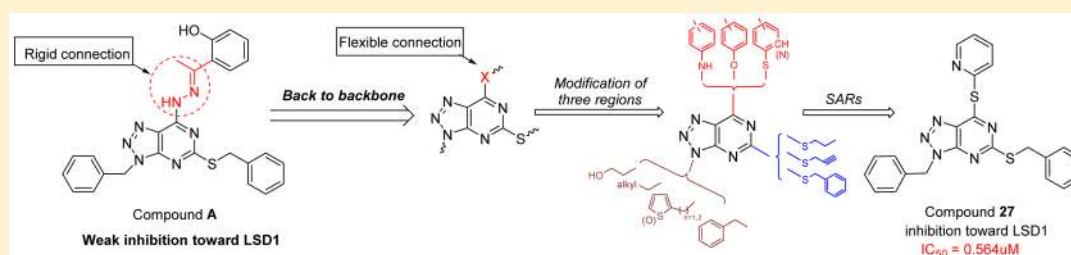


Discovery of [1,2,3]Triazolo[4,5-*d*]pyrimidine Derivatives as Novel LSD1 Inhibitors

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Supporting Information



ABSTRACT: Lysine specific demethylase 1 (LSD1) plays a pivotal role in regulating the lysine methylation. The aberrant overexpression of LSD1 has been reported to be involved in the progression of certain human malignant tumors. Abrogation of LSD1 with RNAi or small molecule inhibitors may lead to the inhibition of cancer proliferation and migration. Herein, a series of [1,2,3]triazolo[4,5-*d*]pyrimidine derivatives were synthesized and evaluated for their LSD1 inhibitory effects. The structure–activity relationship studies (SARs) were conducted by exploring three regions of this scaffold, leading to the discovery of compound 27 as potent LSD1 inhibitor ($IC_{50} = 0.564 \mu\text{M}$). Compound 27 was identified as a reversible LSD1 inhibitor and showed certain selectivity to LSD1 over monoamine oxidase A/B (MAO-A/B). When MGC-803 cells were treated with compound 27, the activity of LSD1 can be significantly inhibited, and the cell migration ability was also suppressed. Docking studies indicated that the hydrogen interaction between the nitrogen atom in the pyridine ring and Met332 could be responsible for the improved activity of 2-thiopyridine series. The [1,2,3]triazolo[4,5-*d*]pyrimidine scaffold can be used as the template for designing new LSD1 inhibitors.

KEYWORDS: [1,2,3]Triazolo[4,5-*d*]pyrimidine, LSD1 inhibitor, migration inhibition, molecular docking

Unlike histone acetylation, phosphorylation, and ubiquitination, which were identified as a dynamic process, histone methylation has long been considered as an irreversible modification until the identification of lysine specific demethylase 1 (LSD1) in 2004.¹ LSD1 can demethylate mono- and dimethylated K4 of histone 3 (H3K4) via flavin adenine dinucleotide (FAD)-dependent enzymatic oxidation, as well as remove mono- and dimethylated K9 of histone 3 (H3K9) through the interaction with other nuclear receptors.^{2,3} In addition, LSD1 could also demethylate nonhistone proteins such as p53, E2F transcription factor, and DNA methyltransferases (DNMTs) and further modulate their activity.^{4–6} By regulating the expression of target genes, LSD1 is closely associated with tumorigenesis, pluripotent stem cells, and neurodegenerative disorders.^{7–9} Particularly, aberrant expression of LSD1 has been associated with malignant tumors such as prostate, gastric, breast, lung cancers, and acute myelocytic leukemia.^{10–12} Downregulation of LSD1 expression by RNAi or inhibition of its activity by small molecules can inhibit the

proliferation of cancer cells.^{13–15} Thus, LSD1 is considered as a promising epigenetic target for anticancer drug discovery.¹⁶

