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Identification of osimertinib (AZD9291) as a lysine specific demethylase 1 inhibitor

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ABSTRACT

Osimertinib (AZD9291) is a third-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) that has been approved for the treatment of EGFR-mutated non-small cell lung cancer (NSCLC). In this study, osimertinib was characterized as a LSD1 inhibitor for the first time with an IC_{50} of 3.98 ± 0.3 μM and showed LSD1 inhibitory effect at cellular level. These findings provide new molecular skeleton for dual inhibitor for LSD1 and EGFR. Osimertinib could serve as a lead compound for further development for anti-NSCLC drug discovery with dual targeting.

1. Introduction

Epigenetics refers to heritable changes in gene expression that arise from changes in chromosomes without alteration of DNA sequence [1]. In the past two decades, the epigenetic post-transcriptional modification mostly focused on the DNA, RNA, and histone [2]. Among them, histone modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation and so on, associate with the gene transcription closely [3]. Historically, histone methylation was believed to be an irreversible process until the histone lysine specific demethylase 1 (LSD1, also known as KDM1A) was first identified by Shi Yang in 2004 [4]. LSD1 is a transcription regulator and functions as co-repressor or co-activator in a target-specific manner and its activity is strictly dependent on the interaction with specific chromatin regulatory complexes [5]. LSD1 is commonly associated with protein complexes containing different transcriptional corepressors, such as HDAC1/2, the REST corepressor (CoREST), CtBP and the nucleosome remodeling, deacetylase (NuRD) complexes [6–9]. In these complexes, LSD1 works as a repressor since it demethylates H3K4me1/2, a transcription activation marker. Conversely, the interaction with androgen (AR) and estrogen (ER) nuclear hormone receptors, converts LSD1 into a transcription activator as it demethylates H3K9me1/2, a transcription repressor mark [10,11]. Besides, LSD1 also regulates directly, by lysine demethylation, the activity or stability of several proteins, such as p53 [12], DNMT1 [13], E2F1 [14], and MYPT1 [15]. Due to the specificity of substrate recognition of LSD1 by forming distinct complex, LSD1 was reported to be an oncogene in several kinds of cancers and considered as a drug target [16–18]. It was reported that LSD1 was overexpressed in lung cancer tissues than normal lung tissues, and the level of LSD1 expression was negatively associated with the total survival time of non-small cell lung cancer (NSCLC) patients [19]. Hence, a large number of medicinal chemists are try to have potent and specific LSD1 inhibitors. Until now, a large number of inhibitors with different chemotypes have been reported and are mainly classified into two types: irreversible inhibitors and reversible inhibitors [20]. And some of them have entered into clinical trials for the treatment of acute myeloid leukemia, such as ORY-1001 and GSK2879552 [21]. Nevertheless, none of the reversible inhibitor has entered into clinical trials successfully.

Currently, targeted therapy with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) has become a standard first-line therapy for the treatment of patients with EGFR-mutated non-small-cell lung cancer (NSCLC) [22,23]. As one of the clinic applied drugs, osimertinib is an orally administration, irreversible binding, third generation EGFR-TKI [24]. It has been approved by the US Food and Drug Administration (FDA) for the treatment of patients with EGFR T790M mutation-positive NSCLC. For NSCLC therapy, LSD1 and EGFR are both important drug targets. Dual targeting of LSD1 and EGFR may be a promising way for the NSCLC treatment. Nevertheless, pharmacology

