Discovery of WS-157 as a highly potent, selective and orally active EGFR inhibitor

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KEY WORDS
WS-157: Tyrosine kinase; EGFR inhibitor; Antitumor activity

Abstract  EGFR tyrosine kinase inhibitor (EGFR-TKI) has been used successfully in clinic for the treatment of solid tumors. In the present study, we reported the discovery of WS-157 from our in-house diverse compound library, which was validated to be a potent and selective EGFR-TKI. WS-157 showed excellent inhibitory activities against EGFR (IC50 = 0.81 nmol/L), EGFR[Δ746-750] (IC50 = 1.2 nmol/L) and EGFR[ΔL858R] (IC50 = 1.1 nmol/L), but was less effective or even inactive against other nine kinases. WS-157 also displayed excellent antiproliferative activities against a panel of human cancer cell lines, and exhibited the ability to reduce colony formation and wound healing the same as gefitinib. We found that WS-157 upon oral administration showed better anti-tumor activity in A431 bearing xenograft mouse models compared to gefitinib. In addition, WS-157 showed better intestinal absorption than gefitinib and had favorable pharmacokinetic properties and microsomal metabolic stability in different species. These studies indicate that WS-157 has strong antitumor activity in vitro and in vivo, and could be used for the development of anti-lung cancer agent targeting EGFR.

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1. Introduction

The human epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases consisting of four members (ErbB1-4). All ErbB family members share a common structure organization that is composed of an extracellular ligand binding domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The binding of their respective ligands to the corresponding ErbB extracellular domains induces homodimerization or heterodimerization of the receptors and subsequent phosphorylation at the multiple tyrosine residues located in the intracellular region, and then phosphorylated tyrosine residues recruit diverse effector proteins to activate multiple signal transduction pathways, including the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Aberrant EGFR activation promotes multiple biological processes, including survival, proliferation, invasion, metastasis, angiogenesis and decreased apoptosis, which play central roles in the progression of tumors.

Blocking or inhibiting signaling pathways with EGFR tyrosine kinase inhibitor (EGFR-TKI) has resulted in development of several novel EGFR-TKI (Fig. 1). The first generation of EGFR-TKI, including gefitinib, erlotinib, and icotinib, are ATP competitive reversible inhibitors for the treatment of non-small-cell lung cancer (NSCLC). Although gefitinib and erlotinib are effective in the treatment of NSCLC, especially in patients with tumors possessing EGFR-sensitive mutants (EGFR<sup>L858R</sup>), its resistance has been observed clinically and was associated with the T790M mutation of EGFR. The second generation EGFR-TKIs, such as afatinib/BIBW2992 and lapatinib, are irreversible inhibitors. Afatinib was approved by FDA in July 2013 for first-line treatment of subjects with EGFR exon 19 deletions or exon 21 (L858R) substitution mutations. Osimertinib, rociletinib and olmutinib are the newly developed “third-generation” EGFR-TKIs that efficiently overcome the EGFR<sup>T790M</sup> drug-resistance mutation while sparing the EGFR wt. Rociletinib and osimertinib exhibited excellent clinical efficacy in NSCLC patients harboring EGFR<sup>T790M</sup> with more than 50% response rates and

![Figure 1](image1.png)

**Figure 1** Representative EGFR inhibitors.

![Figure 2](image2.png)

**Figure 2** Discovery of WS-157 as a potent EGFR inhibitor. WS-157 was identified from our combined diverse compound library based on the random screening against EGFR tyrosine kinase.
less skin and gastrointestinal toxicities than those typically observed for the first generation EGFR TKIs. Recently, osimertinib was approved by FDA for the treatment of patients with metastatic EGFR\[^{T790M}\] mutation positive NSCLC who have progressed on or after EGFR TKI therapy. Unfortunately, resistance to third-generation EGFR inhibitors may occur during treatment and C797S has been shown to be an acquired drug resistance mutation in NSCLC patients with EGFR\[^{T790M}\] mutation. Therefore, it is highly needed for the development of new drugs that overcome EGFR mutations. Recently, the first EGFR allostERIC inhibitor (EAI) EA1045 was reported. This compound, in combination with the antibody cetuximab, inhibits gefitinib. In addition, the IC50 values of this compound against HER4, HER2, EGFR\[^{T790M/L858R}\] and EGFR\[^{T790M}\] were 12, 90, 230 and 348 nmol/L respectively, which were similar to those of gefitinib. For the other nine kinases, gefitinib had low or no inhibitory activity with the IC\(_{50}\) values more than 1000 nmol/L. The result indicates that WS-157 is a highly selective inhibitor.