Since the discovery of LSD1, a large number of inhibitors with different chemotypes have been reported and are mainly classified into two types: (A) Irreversible inhibitors such as *trans*-2-phenylcyclopropylamine (2-PCPA) based LSD1 inhibitor.^{16–19} (B) Reversible inhibitors including (bis)thiourea, amidoxime, hydrazone, pyridine–piperidine hybrids, and other heterocyclic based LSD1 inhibitors.^{20–23} To date, ORY-1001, GSK2879552, and INCB059872 have advanced into clinical trials for the treatment of acute myeloid leukemia.^{9,24,25} Our group has previously reported several classes of LSD1 inhibitors based on different skeletons including dithiocarbamate-triazoles, pyrimidine-thioureas, steroidal-triazoles, and [1,2,4]-triazolo[1,5-*a*]pyrimidines, and some of these inhibitors exhibited potent inhibitory activity against LSD1 and were

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proved to be orally active.^{26–28} Recently, a series of [1,2,3]triazolo[4,5-*d*]pyrimidine derivatives possessing hydrazone moiety were reported by our lab as potent antiproliferative agents. Among them compound A (Figure 1) displayed the

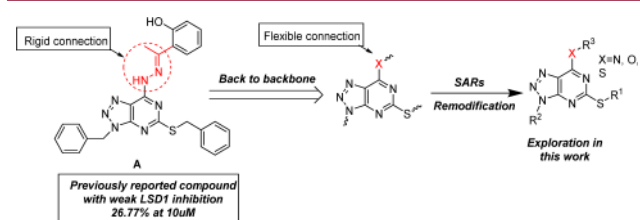


Figure 1. Compound A with weak anti-LSD1 activity and strategy employed for further optimization described in this work.

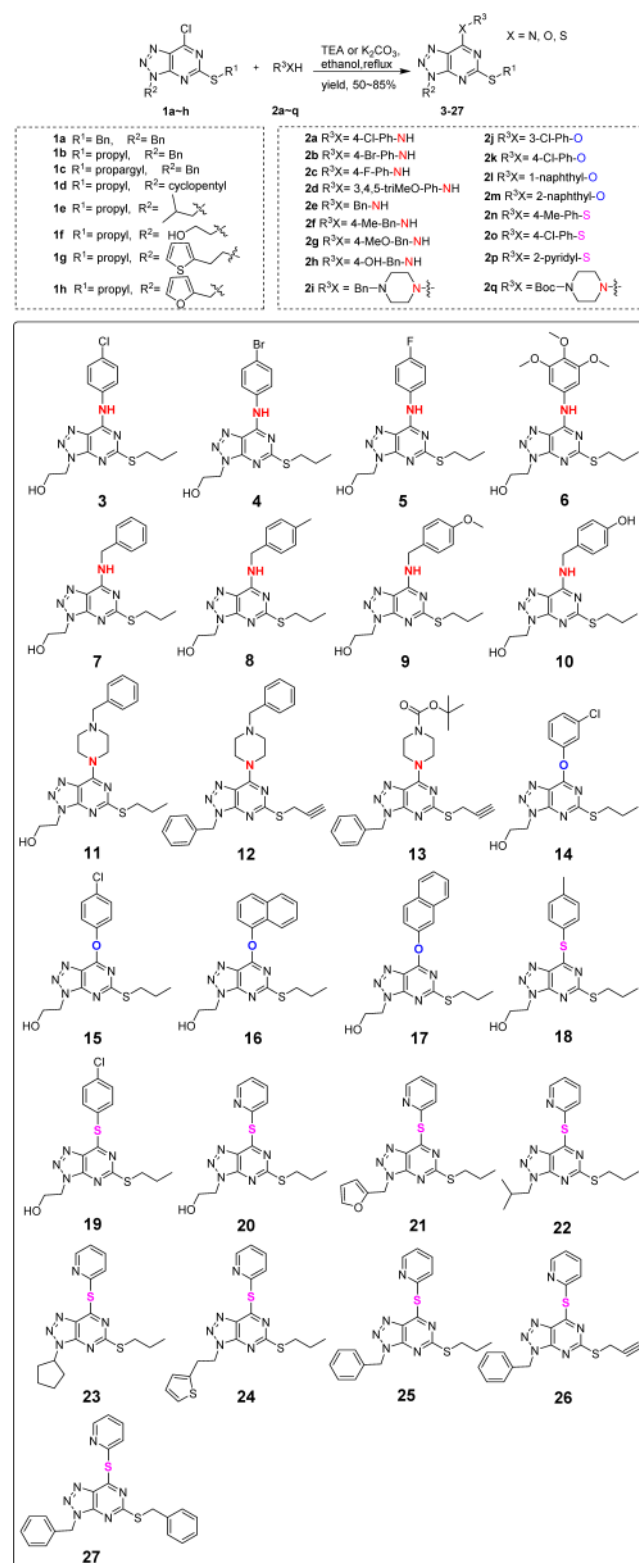
most potent antiproliferative ability, albeit with weak inhibitory activity toward LSD1 (only 26.77% inhibition at 10 μ M).²⁹ As for this weak affinity toward LSD1, docking studies revealed that compound A failed to occupy the active pocket of LSD1 due to the rigid hydrazone moiety. Based on the [1,2,3]-triazolo[4,5-*d*]pyrimidine scaffold, further modification by replacing the hydrazone moiety with flexible groups (R³, X = N, O, S), along with alterations in R¹ and R², were then carried out in this work, aiming to identify more potent LSD1 inhibitors as shown in Figure 1.

