ORIGINAL ARTICLE

Exploration of 5-cyano-6-phenylpyrimidine derivatives containing an 1,2,3-triazole moiety as potent FAD-based LSD1 inhibitors

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Abstract  Histone lysine specific demethylase 1 (LSD1) has become a potential therapeutic target for the treatment of cancer. Discovery and develop novel and potent LSD1 inhibitors is a challenge, although several of them have already entered into clinical trials. Herein, for the first time, we reported the discovery of a series of 5-cyano-6-phenylpyrimidine derivatives as LSD1 inhibitors using flavin adenine dinucleotide (FAD) similarity-based designing strategy, of which compound 14q was finally identified to repress LSD1 with IC50 \( \leq 183 \) nmol/L. Docking analysis suggested that compound 14q fitted well into the FAD-binding pocket. Further mechanism studies showed that compound 14q may inhibit LSD1 activity competitively by occupying the FAD binding sites of LSD1 and inhibit cell migration and invasion by reversing epithelial to mesenchymal transition (EMT). Overall, these findings showed that compound 14q is a suitable candidate for further development of novel FAD similarity-based LSD1 inhibitors.

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Abbreviations: AML, acute myeloid leukemia; EMT, epithelial to mesenchymal transition; ESI, electrospray ionization; FAD, flavin adenine dinucleotide; FBS, fetal bovine serum; HRMS, high resolution mass spectra; IC50, half maximal inhibitory concentration; LSD1, histone lysine specific demethylase 1; MOE, molecular operating environment; ANOVA, analysis of variance; PAINS, pan assay interference compounds; PDB, the Protein Data Bank; RLU, relative light units; SARs, structure–activity relationship studies; TCP, tranylcypromine; VDW, van der Waals.

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1. Introduction

Epigenetic plays a key role in life and controls gene expression and transcription, and contributes to a variety of cellular processes, including cell differentiation, proliferation and migration. Among the diverse epigenetic modifiers, histone lysine specific demethylase 1 (LSD1), the first identified histone demethylase, specifically removes the methyl groups from mono- and di-methylated H3K4 and H3K9 via enzymatic oxidation, and contributes to the downstream gene transcription (1–7). As reported, LSD1 is overexpressed in many human cancers, such as gastric cancer, lung cancer, leukemia, etc., and abrogation of LSD1 leads to the antitumor effect (8–16), which suggests LSD1 as a promising therapeutic target for cancer.

Significant efforts have been made to develop biological active LSD1 inhibitors over the past decades (17–25). So far, six tranylcypromine (TCP)-based LSD1 inhibitors have already advanced into clinical trials for the treatment of acute myeloid leukemia (AML) as well as small-cell lung cancer, including GSK2879552, INCB059872, ORY-2001, ORY-1001, CC-90011 and IMG-728930. In addition to TCP-based inhibitors, multiple LSD1 inhibitors with diverse classes structures, such as polyamine-, peptide-, phenelzine-, pargyline- and triazole-based derivatives, have also been reported (Fig. 1). Nevertheless, most of these inhibitors still perform some shortages. For instance, TCP-based LSD1 inhibitors generally displayed some side effects due to irreversible binding with the endogenous flavin adenine dinucleotide (FAD) and micromolar affinity with many targets in body, such as norepinephrine transporter, dopamine transporter, etc. (26–28). Phenelzine- and pargyline-based derivatives displayed insufficient inhibitory activity of pyrimidine-based derivatives. Moreover, Holshouser et al. (55) and Kutz et al. (56) also reported a class of compounds containing 3,5-diamino-1,2,4-triazoles as novel inhibitors. Further mechanism studies found that compared with parent compounds 7a–g, the introduction of 1,2,3-triazole moiety (10a–g) could effectively improve the inhibitory activity against LSD1 (Table 1). These findings suggest that 1,2,3-triazole group was beneficial to the discovery of 5-cyano-6-phenylpyrimidine-based LSD1 inhibitors.