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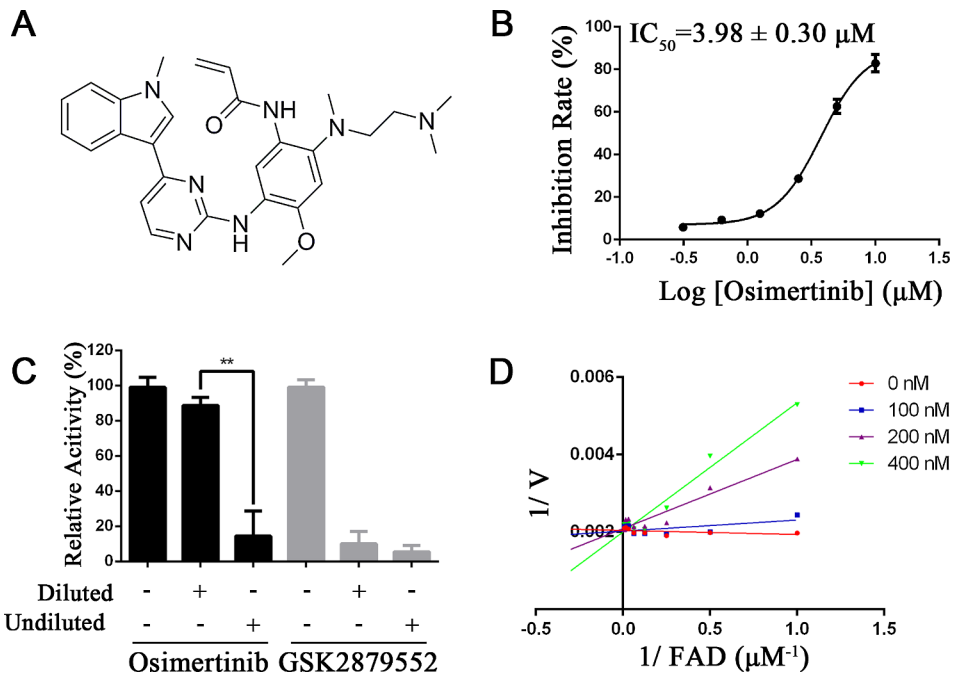

Fig. 1. Inhibitory effect of osimertinib on LSD1. (A) Chemical structure of osimertinib. (B) Inhibitory activity of osimertinib against recombinant LSD1. (C) The reversibility of osimertinib to LSD1 activity was determined by dilution assay. GSK2879552 was used a control. (D) Lineweaver-Burk plots demonstrated that osimertinib is competitive with the LSD1 cofactor FAD. Data are mean ± SD. $^{**}p < 0.01$ was considered statistically highly significant. All experiments were carried out at least three times.

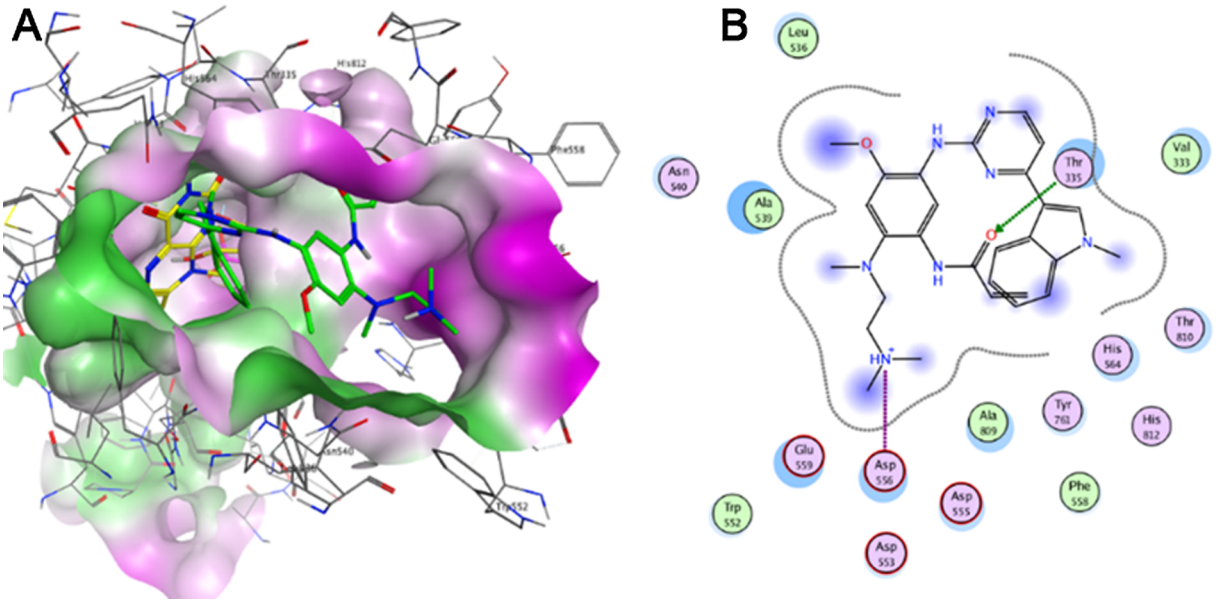

Fig. 2. Binding models of EGFR inhibitor osimertinib within the active site of LSD1 (PDB code: 5L3E). (A) Surface representation of osimertinib (colored in green) in the active site of LSD1 containing FAD (highlighted in yellow). The green and purple color represents hydrophobic and hydrophilic region, respectively. (B) 2D diagram of osimertinib in the active site of LSD1. The green dashed line represents the hydrogen bond, while purple dashed line represents the ion contact. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tool to abrogate LSD1 and EGFR simultaneously is still in shortage. There is still a blank about this kind of inhibitor. In this study, we characterized osimertinib as the first dual inhibitor for LSD1 and EGFR. Meanwhile, osimertinib could inhibit tumour cells proliferation and migration. As the first dual inhibitor, this study provides a new molecular skeleton for the development of potential LSD1 and EGFR inhibitors.