The synthetic route of designed compounds was illustrated in Scheme 1. The intermediates 1a–c and 1f–h were previously reported,²⁹ and the intermediates 1d–e were synthesized following the same procedure as that for compounds 1a–c and 1f–h. The target compounds 3–27 were prepared in 50–85% yield by reacting 1a–h with 2a–q (amines, phenols, thiophenols, and 2-thiopyridine) under alkaline conditions.

All the target compounds were screened for their *in vitro* inhibitory activity toward LSD1,²⁶ and GSK2879552 was chosen as the positive control.³⁰ Results were summarized in Tables 1 and 2. As shown in Table 1, with the exception of compound 5 having weak inhibition, the other aniline derivatives 3, 4, and 6 were found to be inactive toward LSD1 regardless of the substituent groups attached. Among the benzyl amine substituted series, compounds 7 and 8 also showed weak activities. However, compounds 9 and 10 had around 45% of inhibition rate toward LSD1, possibly due to the electron-donating property of methoxyl and hydroxyl groups on the phenyl ring. For piperazine substituted compounds 11–13, the inhibitory effects were not satisfactory as well. Phenol derivatives 14 and 15 with X = O also displayed poor inhibition against LSD1, while compounds 16 and 17 bearing the bulky naphthol groups showed significantly increased potency against LSD1 with an inhibitory rate of 57.52% and 51.73% at 10 μ M, respectively. As for compounds 18 and 19, the introduction of sulfur atom led to a slight improvement in comparison with compounds 14 and 15. Noticeably, the introduction of 2-thiopyridine into the scaffold resulted in a remarkable improvement of activity (compound 20) with inhibitory rate reaching 88.24% at 10 μ M.

Inspired by these results, we then performed further structural modifications by altering R¹ and R² groups while keeping the R³X as 2-thiopyridine unchanged. The results were listed in Table 2. It is evident that compounds 21–27 demonstrated significantly improved but comparable inhibition against LSD1 (IC₅₀ < 2 μ M), regardless of their substitution

Scheme 1. Synthesis of [1,2,3]Triazolo[4,5-*d*]pyrimidine Derivatives



patterns. Among these compounds, the most hydrophobic compound 27 (Log *P* = 5.30) exerted the best activity with an IC₅₀ value of 0.564 μ M. Generally, the hydrophobic groups for R¹ and R² are preferred for the inhibitory activity against LSD1. Also, the Log *P* values are given in Table 2.

