): 149.20 (C-1'), 141.00 (C-1''), 129.70 (C-4'), 128.63 (C-2',-6'/-3',-5'/-3'',-5''), 127.77 (C-2',-6'/-3',-5'/-3'',-5''), 126.26 (C-2',-6'/-3',-5'/-3'',-5''), 126.06 (C-4''), 112.92 (C-2',-6'), 81.63 (N-CH₂-C≡CH), 71.78 (N-CH₂-C≡CH), 68.22 (CH₂-CHOH-CH₂), 57.38 (Ar-N-CH₂-CH), 51.85 (CHO-CH₂-N), 39.52 (N-CH₃), 38.12 (N-CH₂-C≡CH); APPI-MS (Toluol) [m/z] = 295.2 (M-H⁺, 100%); Purity: >99% (4.68 min, M3)

2',4'-dichloro-4-phenylphenol (8a) as example for the general procedure for the synthesis of dichloro-4-phenylphenols—2,4-dichlorophenylboronic acid (1.3 equiv), 4-bromoanisole (3.5 mmol, 1 equiv) and tetrakis(triphenylphosphine)palladium (5 mol% of boronic acid) were suspended in 1.5 ml DMF in a microwave tube. After adding NaHCO₃ (3 equiv) in water (1.5 ml), the tube was sealed and irradiated in a microwave reactor for 10 minutes (100 W, 140 °C). After cooling, the mixture was filtrated over kieselgur and extracted with brine and ethyl acetate. The combined ester phases were evaporated and TLC was performed. The product was then dissolved in dichloromethane (5 ml) and boron tribromide (3 equiv) in dichloromethane (20 ml) was added dropwise under

nitrogen at -30°C . The mixture was stirred for 16 hours at room temperature, quenched with ice, stirred for 30 minutes and extracted with dichloromethane. After evaporation, flash chromatography was performed. Yield (2 steps): 37% of a white solid; $^1\text{H-NMR}$ (δ [ppm]): 7.50-7.49 (m, 1H_{ar} , 3''), 7.34-7.27 (m, 4H_{ar} , 3', 5', 5'', 6''), 6.95-6.90 (m, 2H_{ar} , 2', 6'); $^{13}\text{C-NMR}$ (δ [ppm]): 155.33 (C-1'), 138.59 (C-1''), 133.25 (C-2''/-4''), 133.24 (C-2''/-4''), 132.01 (C-3''), 130.81 (C-4'), 130.72 (C-3', -5'), 129.64 (C-5''), 127.08 (C-6''), 115.03 (C-2', -6')

General procedure for the synthesis of aryloxymethyl oxiranes (9a–9k)—The phenol was dissolved in 3 ml of acetone and 2 equivalents of potassium carbonate were added in a microwave tube. After epichlorohydrin was added, the tube was sealed and irradiated in a microwave reactor for 10 minutes at 80°C with 100 W. After TLC control, purification with flash chromatography was performed. In case of little product turnover, the irradiation was prolonged with one or several additional cycles under TLC control. Since oxiranes are well studied, $^1\text{H-NMR}$ spectra were compared with literature spectra to assure identity and suitable purity.

2-((4-Biphenyloxy)methyl)oxirane (9a)—From 4-phenylphenol. The mixture was irradiated for 60 min, 100 W, 80°C . Yield: 59% of a white solid; $^1\text{H-NMR}$ ($\text{DMSO-}d_6$, δ [ppm]): 7.65-7.57 (m, 4H_{ar} , 3', 5', 2'', 6''), 7.46-7.40 (m, 2H_{ar} , 3'', 5''), 7.34-7.29 (m, 1H_{ar} , 4''), 7.08-7.03 (m, 2H_{ar} , 2', 6'), 4.38 (dd, 1H, Ar-O-CH_2 , $^2\text{J} = 11.4\text{ Hz}$, $^3\text{J} = 2.6\text{ Hz}$), 3.88 (dd, 1H, Ar-O-CH_2 , $^2\text{J} = 11.4\text{ Hz}$, $^3\text{J} = 6.5\text{ Hz}$), 3.38-3.33 (m, 1H, $\text{CH}_2\text{-CHO-CH}_2$), 2.86 (dd, 1H, $\text{CH-CH}_2\text{-O}$, $^2\text{J} = 5.0\text{ Hz}$, $^3\text{J} = 4.2\text{ Hz}$), 2.73 (dd, 1H, $\text{CH-CH}_2\text{-O}$, $^2\text{J} = 5.0\text{ Hz}$, $^3\text{J} = 2.7\text{ Hz}$).