2. Experimental

2.1. Inhibitory evaluation of osimertinib against LSD1 and mechanism of action studies

LSD1 inhibition assay, dilution assay and competitive analysis were carried out following our previously reported method [25].

2.2. Flow cytometric analysis of CD86 expression

Cells were seeded into 6-wells plate (Nest Biotechnology, China) at 2×10^5 cells per well. After 24 h incubation, the cultured medium was removed and replaced with fresh medium containing the osimertinib at different concentrations for another 48 h. Then, the cells were harvested and the CD86 antibody was used according to the manufacturer's instructions. Ten thousand events were collected for each sample and analyzed by flow cytometry (BD Bioscience, USA).

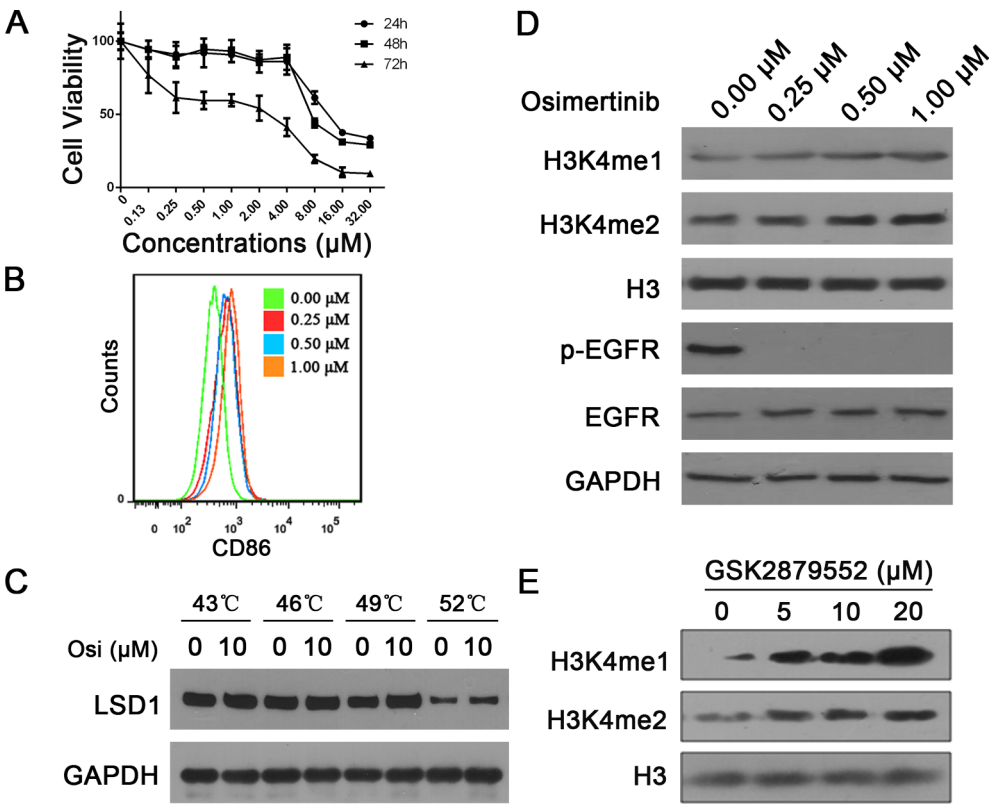

Fig. 3. Inhibitory effect of osimertinib against LSD1 in NCI-H1975 cells. (A) NCI-H1975 cell viability of osimertinib treatments relative to the controls determined by the MTT assay; (B) The expression of CD86 in NCI-H1975 cells with indicated concentrations treatment of osimertinib; (C) Enhancement of thermal stability of LSD1 protein by osimertinib in NCI-H1975 cells. Protein levels of LSD1in NCI-H1975 cells treated with osimertinib at 10 μM or DMSO for 1 h, followed by heating at different temperatures for 3 min were examined by western blotting analysis. (D) The expression of H3K4me1, H3K4me2, H3, p-EGFR, EGFR and GAPDH in NCI-H1975 cells with indicated concentrations treatment of osimertinib; (E) The expression of H3K4me1, H3K4me2 and H3 in NCI-H1975 cells with indicated concentrations treatment of GSK2877552. GAPDH or H3 was used as a loading control.