Table 1. Inhibition of Compounds 3–20 toward LSD1

compd	inhibition at 10 μM^a (%)	compd	inhibition at 10 μM^a (%)
3	inactive	12	27.16
4	inactive	13	inactive
5	10.18	14	15.70
6	inactive	15	11.38
7	23.25	16	57.52
8	14.31	17	51.73
9	45.45	18	28.29
10	44.86	19	38.16
11	inactive	20	88.24

GSK2879552 $\text{IC}_{50} = 0.041 \pm 0.006 \mu\text{M}$

^aData are represented as inhibition % at 10 μM . All experiments were independently carried out at least three times.

Table 2. Inhibition of Compounds 20–27 toward LSD1

compd	inhibition at 10 μM^a (%)	IC_{50} (μM)	Log P^b
20	88.24	2.633 ± 0.401	2.38
21	97.17	0.758 ± 0.003	3.86
22	94.11	0.960 ± 0.003	4.34
23	95.63	1.153 ± 0.062	4.69
24	90.79	0.717 ± 0.025	4.58
25	99.98	0.685 ± 0.062	4.70
26	95.91	0.678 ± 0.250	3.82
27	98.40	0.564 ± 0.003	5.30

GSK2879552

0.041 ± 0.006

^aData are represented as mean \pm SD. All experiments were independently carried out at least three times. ^bLog P values were predicted at <http://molsoft.com/mpropr/>.

Next, we examined inhibitory effects of compound 27 against MAO-A/B as LSD1 shares similar amine acid sequence with MAO-A/B (Figure 2).^{16,26} We found that compound 27 may

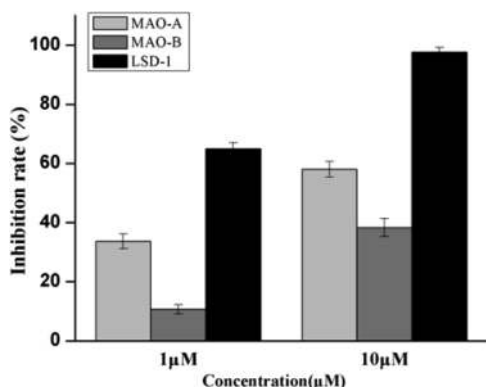


Figure 2. Inhibitory activities of compound 27 against LSD1 and MAO-A/B. Data are mean \pm SD. All experiments were independently carried out at least three times.

inactivate MAO-A 59% at 10 μM and 34% at 1 μM . Meanwhile, we also observed that compound 27 may inactivate MAO-B 39% at 10 μM and 11% at 1 μM . These results indicated that compound 27 had certain selectivity to LSD1 over MAO-A/B. To evaluate the reversibility of compound 27 for LSD1, the dilution assay were performed (Figure 3). Our results suggested that 80-fold dilution of the LSD1/compound 27 mixture resulted in the recovery of LSD1 activity, indicating that compound 27 may interact noncovalently with the enzyme. However, in the presence of the covalently binding inhibitor

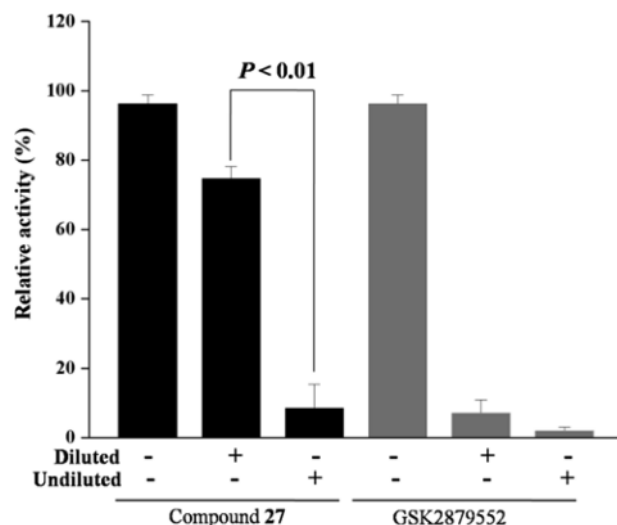


Figure 3. Reversibility of compound 27 was determined by dilution assay, and GSK2879552 was used as control. Data are mean \pm SD. $p < 0.01$ was considered statistically highly significant. All experiments were independently carried out at least three times.