2. Results

2.1. Synthesis of WS-157

As shown in Scheme 1, the synthesis of WS-157 started from commercially available compound 1; chlorination of compound 1 in POCl\(_3\) gave compound 2, which then reacted with 3-ethynylaniline to form WS-157 in the presence of TEA.

2.2. WS-157 potently and selectively inhibits EGFR kinase activity in vitro

In order to determine the selectivity profile of WS-157, the effects of WS-157 on the enzymatic activity of a panel of 16 tyrosine kinases consisting of EGFR, EGFR\[^{T746\rightarrow\text{750}}\], EGFR\[^{L858R}\], EGFR\[^{T790M/L858R}\], EGFR\[^{T790M}\], FGR1, HER4, KDR (VEGFR2), PDGFR\(\alpha\) (PDGFR\(\alpha\)), PDGFR\(\beta\) (PDGFR\(\beta\)), IGF1R, SRC, FLT1, MET, KIT and HER2 were examined. Gefitinib was chosen as reference drug (Table 2). The results indicated that WS-157 showed excellent inhibitory activities against EGFR (IC\(_{50}\) = 0.81 nmol/L), EGFR\[^{T746\rightarrow\text{750}}\] (IC\(_{50}\) = 1.2 nmol/L) and EGFR\[^{L858R}\] (IC\(_{50}\) = 1.1 nmol/L), which was more potent than gefitinib. In addition, the IC\(_{50}\) values of this compound against HER4, HER2, EGFR\[^{T790M/L858R}\] and EGFR\[^{T790M}\] were 12, 90, 230 and 348 nmol/L respectively, which were similar to those of gefitinib. For the other nine kinases, WS-157 had low or no inhibitory activity with the IC\(_{50}\) values more than 1000 nmol/L. The result indicates that WS-157 is a highly selective inhibitor.

### Table 1 Kinases and control compounds.

| Kinase | Manufacturer | Item number | Lot number |
|--------|--------------|-------------|------------|
| EGFR   | Carna        | 08-115      | 13CBS-0005X|
| EGFR\[^{T790M}\] | Carna | 08-527 | 11CBS-1129H |
| EGFR\[^{L858R}\] | Carna | 08-502 | 13CBS-0537F |
| EGFR\[^{T790M/L858R}\] | Carna | 08-510 | 12CBS-0765H |
| EGFR\[^{T790M}\] | Carna | 08-194 | 08CBS-0510N |
| FGR1   | Carna        | 08-133      | 12CBS-0123K|
| HER4   | Carna        | 08-118      | 08CBS-0652J|
| KDR (VEGFR2) | Carna | 08-191 | 13CBS-0442H |
| PDGFR\(\alpha\) (PDGFR\(\alpha\)) | Carna | 08-156 | 11CBS-1067P |
| PDGFR\(\beta\) (PDGFR\(\beta\)) | Carna | 08-157 | 09CBS-1164P |
| IGF1R  | Carna        | 08-158      | 12CBS-0573J|
| SRC    | Carna        | 08-016      | 15CBS-0769C|
| FLT1   | Carna        | 08-141      | 09CBS-0414H|
| MET    | Carna        | 08-189      | 09CBS-0902J|
| KIT    | Carna        | 08-151      | 10CBS-1188P|
| HER2   | Carna        | 08-173      | 10CBS-1134K|
| Afinatin | Selleckchem | S1011   | lot10      |
| AZD9291 | Selleckchem | S7297   | lot07      |
| BGF398 | Selleckchem | S2183   | NVP-BG398 |
| Nintedanib | Selleckchem | S1010 | S101004   |
| Saracatinib | Selleckchem | S1006 | lot03      |
| Staurosporine | Selleckchem | S1421 | S142105   |
| DMSO   | Sigma       | D8418-1L    | SHBG3288   |