2.3. Cellular thermal shift assay

Cellular Thermal Shift Assay was performed according to the reported method [26,27]. Briefly, cells (5×10^5 per sample) were treated with 10 μM osimertinib or with DMSO for 1 h, washed with PBS three times, and dissolved in 50 μL PBS supplemented with a protease inhibitor, followed by heating at the indicated temperatures. 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 blots. GAPDH was used as the control. Results are representative of three independent experiments.

2.4. Wound healing assay

Cells were seeded in a 6-wells plate (CORNING, USA) at 2×10^5 per well. After 24 h incubation, the cell surface was scratched using a 10 μL pipette tip. Then, cells were cultured with fresh medium containing 1% FBS plus different concentrations of osimertinib for 48 h. Finally, cells were photographed on an inverted microscope.

2.5. Transwell assay

For the migration assay, 400 μL medium without FBS, different concentrations of osimertinib and 8000 cells were added to each upper chamber. In the lower chamber, 600 μL medium with 20% FBS was used as chemoattractant. After incubation for 48 h, both chambers were washed by PBS for three times. After staining with Hoechst 33258 (10 μg/mL) and wash the chamber twice, migrated cells were detected and numbered using high content screening system (ArrayScan XTI, Thermo Fisher Scientific, MA).

2.6. Western blot analysis

Cells were seeded and treated with 0, 0.25, 0.5 and 1 μM of osimertinib for 48 h, then cells were collected and lysed by radio immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% Triton X-100) with the complete proteinase inhibitor cocktail (Roche, Switzerland) for 30 min. After centrifugation of 12,000 rpm for 10 min at 4 °C, supernatant was collected and the protein concentration was detected using a bicinchoninic acid (BCA) assay kit (Beyotmie Biotechnology, China). After added with loading buffer, cell lyses were denatured for 10 min at 100℃ for SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose (NC) membranes. The membranes were blocked with 5% skim milk at room temperature for 2 h, then incubated overnight at 4 °C with primary antibodies. After washing the membrane with TBST (TBS, 0.05% Tween-20)/TBS three times (5 min per wash), membranes were incubated with the secondary antibody (1:5000) at room temperature for 2 h. Finally, the blots were washed in TBST/TBS. The antibody-reactive bands were revealed by enhanced chemiluminescence (ECL) and exposed on radiographic film.

2.7. Statistical analysis

All these data were presented as means ± SD. Comparison of difference between two groups was evaluated by the Student's *t*-test. The difference between more than two groups was determined by one-way ANOVA (Prism GraphPad). P value less than 0.05 was considered as significant.

3. Results and discussion

3.1. In vitro inhibition properties of osimertinib to LSD1 recombinant

Osimertinib is an orally administrated, irreversible binding, third generation EGFR TKI. It forms a covalent bond with residue C797 in the EGFR kinase domain and has been approved by FDA for the treatment of patients with EGFR T790M mutation-positive NSCLC. In our study, we characterized osimertinib (Fig. 1A) as the first dual inhibitor of