To explore the correlation between the introduction of 1,2,3-triazole and the improvement of LSD1 inhibitory activity, the proposed binding modes of compound 10a in LSD1 were analyzed using the Molecular Operating Environment (MOE, version 2015.10) package. The docking results indicate that compound 10a fitted well into the FAD binding pocket of LSD1 (Fig. 2A). The triazole formed a hydrogen bond with Val288, and the cyano group connected to pyrimidine formed a key hydrogen bond with Lys661 bridged by water1032 as Lys661 is essential for LSD1 activity, which may form a hydrogen bond with the N5 atom of FAD via a conserved water molecule. In addition, the phenyl group connected with pyrimidine was found to be located in the hydrophobic pocket surrounded by Tyr761, Arg316, Lys661, Trp751 and Leu659, while the trifluoromethyl-benzene hydrophobic interactions with Tyr761, Ala539, Thr810, Val811 and Val333 (Fig. 2B). These findings reveal that the introduction of 1,2,3-triazole may inhibit the activity of LSD1 by stabilizing the conformation of series I derivatives to compete with FAD in LSD1.

Based on the above findings, to further investigate the influence for increasing similarity of target compounds with FAD on LSD1 inhibitory activity, series II derivatives containing hydrogen bond donor and receptor groups between 1,2,3-triazole and pyrimidine ring were designed and synthesized. Their inhibitory activities were listed in Table 2. To our delight, compared with series I derivatives, compounds 14a–e with the extension of the side chain exhibit stronger inhibitory activity against LSD1, suggesting that the increased activity of LSD1 may be related to their structural similarity to FAD. Particularly, compound 14e displayed significantly increased inhibitory activity against LSD1 with the
inhibitory rate of 89.2% at 1 μmol/L, about 50-fold more potent than of compound 10.

The exciting discovery prompted us to further explore the SARs of series II derivatives. Their inhibition results were summarized in Table 3. Majority of these compounds showed excellent activity with IC50 value ranging from 183 nmol/L to 5.62 μmol/L. The SARs found that in terms of the para-methyl substitution in R2, compounds (14b, 14d and 14e) with an electron-withdrawing or -donating group in R1 resulted in a similar inhibitory activity against LSD1 compared with compound 14a. However, compound 14c with para-chlorine and compound 14f with para-isopropyl substitution in R1 have significantly increased inhibitory activity, which were about 3.5- and 5-fold stronger than that of compound 14a, respectively. These findings indicate that the types of substituents have a more significant effect on the activity than the electrical properties of substituents at R1. Furthermore, except for compound 14j, compounds 14g and 14i with ortho-electron-donating substitution displayed more potent activity than corresponding compound 14h with electron-withdrawing group. Interestingly, compounds 14f, 14l and 14m with para-substitution at aromatic ring in R2, showed improved inhibitory activity against LSD1 compared with ortho-substituted analogue and unsubstituted aromatic ring in R2, excitingly, except for compound 14n. Compound 14p with electron-withdrawing exhibited better activity compared to compounds 14b and 14o. In particular, the compound 14q with meta-trifluoromethyl substituent at aromatic ring in R2, exhibited remarkable inhibitory activity against LSD1 with an IC50 value of 183 nmol/L. It is worth noting that compounds 14n and 14r also showed excellent inhibitory activity, which is worthy of our further study of SARs. The IC50 values of compounds 14n and 14r against LSD1 are 287 and 387 nmol/L, respectively. Compound 14n, with a para-methyl in R1 and para-methoxy in R2, showed better inhibitory activity compared with both its para-isopropyl in R1 (14m) and ortho-methoxy in R2 (14o) analogues. A similar trend was also observed (14a–e, 14g vs. 14f). The comparison of the influence of electronic-effect in compounds 14p–r and 14v on the LSD1 inhibitory activity is distinct. It reveals that simultaneous replacement of R1 and R2 (14v) with electron-withdrawing group resulted in a dramatically decreasing of LSD1 inhibitory activity, whereas replacement R1 with electron-donating group and R2 with electron-withdrawing exhibited excellent inhibitory activity. We also found that the electronic-effect on the activity of compounds 14c, 14n,
and 14r is completely different, and we speculate that this result is due to the synergistic effect of the groups at two positions. In addition, compounds 14t with meta-chlorine substitute at R₂ exhibited better inhibitory activity than corresponding compound 14s with para-chlorine substitution. These results indicate that the substitution pattern and electronic-effect at R₂ are critical for inhibitory activity against LSD1. SAR was summarized and indicated in Fig. 3.