### Table 2 Differential inhibitory activities of WS-157 and gefitinib against a panel of tyrosine kinases.

| Kinase | WS-157 (nmol/L) | Gefitinib (nmol/L) |
|--------|-----------------|--------------------|
| EGFR   | 0.81±0.01       | 0.96               |
| EGFR\[^{T746\rightarrow\text{750}}\] | 1.23±0.03       | 1.43±0.04           |
| EGFR\[^{L858R}\] | 1.05±0.01       | 1.268±0.02        |
| EGFR\[^{T790M/L858R}\] | 208.9±0.57      | 296.4±0.99         |
| EGFR\[^{T790M}\] | 344.05±20.72    | 70.56±1.28        |
| FGR1   | >10,000         | >10,000            |
| HER4   | 10.98±0.78      | 10.55±0.17        |
| KDR (VEGFR2) | 4384.50±135.06 | 5465.5±122.33    |
| PDGFR\(\alpha\) (PDGFR\(\alpha\)) | 3446.50±210.01 | 2040.7±77.78   |
| PDGFR\(\beta\) (PDGFR\(\beta\)) | 1180±113.40    | 1506±2.83        |
| IGF1R  | >10,000         | >10,000            |
| SRC    | 5210±368.08     | 3403.5±17.68      |
| FLT1   | 5020.50±153.44  | >10,000            |
| MET    | >10,000         | 9725±961.67       |
| KIT    | >10,000         | >10,000            |
| HER2   | 90.82±12.45     | 78.03±1.78        |

Scheme 1 Synthesis of WS-157. Reagents and conditions: (a) POCl\(_3\), 90 °C, 5–6 h, reflux; (b) EtOH, TEA, r.t., 3 h.
that targets EGFR family members, particularly EGFR, EGFR\(^{[746-750]}\) and EGFR\(^{[L858R]}\).

### 2.3. WS-157 suppresses the biochemical pathways in human EGFR wt cell lines

The PI3K/AKT pathways are the downstream signaling transduction of EGFR and is critical for tumor proliferation, survival and response to exterior stimuli\(^2\). We next tested the ability of WS-157 to inhibit the phosphorylation of EGFR and its downstream AKT in human A431 cell, with gefitinib as a reference drug. As shown in Fig. 3A, exposure of cells to the growth factor EGF rapidly increased the level of cellular p-EGFR and p-AKT, but WS-157 could dramatically and dose-dependently inhibit EGF-induced p-EGFR and p-AKT similar to gefitinib. Moreover, 1.0 \(\mu\)mol/L of WS-157 or gefitinib almost completely prevented the p-EGFR and p-AKT stimulated by EGF (Fig. 3A). Similar results were obtained for additional EGFR wt cell lines of other cancer types, including NSCLC and gastric cancer (Fig. 3B–F). Then, we detected the effect of WS-157 in EGFR\(^{[T790M/E855K]}\) cell line NCI-H1975 and found that WS-157 and gefitinib did not prevent p-EGFR and p-AKT (Fig. 3G), which indicated that WS-157 was an EGFR wt inhibitor. As gefitinib is a well-known first generation reversible EGFR inhibitor, we also explored whether

![Figure 3](https://example.com/figure3.png)

**Figure 3** WS-157 suppressed the biochemical pathways in different cell lines. (A) A431 cells, (B) A549 cells, (C) BGC-823 cells, (D) MGC-803 cells, (E) SGC-7901 cells and (F) NCI-H1975 cells were starved for 24 h, treated with increasing concentrations of WS-157 or gefitinib for 1 h, and exposed to EGF (50 ng/mL) for 10 min. (G) A549 cells were treated with WS-157 or gefitinib for 2 h and incubated for 0–24 h after removal of WS-157 or gefitinib. The cell lysates were collected for Western blotting with the indicated antibodies. The representative data of at least three independent experiments are shown, \(^*P < 0.05\), \(^{**}P < 0.01\), \(^{***}P < 0.001\), normal control vs. EGF-stimulated control (mean ± SD, \(n = 3\)); \(^{$P < 0.05$\), \(^{#P < 0.01$\), \(^{##P < 0.001$\ vs. EGF-stimulated control (mean ± SD, \(n = 3$).
inhibitory activities of WS-157 was reversible. Cells were washed to remove the unbound compounds after treatment with WS-157 for 2 h and incubated in fresh medium 0–24 h. The results indicated that inhibition of p-EGFR and p-AKT were gradually recovered after removal of WS-157. All these data suggest that WS-157 is a highly selective and reversibly EGFR inhibitor.