GSK2879552,³⁰ LSD1 activity cannot be recovered after dilution. These results indicated the reversibility of compound 27.

Besides, we also evaluated its enzymatic activity in LSD1 overexpressed gastric cancer cell line MGC-803.²⁶ As indicated in Figure 4, MGC-803 cells were seeded in a 96-well plate and

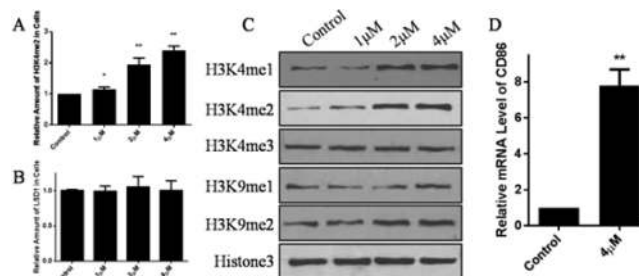


Figure 4. Inhibitory effect of compound 27 against LSD1 in MGC-803 cells. (A) High content analysis of the LSD1 inactivation status at indicated concentration using H3K4me2 as an indicator. (B) High content analysis of LSD1 expression with indicated treatment. (C) Western blot analysis of different histone modifications at indicated concentration. (D) Relative mRNA level of CD86 in MGC-803 cells with 4 μM compound 27 treatment. Data are mean \pm SD. * $p < 0.05$ was considered significant; ** $p < 0.01$ was considered statistically significant. All experiments were carried out at least three times.

treated with compound 27 for 5 days. Then the cells were subjected to immunofluorescence with H3K4me2 antibody, which indicated the activity of LSD1 in cells. Meanwhile, 4',6-diamidino-2-phenylindole (DAPI) was also applied for cell counting. By analyzing each well with 12 sights of immunofluorescence with high content analysis, intensity of H3K4me2 was evaluated, and the ratio of the intensity of H3K4me2 to cell number indicates the activity of LSD1. As can be seen from Figure 4A, treatment with compound 27 dose-dependently led to the significant accumulation of H3K4me2, the substrate of LSD1, without impact on the expression of LSD1 (Figure 4B). To further support its cellular activity, proteins of cells treated with compound 27 for 5 days were

extracted and subjected to Western blot. As indicated in Figure 4C, both H3K4me1/2 were accumulated after treatment with compound 27. Nevertheless, the amount of H3K9me1/2 and H3K4me3 was unchanged, indicating that inactivation of LSD1 by compound 27 had no impact on the activity of other histone methyltransferase or histone demethylase. As CD86 is a surrogate cellular biomarker for LSD1 activity,³¹ RT-qPCR analysis of CD86 was also performed when cells were treated with compound 27, and significant induction of CD86 mRNA was observed (Figure 4D). Hence, we confirmed the cellular inhibitory effect of compound 27 against LSD1 in MGC-803 cells.