To further explore the effect of 1,2,3-triazole group activity, compounds 16a–d were synthesized and evaluated on their LSD1 inhibitory activity. As shown in Table 4, compounds 16a–d without 1,2,3-triazole moiety exhibited the complete loss of inhibitory activity.

### Table 1
Inhibitory results of target compounds 7a–g and 10a–g against LSD1.

| Compd. | R₁   | R₂       | IC₅₀ (μmol/L) | Compd. | R₁   | R₂       | R₃     | IC₅₀ (μmol/L) |
|--------|------|----------|---------------|--------|------|----------|--------|---------------|
| 7a     | H    | m-CF₃    | >64           | 10a    | H    | m-CF₃    | o-Cl   | 0.407 ± 0.021 |
| 7b     | H    | p-Cl     | >64           | 10b    | H    | p-Cl     | o-Cl   | 0.628 ± 0.027 |
| 7c     | H    | m-Cl     | >64           | 10c    | H    | m-Cl     | o-Cl   | 6.310 ± 0.719 |
| 7d     | H    | p-F      | >64           | 10d    | H    | p-F      | o-Cl   | 3.227 ± 0.509 |
| 7e     | H    | o-Cl     | >64           | 10e    | H    | o-Cl     | p-CH₃  | 3.740 ± 0.573 |
| 7f     | H    | p-CH₃    | >64           | 10f    | H    | p-CH₃    | p-CH₃  | 40.408 ± 1.606 |
| 7g     | H    | p-OCH₃   | >64           | 10g    | H    | p-OCH₃   | o-Cl   | 1.433 ± 0.516 |

*Data are displayed as mean ± SD. All experiments were performed three times at least.
activity, which further indicated that 1,2,3-triazole is an essential moiety for LSD1 inhibitory activity.

2.3. Molecular docking study

The possible binding mode of compound 14q in LSD1 was predicted using MOE. The docking results suggested that the most potent compound 14q could be well docked into the FAD-binding pocket. Considering the detailed binding mode, we selected the top docking conformation based on the docking score and SARs in combination mode (Fig. 4A). Through the superposed conformation of compound 14q and FAD with LSD1 (Fig. 4B), it was found that they performed similar modes of action, and compound 14q fitted into the FAD-binding pocket appropriately. The cyano group connected with pyrimidine formed a key hydrogen bonding interaction with Lys661 bridged by water1032, meanwhile, this cyano group may also form a hydrogen bond with Met332. The triazole ring formed a hydrogen bond with Val288. For bisamide moiety, the nitrogen and oxygen formed two hydrogen bonds with Thr624 and Arg316. Additionally, compound 14q formed extensive van der Waals (VDW) interactions and hydrophobic interactions. The benzyl group connected with pyrimidine located in the hydrophobic pocket surrounded by Tyr761, Leu659, Lys661, Trp751 and the side chain of Arg316, while having a face to face π–π stacking with Tyr761 and Trp751. The trifluoromethyl-benzene group formed hydrophobic interactions with Tyr761, Ala539, Thr810, Val811 and Val333, and the

Figure 2  (A) The action mode of FAD (yellow) with LSD1 (PDB:2DW4); (B) the proposed binding mode of compound 10a (yellow) in LSD1. Residues are shown as white lines, water is shown as red ball and H-bonds are depicted in green dotted line, respectively.

Table 2  Inhibitory activity of target compounds against LSD1.

| Compd. | R1 | R2 | R3 | Inhibition rate (%) at 1 μmol/L |
|--------|----|----|----|-------------------------------|
| 10h    | H  | p-CH3 | o-Cl | 21.3                          |
| 14a    | H  | p-CH3 | p-Cl | 100                           |
| 14b    | p-CH3 | p-CH3 | o-Cl | 83.7                          |
| 14c    | p-Cl | p-CH3 | p-Cl | 94.8                          |
| 10j    | p-Cl | p-CH3 | o-Cl | 91.4                          |
| 14c    | p-Cl | p-CH3 | p-Cl | 39.8                          |
| 14c    | p-Cl | p-CH3 | p-Cl | 91.4                          |
| 14d    | p-Br | p-CH3 | o-Cl | 8.4                           |
| 14d    | p-Br | p-CH3 | p-Cl | 46.3                          |
| 10l    | m,p,m-tri-OCH3 | p-CH3 | o-Cl | 2.2                           |
| 14e    | m,p,m-tri-OCH3 | p-CH3 | p-Cl | 89.2                          |

*All experiments were performed three times at least.