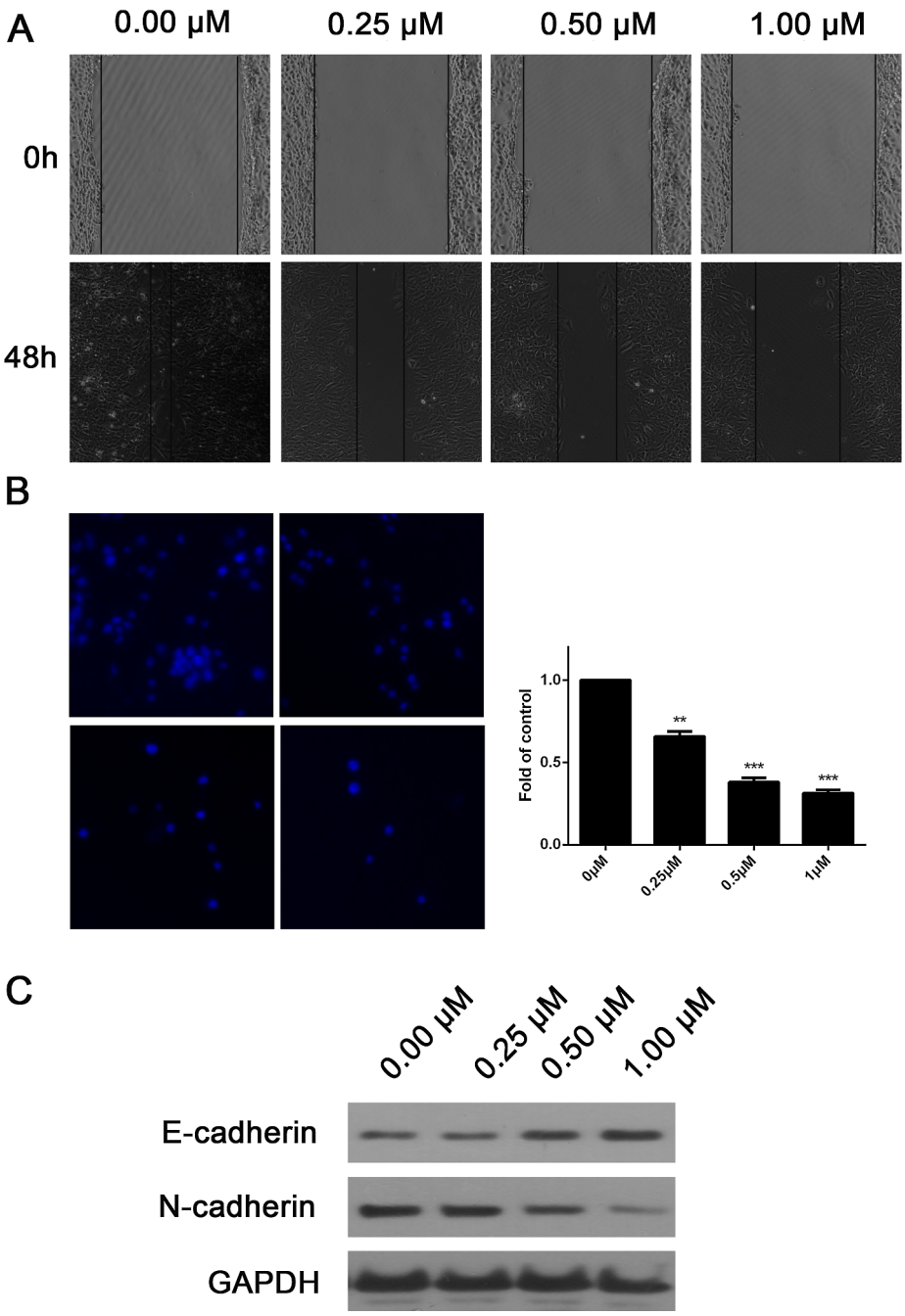

Fig. 4. Inhibitory effect of osimertinib on NCI-H1975 cell migration. Wound healing (A) and transwell (B) assays performed using osimertinib at different concentrations and different time intervals. (C) The protein expression of E-Cadherin, N-Cadherin and GAPDH in NCI-H1975 cells were determined after 48 h treatment of various concentrations of osimertinib. $^{*}p < 0.05$ and $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control. All experiments were performed at least three times.

LSD1 and EGFR with an IC_{50} (LSD1) of 3.98 ± 0.30 μM (Fig. 1B) using our previously reported method [25].

To test the reversibility of osimertinib for LSD1, a dilution assay was used. The LSD1 recombinant was incubated with 60 μM osimertinib first, then the mixture was diluted 80 folds to test the activity of LSD1. We found that dilution of the LSD1/osimertinib mixture by 80 folds resulted in the recovery of LSD1 activity. However, in the presence of the covalently binding inhibitor GSK2879552, LSD1 activity cannot be recovered after dilution (Fig. 1C). These results indicate osimertinib may interact noncovalently with the enzyme.

Next, the competitive analysis was performed to test the hypothesized binding pocket of osimertinib on LSD1. With classic Lineweaver–Burk plots [28], we characterized that osimertinib was a competitive inhibitor over LSD1 cofactor FAD (Fig. 1D). These results indicate that osimertinib may penetrate into the cavity in LSD1 where FAD stands and reduce the recombinant activity. It is not so common to create a FAD competitor. But there are some published FAD competitors, for example, Quinine [29], mepacrine, chlorpromazine [30], triazole-dithiocarbamate based LSD1 inhibitors [25]. Hence, although the structure of osimertinib is not similar to that of FAD, it is still possibly for it to act as FAD competitor or FAD ejector.