Inactivation of LSD1 may abrogate the gastric cancer cell proliferation and migration.²⁶ Antiproliferative evaluation of compound 27 against MGC-803 cells was performed with MTT assay.²⁶ When cells were treated with compound 27 for 5 days, the IC_{50} value of compound 27 was $7.64 \pm 0.59 \mu\text{M}$, while GSK2879552 with high potency against LSD1 ($IC_{50} = 41 \text{ nM}$) presented weak cytotoxic activity toward MGC-803 ($IC_{50} > 125 \mu\text{M}$). As reported, the majority of cell lines were insensitive to growth inhibition by GSK2879552, and the cytotoxicity of LSD1 inhibitors is closely associated with the levels of DNA methylation, as observed in small cell lung carcinoma (SCLC).³⁰ Cell migration ability was evaluated by transwell experiment coupled with high content analysis. For this experiment, three low concentrations of compound 27 were applied to MGC-803 cells for 24 h treatment. As shown in Figure 5A, such lower concentration of compound 27 can

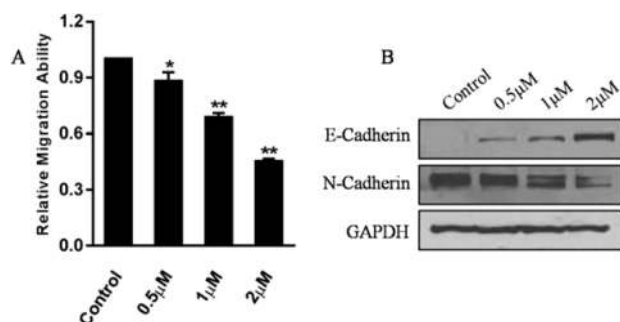


Figure 5. Inhibitory effect of compound 27 against MGC-803 cells migration. (A) Treatment with compound 27 at indicated concentrations resulted in the decreased migration ability of MGC-803 cells. (B) Expression of E-Cadherin and N-Cadherin when cells were treated with compound 27 at indicated concentrations. ** $p < 0.01$ was considered statistically significant. Data are mean \pm SD. All experiments were carried out at least three times.

inhibit the migration of MGC-803 cells significantly, and further Western blot analysis also indicated the accumulation of epithelial cell marker E-Cadherin and decreasing amount of mesenchymal cell marker N-Cadherin induced by compound 27 (Figure 5B). All these results indicated that compound 27 may be a promising scaffold for developing new LSD1 inhibitor.

In order to understand the important role of 2-thiopyridine in determining the inhibitory activity toward LSD1, we chose structurally similar compounds 18 and 20 for the docking studies to rationalize the remarkable difference in the activity toward LSD1 using the MOE2015 software (PDB: 2V1D).³² With the lowest docking energy in the active site of LSD1, the triazole ring and hydroxy group in compound 20 formed hydrogen bonds with Arg316 (Figure 6A,B). In addition to the H-bond, Tyr761 had an arene-H interaction with the triazole