Table 3  Inhibitory results of target compounds 14a–w against LSD1.

| Compd. | R1 | R2 | IC50 (μmol/L) |
|--------|----|----|---------------|
| 14a    | H  | p-CH3 | 1.880/C60.272 |
| 14b    | p-Cl | p-CH3 | 1.435/C60.150 |
| 14c    | p-Cl | p-CH3 | 0.531/C60.002 |
| 14d    | p-Br | p-CH3 | 3.822/C60.582 |
| 14e    | m,p,m-tri-OCH3 | p-CH3 | 1.262/C60.100 |
| 14f    | p-CH(CH3)2 | p-CH3 | 1.632/C60.002 |
| 14g    | p-CH(CH3)2 | o-CH3 | 3.822/C60.582 |
| 14h    | p-CH(CH3)2 | o-Cl | >10            |
| 14i    | p-CH(CH3)2 | o-OH | 5.041/C60.702 |
| 14j    | p-CH(CH3)2 | o-OCH3 | >10            |
| 14k    | p-CH(CH3)2 | H  | >10            |
| 14l    | p-CH(CH3)2 | p-F | 5.620/C60.215 |
| 14m    | p-CH(CH3)2 | p-OCH3 | 0.611/C60.002 |
| 14n    | p-CH3 | p-OCH3 | 0.287/C60.002 |
| 14o    | p-CH3 | o-OCH3 | 1.533/C60.271 |
| 14p    | p-CH3 | m-Cl | 0.232/C60.002 |
| 14q    | p-CH3 | m-Cl | 1.803/C60.003 |
| 14r    | m,p,m-tri-OCH3 | m-Cl | 0.387/C60.002 |
| 14s    | m,p,m-tri-OCH3 | p-Cl | 0.965/C60.003 |
| 14t    | m,p,m-tri-OCH3 | m-Cl | 0.635/C60.190 |
| 14u    | p-Cl | p-CH(CH3)2 | 1.234/C60.091 |
| 14v    | p-Br | m-Cl | >10            |
| 14w    | p-Br | p-CH(CH3)2 | 1.351/C60.130 |
| GSK-LSD1 | — | — | 0.022/C60.0008 |

*Data are displayed as mean ± S.D. All experiments were performed three times at least. ‒Not applicable.
pyridine performed hydrophobic interactions with Trp756, Val590, Ile284, Val629 and Leu625. All these interactions stabilized the binding conformation of compound 14q with LSD1.

2.4. Compound 14q inhibits LSD1 selectively and competitively in a time dependent and reversible manner

As compound 14q was identified to inhibit LSD1 potently, selectivity of compound 14q against homologous proteins of LSD1 remains to be answered. Hence, compound 14q was subjected to MAO-A/B assay, and results indicated that compound 14q cannot inhibit MAO-A/B (inhibitory rate against MAO-A/B less than 5% at 100 μmol/L), suggesting that compound 14q is a selective LSD1 inhibitor. After that, further mechanism study was carried out to reveal the potential binding manner of compound 14q against LSD1 recombinant. Firstly, dialysis experiment was carried out to investigate whether compound 14q may bind to LSD1 recombinant in a reversible manner or irreversible manner. As indicated in Fig. 5A, compound 14q may inhibit LSD1 in a reversible manner as dialysis mediated depletion of compound 14q can rescue the activity of LSD1. To further confirm this result, centrifuge experiment and dilution assay were also carried out. With the aid of 10 kDa ultracentrifuge filter, reversible compound was supposed to be removed from LSD1 by centrifuge. So, compound 14q was characterized as a reversible LSD1 inhibitor as split of compound 14q by ultracentrifuge may rescue the activity of LSD1 either (Fig. 5B). And 80-fold dilution of compound 14q/LSD1 mixture, which led to the lower concentration of compound 14q in the enzyme reaction system, also resulted in the recovery of LSD1 enzymatic activity (Fig. 5C), indicating that compound 14q was a reversible LSD1 inhibitor as proved by Fig. 5A and B. Then, time-dependent assay was also performed, and results showed that compound 14q inhibited LSD1 in a time dependent manner (Fig. 5D). As compound 14q could well penetrate into the FAD-binding pocket of LSD1, classic Lineweaver–Burk plots was applied and results showed that compound 14q was a competitive LSD1 inhibitor against FAD (Fig. 5E). Further recombinant-based thermal shift assay firmed that compound 14q can stabilize LSD1 dose dependently (Fig. 5F), which suggests that compound 14q can bind to LSD1 directly.