5.4 Cellular studies

Cell proliferation assay—Cells were seeded in 96-well tissue culture plates at a density of 5000 per well. After 24 h of incubation, diluted compounds or DMSO vehicle were added to each well to a total volume of 100 μL . Compounds were diluted from 100 $\times$ stock solutions in DMSO. Inhibitors were compared to DMSO vehicle only and three replicates per concentration were used. Growth inhibition was determined using the CellTiter 96Aqueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Data was plotted as absorbance units against logarithm of compound concentration using GraphPad Prism 4.02. 50% Growth inhibition (GI_{50}) was determined as compound concentration required to reduce the number of metabolic active cells by 50% compared to DMSO control.

Western blotting—MCF7 cells obtained from the American Type Culture Collection (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were depleted of hormone for 96h using charcoal-stripped hormone-free medium (Gibco, Grand Island, NT) and 10% charcoal-dextran-treated FBS (Thermal Scientific Hyclone, Logan, UT). During hormone depletion, cells were pretreated with the indicated amount of LSD1 inhibitors (or vehicle control DMSO) for 24h. Upon harvest, cells were washed once with cold PBS, and then lysed with cold NTEP buffer (150 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA, and 0.1% NP-40). The cell lysate was

lyzed on ice for 30 minutes, and then centrifuged at 14,000 rpm for 15 minutes. The supernatant was collected, and 50 micrograms of protein was incubated with 6× SDS-PAGE loading buffer at 95°C for 5 minutes before loading to a 10% SDS-PAGE gel for Western blot analysis. The H3K4me2 (Active Motif, Carlsbad, CA) and H3 (Abcam, Cambridge, MA) antibodies were used in the assays. The band size and intensity for each signal was quantified using Adobe Photoshop and normalized to its loading control (Histone H3). The relative density value of DMSO-treated lysate was set as 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

LSD1	Lysine specific demethylase 1
H3	Histone H3
FAD	Flavin adenine dinucleotide
MAO	Monoamine oxidase
HRP	Horseradish peroxidase
APPI	Atmospheric pressure photo ionisation
ACN	Acetonitrile
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
ATCC	American type culture collection
SE	standard error

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Figure 1. Dimethylated lysine 4 in histone H3 (H3K4me2) as native substrate of LSD1. The figure shows the terminal 21 amino acids of the H3 histone tail.

Figure 2. Global H3K4 hypermethylation after incubation with the inhibitors **4b** and **5a** for 24 hours in MCF7 cells, see experimental for details. The numbers below the H3K4me2 lane represent relative intensities of bands for dimethylated H3K4, each normalized to loading control.

Figure 3.
Docking result for compound **4a** at LSD1. The cofactor FAD is colored orange and interacting amino acid residues are colored green. Hydrogen bonds are shown as dashed lines.

T5342129 (**5a**)

T5342128

Figure 4.
Similarity based virtual screening results

Figure 5. Putative binding modes of the most active compounds belonging to the aryloxy propylamine dataset. FAD cofactor is shown as a orange sticks. The interacting amino acid residues are colored dark green and the hydrogen bonds are represented as black-dashed lines.