3.2. Molecular docking studies

In order to rationalize the potency of osimertinib against LSD1, the LSD1-CoREST1 in complex with quinazoline-derivative reversible

inhibitor (PDB code: 5L3E) was downloaded from the RCSB protein database bank (PDB) [31] and used as the docking receptor after the structural preparation, protonation, and energy minimization. Osimertinib was protonated and energetically minimized (force field: Amber10: EHT) according to the default settings, followed by the conformation search. The docking procedures were performed according to the default settings. As shown in Fig. 2A, osimertinib (colored in green) occupied the active site of LSD1, but was away from the FAD (colored in yellow). The protonated N,N-dimethyl group formed ion contact (shown in purple dashed line) with Asp556, the carbonyl oxygen atom of the amide group had a hydrogen bond interaction (showed in green dashed lines) with Thr335 (Fig. 2B). It also should be noted that the 3D structural features of osimertinib may have critical roles in the anti-LSD1 activity. To conclude, osimertinib could be potentially used as a template to design new LSD1 inhibitors.

3.3. Cellular inhibitory effect of osimertinib against LSD1

After we have confirmed the LSD1 inhibitory effect in recombinant level, cellular activity of osimertinib against LSD1 was also investigated for the following study. First, the antiproliferation effect of osimertinib on NCI-H1975 cells was measured by the MTT assay. Treatment of osimertinib with its increasing concentrations resulted in dose- and time-dependent reductions in NCI-H1975 cell viability: the IC_{50} values were 13.29 ± 0.97, 9.97 ± 1.03, and 1.51 ± 0.37 μM at 24, 48, and 72 h, respectively (Fig. 3A). CD86, a surrogate cellular biomarker for LSD1 activity [32], was also performed when NCI-H1975 cells were treated with osimertinib for 48 h, and CD86 expression was increased (Fig. 3B). To assess the target engagement of osimertinib in cells, we next employed the cellular thermal shift assay (CETSA) [26]. As shown in Fig. 3C, cellular LSD1 protein was degraded at 49 °C and 52 °C in NCI-H1975 cells treated with DMSO. The thermal stability of LSD1 protein was clearly enhanced by osimertinib at 49 °C and 52 °C. H3K4me1/2, which indicated the activity of LSD1 in cells, p-EGFR and EGFR was detected after treatment of different concentrations of osimertinib. Osimertinib treatment induced a significant dose-dependent increase in the accumulation of H3K4me1/2 (Fig. 3B) in cells. Meanwhile, all the concentrations of osimertinib we choose could inactive EGFR for the strongly decreased p-EGFR (Fig. 3B). These data suggest that osimertinib engages LSD1 proteins in cells. Hence, we confirmed the cellular inhibitory effect of osimertinib against LSD1 in NCI-H1975 cells.

3.4. Effect of osimertinib on cell migration

As reported, LSD1 was involved in epithelial-mesenchymal transition (EMT), which contributed for the migration and invasion abilities of cancer cells [33,34]. So we evaluated the migration ability of NCI-H1975 cells by wound healing and transwell assay. As shown in Fig. 4A, the monolayer of NCI-H1975 cells were scratched following indicated treatment, osimertinib inhibited the wound healing obviously. To further confirm the cell migration inhibitory effect of osimertinib, cell migration ability was evaluated by transwell experiment coupled with high content analysis. For this experiment, different concentrations of osimertinib were applied for 48 h treatment. As shown in Fig. 4B, even 0.25 μM of osimertinib can inhibit the migration of NCI-H1975 cells significantly, and further western blot (Fig. 4C) also indicates the accumulation of epithelial cell marker E-Cadherin and decreasing amount of mesenchymal cell marker N-Cadherin induced by osimertinib. All these results suggest that osimertinib may be a promising leader compound for further development as a dual inhibitor of LSD1 and EGFR for anti-NSCLC drug discovery.

4. Conclusion

In summary, we have first characterized osimertinib, EGFR inhibitor, as the inhibitor of LSD1 (IC_{50} = 3.98 μM). Meanwhile, osimertinib showed cellular inhibitory activity against LSD1. As a target of osimertinib, LSD1 plays key roles in cancer progression and development. Osimertinib could inhibit NCI-H1975 cells migration. Further structure-activity relationship (SAR) study on osimertinib is necessary to identify potent and selective dual inhibitor of LSD1 and EGFR.

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Conflict of interest

The authors declare no conflicts of interest.

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