2.5. Compound 14q can inhibit LSD1 and reverse EMT cells

As compound 14q was confirmed as an FAD competitive LSD1 inhibitor in recombinant level, its cellular activity was further investigated in MGC-803 cell line, the one we chose previously. First of all, cell viability was investigated when cells were treated with compound 14q for 5 days, and results indicated that compound 14q could inhibit the proliferation of MGC-803 cells with IC50 = 6.623 ± 0.359 μmol/L. Then, further cellular thermal shift assay was applied to confirm the target engagement of compound 14q in cells. Result in Fig. 6A clearly suggested that compound 14q can bind to LSD1 directly.
indicating cellular target engagement of compound 14q in MGC-803 cells. To determine the inhibitory effect of our compound against LSD1 in cells, amount of four reported LSD1 substrates H3K4me1/2 and H3K9me1/2 were analyzed as well as H3K4me3, a substrate of JMJC containing demethylase. As shown in Fig. 6B, the amount of H3K4me1 and H3K4me2 showed a dose-dependent accumulation in the presence of compound 14q for 5 days, while expressions of H3K9me1/2 and H3K4me3 were kept without change (Fig. 6B). These results indicated that compound 14q can inhibit LSD1 in cells. As LSD1 can promote cell migration with
diverse mechanism, for example, LSD1 may form a complex with Snail and act as a suppressor on the promoter activity of E-cadherin, and LSD1 can also be integrated with the SIN3A/HDAC complex and target metastasis relating genes, then the abrogation of LSD1 genetically or pharmacologically can suppress cell migration and invasion, and activity of compound 14q against cell migration and invasion was investigated either. As the transwell and matrigel-coated transwell experiment showed in Fig. 6C and D, different concentrations of compound 14q can suppress the migration and invasion of MGC-803 cells in a concentration-dependent manner for 24 treatments. Studies have shown that LSD1 plays a very important role in the EMT process, hence, selected biomarkers of EMT process were investigated after compound 14q treatment. As shown in 6E, 24 h treatment of compound 14q leads to the accumulation of E-cadherin, the epithelial cell marker, and less amount of N-cadherin and vimentin, the mesenchymal cell marker, in a dose-dependently manner, while the amount of Snail was kept constant. All of these results indicated that compound 14q can suppress EMT progression and inhibit cell invasion and migration in cells.

3. Conclusions

In this study, we have designed and synthesized 5-cyano-6-phenylpyrimidine derivatives containing a triazole group following FAD similarity-based designing strategy. The inhibitory activity of all compounds towards LSD1 was evaluated. SARs studies showed that compound 14q exhibited the most potent inhibitory activity against LSD1 with IC50 of 183 nmol/L. With the aid of dialysis experiment, ultracentrifuge experiment and dilution assay, compound 14q was confirmed to inhibit LSD1 in a reversible manner, further experiments also indicated that compound 14q may inhibit LSD1 in a time-dependent manner in 30 min. Based on these findings, compound 14q was characterized as an FAD competitive LSD1 inhibitor, and may bind LSD1 closely. Importantly, cellular experiment also indicated that compound 14q can contribute to the accumulation of mono-, and di-methylation of H3K4 and H3K9. Furthermore, with the treatment of compound 14q, EMT can be reversed in gastric cancer cells and cell migration and invasion ability can also be inhibited. So, our findings indicated that these 5-cyano-6-phenylpyrimidine derivatives could be served as candidates of LSD1 inhibitors for further development with FAD similarity-based designing strategy.

4. Experimental

4.1. General information

Reagent and solvent were purchased from commercial sources and were used without further purification. 1H NMR and 13C NMR spectra were determined on a Bruker 400 and 100 MHz spectrometer (Ettlingen, Germany), respectively. High resolution mass spectra (HRMS) were recorded on a Waters Micromass spectrometer (Milford, MA, USA) by electrospray ionization (ESI).