Scheme 1. Synthesis of the lysine-derived LSD1 inhibitors^a

^aConditions: (a) benzoyl chloride, NaOH, H₂O. (85%) (b) H₂, Pd/C, MeOH. (83%) (c) R-CH≡C-CH₂-Br, LiOH•H₂O, DMF. (8–33%) (d) Na₂[Fe(CN)₅NO], NaOH, H₂O, 60 °C. (94%) (e) benzyl bromide, Et₃N, acetone. (35%) (f) mesyl chloride, pyridine, CH₂Cl₂. (g) NaI, acetone. (79%) (h) R-NH-CH₂-C≡CH, LiOH•H₂O, DMF. (34–48%)

Scheme 2. Synthesis of 3-alkoxy benzamides^a

^aConditions: (a) Cl-(CH₂)₃-Br, K₂CO₃, acetone. (75%) (b) LiOH, H₂O₂, THF. (85%) (c) R-NH₂, IBCF, 4-methylmorpholine, THF, -15 °C. (56–67%) (d) NaI, 2-butanone. (98–99%) (e) H₂N-CH₂-C≡CH, LiOH•H₂O, DMF. (19–29%)

Scheme 3. Synthesis of 3-alkoxy anilides^a

^aConditions: (a) Cl-(CH₂)₃-Br, NaH, DMF, 0 °C. (49%) (b) benzoyl chloride, 1,4-dioxane, toluene. (41%) (c) NaI, 2-butanone. (99%) (d) R-NH-CH₂-C≡CH, LiOH•H₂O, DMF. (22–33%)

Scheme 4. Synthesis of biaryl phenols as starting materials for further syntheses (**8a-b**)^a^aConditions: (a) $Pd(PPh_3)_4$, $H_2O:DMF$ 1:1, $NaHCO_3$, microwave, $140\text{ }^\circ C$, 100 W, 10 min.(b) BBr_3 in dichloromethane, $-30\text{ }^\circ C$ to room temperature (rt), 16 h at rt. (27–37%)

Scheme 5.

Synthesis of **5a** as an example for synthesis of 1-aryloxy-3-(prop-2-ynylamino)-propan-2-ols (**5a–5r**) from the corresponding oxiranes (**9a–k**).^a

^aConditions for each compound described in supporting material. (a) K₂CO₃, epichlorohydrin, microwave (80–140 °C, 100–200 W, 10–60 min). (18–62%) (b) Ca(SO₃CF₃)₂, acetone, propargylamine, microwave (80–100 °C, 50–100 W, 10–15 min). (3–94%)

Scheme 6.

Synthesis of aza-analogues (**6a** (R = H), **6b** (R = CH₃))^a

^aConditions: (a) Di-*tert* butyldicarbonate, THF, NaHCO₃, reflux, 3 h. (92%) (b) iodomethane, microwave (150 °C, 300 W, 5 min). (60%) (c) Ca(SO₃CF₃)₂, acetonitrile, epichlorohydrin, microwave (90 °C, 100 W, 15 min). (7–55%) (d) Ca(SO₃CF₃)₂, acetonitrile, microwave (90 °C, 100 W, 15 min). (52%)

A

Pargyline

Deprenyl

B **Chart 1.**

Known propargylamine LSD1 inhibitors. (A) Inhibitors of MAO B that carry a propargylamine group and weakly inhibit LSD1, (B) Oligopeptide inhibitor derived from the first 21 amino acids of the LSD1 substrate H3 that is propargylated at the ϵ -amino group of lysine 4.

Table 1*In-vitro* inhibition of LSD1

Compound	Inhibition of LSD1	Inhibition of MAO B	Inhibition MAO A
	% inhibition @ conc. [μ M] or IC ₅₀ $\pm$ SE [μ M]	% inhibition @ conc. [μ M] or IC ₅₀ $\pm$ SE [μ M]	% inhibition @ conc. [μ M]
1a	143.6 $\pm$ 16.1	n.i. ^a @ 500 μ M	n.i. @ 100 μ M
1b	131.9 $\pm$ 15.7	51% @ 500 μ M	45% @ 100 μ M
2a	26% @ 100 μ M	67% @ 500 μ M	n.t. ^b
2b	42% @ 100 μ M	n.i. @ 500 μ M	36% @ 100 μ M
3a	41% @ 100 μ M	17.0 $\pm$ 1.0	100% @ 100 μ M
3b	36% @ 100 μ M	5.7 $\pm$ 0.2	n.t.
4a	184.2 $\pm$ 16.0	0.2 $\pm$ 0.01	62% @ 10 μ M
4b	93.1 $\pm$ 12.8	0.1 $\pm$ 0.002	73% @ 10 μ M