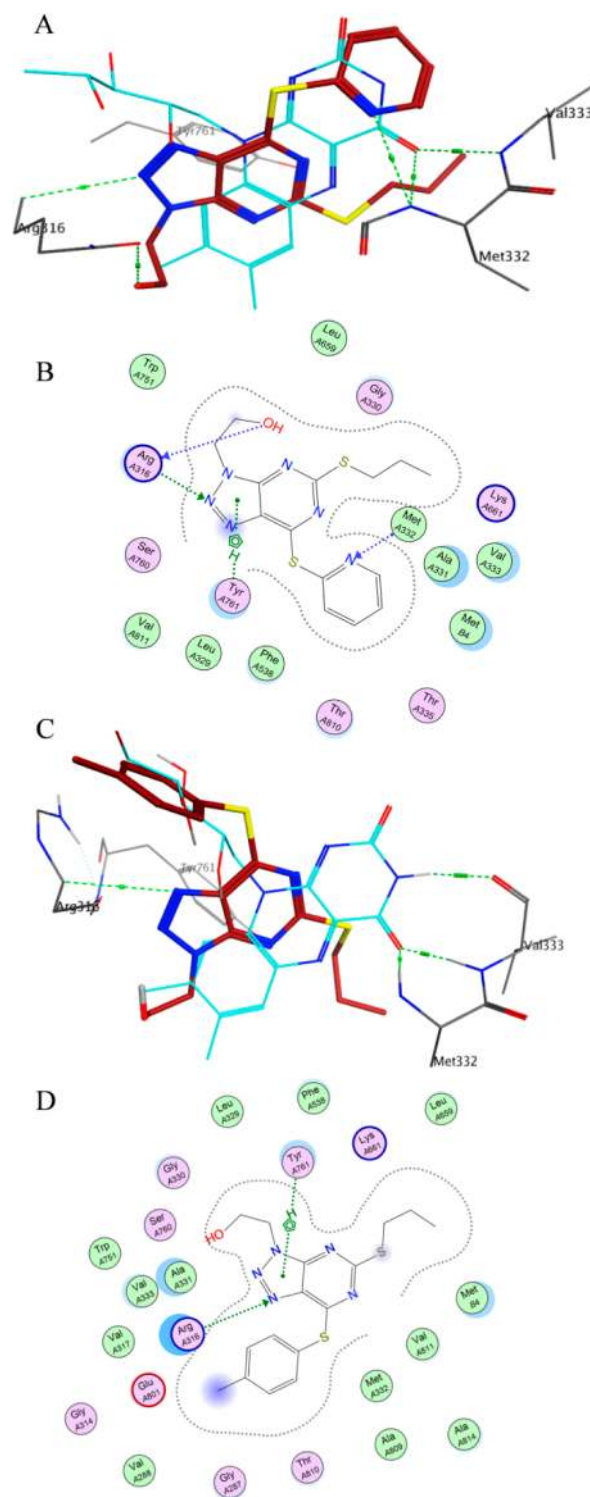


Figure 6. Binding modes of compounds 18 and 20 in the active site of LSD1/CoREST complex (PDB: 2V1D). For clarity, only key residues (in gray) in the active site of LSD1 are shown, together with the cofactor FAD (light blue stick model). Compounds 18 and 20 are highlighted in brown tube model, and hydrogen bonds are shown as green dashed lines. (A,B) Three- and two-dimensional binding modes of compound 20 in LSD1 active site. (C,D) Three- and two-dimensional binding modes of compound 18 in LSD1 active site.

ring. Besides, the nitrogen atom in the pyridine ring formed hydrogen interaction with Met332, which also had a hydrogen interaction with the flavin ring of FAD. For the docking

prediction of compound **18** with benzene ring substitution, the results (Figure 6C,D) showed that there were only interactions between the triazole ring and residues Arg316 and Tyr761 and that no interaction was observed around the benzene ring attached to the 2-position of pyrimidine ring. Therefore, the increased activities of 2-thiopyridine series may be due to the favorable interaction of the pyridine ring with Met332.

In summary, we have designed and synthesized a novel class of [1,2,3]triazolo[4,5-*d*]pyrimidine derivatives as potent LSD1 inhibitors. Among them, compound **27** exhibited the most anti-LSD1 potency, as well as modulated cancer cell growth and migration in vitro at lower concentrations. SAR investigation suggested that R³X group was important for the LSD1 inhibition, and the replacement of the C atom in phenyl ring with the N atom significantly affected the inhibitory activity against LSD1. In addition, the potency of 2-thiopyridine series was also rationally explained by docking studies through forming interactions with residues Arg316 and Tyr761, especially with Met332. Thus, [1,2,3]triazolo[4,5-*d*]pyrimidine core may serve as an attractive structure point for the discovery of potent LSD1 inhibitors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00423.

Experimental sections, characterization and ¹H and ¹³C NMR spectra of the synthesized compounds (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

LSD1, lysine specific demethylase 1; MAO, monoamine oxidase; FAD, flavin adenine dinucleotide; DNMTs, DNA methyltransferases; 2-PCPA, *trans*-2-phenylcyclopropylamine;

SAR, structure–activity relationship; DAPI, 4',6-diamidino-2-phenylindole; SCLC, small cell lung carcinoma

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