4.2. General procedure for the synthesis of compounds 6a–f and 15a–d

2-Mercapto-dihydroyrimi derivatives 4a–f (1 mmol), propargyl bromide (1 mmol) were dissolved in dry dioxane and stirred under refluxed for 3 h. Up completion of the reaction, phosphorous oxychloride was added dropwise with stirring, the mixture was kept the temperature for 1 h, and then quenched with water. The solid was filtered off, washed with water, dried and crystallized from aqueous ethanol to yield the pure product. Compounds 15a–d were also synthesized from compounds 4a–d and 11 following the same procedure.

4.3. General procedure for the synthesis of compounds 9a–f and 13a–f

Compounds 6a, c–f (5 mmol), azide derivatives 8 (5 mmol), CuSO4·5H2O (62 mg, 0.25 mmol), sodium ascorbate (100 mg, 0.5 mmol), THF (20 mL) and H2O (20 mL) were added to a round-bottom flask and stirred at room temperature. TLC indicated completion of the reaction. Water was added and the reaction mixture was extracted with EtOAc. The organic layers were washed with brine, dried over anhydrous Na2SO4 and concentrated under vacuum, and the crude product was then further purified by recrystallized from acetone to give the pure product. Compounds 13a–f were also synthesized from compounds 6a–f and 12 following the same procedure.

4.4. General procedure for the synthesis of compounds 7a–g, 10a–l, 14a–w and 16a–d

Appropriate amine and equimolar amount of compounds 6a–f, 9a–f, 13a–f and 15a–d were in the solvent of ethanol under 80 °C for 3 h. Upon completion, the precipitated product was filtered off and washed with ethanol for three times, which then recrystallized with ethanol to yield the pure product.

4.5. Cell cultures

The human gastric cancer cell line MGC-803 was obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells are cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and incubated at 37 °C and 5% CO2.

4.6. LSD1 enzymatic assay

The inhibition of LSD1 activity by compound 14q was evaluated according to our publication. pET-28b-LSD1 (full length) was transfected into BL21 (DE). Then, the protein was induced with 0.25 μmol/L IPTG following sonication and purified with Ni-NTA (Qiagen, Tübingen, Germany), Resource Q (GE, Pittsburgh, PA, USA) and Sephacryl S-200 HR (GE, Pittsburgh, PA, USA). Then 5 nmol/L recombinant LSD1 and 25 nmol synthesized H3K4me2 peptide were incubated with the compounds with additional 50 nmol FAD, 20 nmol Amplex Red and 5.5 U/mL horseradish peroxidase for 30 min. Finally, the fluorescence intensity was read at ex 530 nm and ex 590 nm using EnVision Plate Reader (PerkinElmer, Waltham, MA, USA) to calculate the inhibition rate. The dilution assay was performed following publication. First, LSD1 recombinant was incubated compound 14q, GSK-LSD1, or DMSO for 1 h. Then we diluted the reaction system for 80 times. Finally, the above stated method was applied to detect the activity of LSD1 before and after dilution.

In the ultrafiltration experiment, LSD1 recombinant was incubated with a concentration of 20-fold IC50 inhibitor. The mixture was then added to a 10 kDa cut-off ultrafiltration tube (Millipore, Darmstadt, Germany) for centrifugation to remove the...
unbound compound. Finally, reversibility of the compound was evaluated by LSD1 assay for the upper chamber reaction system.

In the dialysis experiment, after incubation of the recombinant LSD1 and compound 14q for 1 h at 37 °C, we dialedyzed the reaction system against 50 mmol/L HEPES buffer (pH 8.0) for 24 h at 4 °C, and the buffer was replaced every 12 h, and the reversibility of the compounds was evaluated based on the activity of LSD1 in the dialysis tube.

For the competitive analysis, demethylase activity of LSD1 was assessed in the presence of diverse concentrations of compound at a specific concentration of histone substrate and diverse concentrations of FAD, and kinetics values were obtained using Lineweaver–Burk plots made by GraphPad 6.0.