^a n.i.: no inhibition;^b n.t.: not tested

Table 2

In-vitro inhibition of LSD1

Basic structure:						
number	R1	R2	R3	X	Inhibition of LSD1 [%@conc. [μM]] or IC ₅₀ ± SE [μM]	Inhibition of MAO A [%@conc. [μM]] or IC ₅₀ ± SE [μM]
pargyline						
					1 mM ⁷ 23% @ 100 μM	3.39. ± 0.66
5a	4-Ph-Ph	H	CH ₂ -C≡CH	O	44.0 ± 2.2	0.55 ± 0.06
5b	4-Ph-Ph	CH ₃	CH ₂ -C≡CH	O	22.7% @ 100 μM	n.i. @ 10 μM
5c	1-Naphthyl	CH ₃	CH ₂ -C≡CH	O	23.4 % @ 100 μM	n.t. ^a
5d	2-Naphthyl	CH ₃	CH ₂ -C≡CH	O	51.7 ± 2.4	n.t.
5e	2-Naphthyl	H	CH ₂ -C≡CH	O	92.2 ± 2.3	65% @ 10 μM
5f	6-Bromo-2-naphthyl	CH ₃	CH ₂ -C≡CH	O	22.2 ± 2.1	n.t.
5g	2-Ph-Ph	H	CH ₂ -C≡CH	O	36.3% @ 200 μM	5% 10 μM
5h	2-Ph-Ph	CH ₃	CH ₂ -C≡CH	O	41.8% @ 100 μM	n.t.
5i	4'-Br-4-Ph-Ph	H	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
5j	2',4'-Cl ₂ -4-Ph-Ph	H	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
5k	3',5'-Cl ₂ -4-Ph-Ph	H	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
5l	4-MeOCO-Ph	CH ₃	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
5m	4-MeOCO-Ph	H	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
5n	4-MeOCOCOCH ₂ -Ph	CH ₃	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
5o	4-MeOCOCOCH ₂ -Ph	H	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
5p	4-MeOCO(CH ₂) ₂ -Ph	CH ₃	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
5q	4-NH ₂ CO-Ph	H	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
5r	4-NH ₂ CO-Ph	CH ₃	CH ₂ -C≡CH	O	n.i. @ 100 μM	n.t.
6a	4-Ph-Ph	H	CH ₂ -C≡CH	NH	n.i. @ 100 μM	n.t.

Basic structure:							
number	R1	R2	R3	X	Inhibition of LSD1 [%@conc. [μM]] or IC ₅₀ ± SE [μM]	Inhibition of MAO B [%@conc. [μM]] or IC ₅₀ ± SE [μM]	Inhibition of MAO A [%@conc. [μM]] or IC ₅₀ ± SE [μM]
6b	4-Ph-Ph	H	CH ₂ -C≡CH	NCH ₃	n.i. @ 100 μM	n.t.	n.t.
7a	4-Ph-Ph	H	Cyclopropyl	O	n.i. @ 100 μM	100% @ 10 μM	100% @ 10 μM
7b	2-Naphthyl	H	Cyclopropyl	O	n.i. @ 100 μM	94% @ 10 μM	n.t.
7c	6-Bromo-2-naphthyl	H	Cyclopropyl	O	111.4 ± 15.5	100% @ 10 μM	100% @ 10 μM
7d	2-Ph-Ph	H	Cyclopropyl	O	39.0% @ 100 μM	45% @ 100 μM	n.t.

^a n.t.: not tested