4.7. MAO-A/B enzymatic assay

The MAO-A and -B were purchased from Active Motif (Cat#31502, Cat#31503, Carlsbad, CA, USA). Biochemical Kits were purchased from Promega (MAO-Glo Assay, Madison, WI, USA). Inhibition assay was carried out according to the manufacturer’s protocol. The tested compound solution was transferred into a 384-well plate by Mantis (Formulatrix, Bedford, MA, USA) in triplicate, then incubated with 10 μL of recombinant MAO-A or -B solutions at room temperature for 15 min (the final concentration was 15 and 20 nmol/L, respectively), followed by adding 10 μL of luciferin derivative substrate (the final concentration was 10 μmol/L) to initiate the reaction. After incubation for 60 min at room temperature, the reporter luciferase detection reagent (20 μL) was added and incubated with each reaction for an additional 20 min. Relative light units (RLU) were detected using EnVision Plate Reader (PerkinElmer, Waltham, MA, USA).

4.8. Cell viability assay

The MTS method was applied to the measurement of cell proliferation according to the manufacturer’s instruction (Promega, Madison, WI, USA). Each treatment was performed triplicate at least.

4.9. Western blotting

Western blot was performed with the total lysates using RIPA buffer and total histone was extracted using a histone extraction kit (Epigentek, Farmingdale, NY, USA). Same amounts of protein were subjected to SDS-PAGE, and then transferred to 0.2 μm nitrocellulose membranes (PALL, Cortland, NY, USA). After blocking with 5% nonfat dry milk PBS solution, the membranes were incubated at 4 °C with respective antibody overnight, followed by incubation with a secondary antibody conjugated with horseradish peroxidase. Finally, the blot was visualized by enhanced chemiluminescence kit (Thermo Fisher, Rockford, IL, USA).

Recombinant-based thermo stability assay: LSD1 recombinant was incubated with indicated concentrations of compound for 1 h, then the mixture was heat at 53 °C using PCR instrument (SensoQuest, Göttingen, Germany) for 3 min, after incubation, the mixture was subject to Western blotting analysis.

Cellular based thermo stability assay: cells (5 × 10^5) were treated with indicated compound or with DMSO for 1 h, washed with PBS three times, and dissolved in 50 μL of PBS supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany), followed by heating at the indicated temperatures in a PCR instrument (SensoQuest). Treated cells were then subjected to snap-freezing in liquid nitrogen and thawed on ice for 3 cycles. The protein levels of LSD1 in equal amounts of the supernatant were examined by Western blotting. GAPDH was used as the control.

4.10. Transwell assay

For the migration assay, Costar® Transwell (Corning, Lowell, MA, USA) cell culture chamber (8.0 μm pore size for the porous membrane) was used. Cells and compounds were seeding into the upper chambers containing 1% FBS. Meanwhile, 600 μL medium with 20% FBS was used as chemoattractant in the lower chamber. After incubation for 24 h, cells passing through the membrane were quantified with high content screening using Hoechst 33,258 for cell counting.

For invasion assays, cells were placed in upper chamber coated with Matrigel (Life, Bedford, MA, USA), then the experiment was performed the same as stated above.

4.11. Statistical analyses

Data were expressed as the mean ± S.D. The significance of the difference between different groups was determined with analysis of variance (ANOVA) and Student t test, which was used to compare the difference between the two groups by SPSS 17.0. Results were considered statistically significant at *P < 0.05, **P < 0.01 was considered highly significant. Date are mean ± S.D. All experiments were carried out at least three times.

4.12. Molecular modeling studies

All molecular modeling studies were performed with MOE Version 2015.10. A number of X-ray crystal structures of LSD1 were available from the RCSB protein data bank. At the present, the highest solution crystal structure of LSD1 (PDB:2DW4) without any ligand exact its cofactor FAD was selected as the docking template. The protein was prepared with Quickprep module of MOE 2015.10 under default Amber 10:EHT forcefiled. Here, the waters expect water 1032 were deleted, the hydrogen atoms were added, and residues were protonated with pH = 7. The structures of compounds 10a and 14q were executed by energy minimization and conformation search. Finally, the compounds were docked into the FAD-binding active site of LSD1 by flexible docking with default parameters.

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Author contributions

Lijing Ma contributed to the conceptualization and methodology. Haojie Wang contributed to the investigation, original draft.
writing, review and editing. Yinhua You contributed to the validation and visualization. Yuejiao Liu contributed to the formal analysis and visualization. Feifei Yang contributed to the data curation. Yichao Zheng contributed to the supervision, original draft writing, review and editing, project administration. Hongmin Liu contributed to the supervision, funding acquisition, and resources.

Conflicts of interest
The authors declare no conflicts of interests.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.02.006.

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