2.4. Effects of WS-157 on tumor cell proliferation, clone assay and migration

Having established that WS-157 can selectively inhibit EGFR, we investigated the effects of WS-157 on tumor cell proliferation, clone assay and migration. SRB assays was used to examine the proliferative inhibition of WS-157 on 12 tumor cell lines that possess distinct levels of EGFR protein, with gefitinib as a reference drug (Fig. 4). As shown in Fig. 4A, WS-157 showed varied antiproliferative activities against the tested tumor cells, and for most of the cells, the IC50 values of WS-157 were similar to those of gefitinib. Particularly, WS-157 remarkably inhibited the proliferation of PC-9 and A431 cells with the IC50 values less than 1.0 μmol/L (the inhibitory cures of WS-157 against PC-9, A431 and GES-1 have been provided in Supporting Information Fig. S1). As shown in Fig. 4B‒D, the results suggest that WS-157 had excellent inhibitory activities on cell proliferation and migration. Particularly, WS-157 almost completely inhibited proliferation and migration of PC-9 at 0.1 μmol/L.

2.5. WS-157 inhibits growth of A431 human tumor xenografts

Given its encouraging in vitro antitumor activity, we examined the antitumor efficacy of WS-157 in xenograft mouse models of A431 overexpressing EGFR wt. According to the reported data, oral administration of gefitinib (10 mg/kg body weight once daily) could induce tumor regression, while WS-157 (10 mg/kg/day) showed slightly better anti-tumor activity compared to gefitinib (Fig. 5A and B). However, the tumor burden in nude mice was nearly completely suppressed by WS-157 (40 mg/kg/day) (Fig. 5A–C). Meanwhile, body weights of nude mice measured concurrently showed no obvious differences during the treatment, demonstrating that WS-157 was well tolerated (Fig. 5D). We also examined the effect of WS-157 on the EGFR signaling pathway in tumor extracts. As shown in Fig. 5E and F, p-EGFR and p-AKT were clearly inhibited by WS-157 or gefitinib.

2.6. Caco-2 cell permeability

Caco-2 cell monolayer has become a commonly used in vitro model for intestinal absorption of drugs. As shown in Table 3, the \( \text{P}_{\text{app}} \) (A-B) value of metoprolol was 40.04, and it was used as a high permeable control. On the contrary, the \( \text{P}_{\text{app}} \) (A-B) value of atenolol was 0.66, which was considered as a low permeable control. The \( \text{P}_{\text{app}} \) (B-A)/\( \text{P}_{\text{app}} \) (A-B) value of erythromycin was 240.3, which indicated that more erythromycin was transported from BL to AP. Therefore, erythromycin was used as an efflux

![Figure 4](image-url)
substrate. The $P_{\text{app}}$ (A–B) values of WS-157 and gefitinib were 9.13 and 8.37, respectively. The $P_{\text{app}}$ (B–A)/$P_{\text{app}}$ (A–B) values of WS-157 and gefitinib were 2.51 and 1.85, respectively.

2.7. Metabolic stability

WS-157 was incubated individually with different species to assess the metabolic stability of interspecies difference. We further compared the different metabolism between WS-157 and gefitinib in liver microsome in this study. As shown in Fig. 6, both WS-157 and gefitinib were the most stable in the dog liver microsome, followed by human, rat, mouse and monkey. It was indicated that the metabolic stability of WS-157 and gefitinib was similar, except in rat. The $t_{1/2}$ of WS-157 and gefitinib in rat liver microsome was 11.78 and 21.16, respectively, which indicated that WS-157 was less stable than gefitinib in rat liver microsome (Table 4).

2.8. Pharmacokinetic study

In order to investigate the pharmacokinetic characteristics of WS-157, we determined the plasma concentration of WS-157 in rats after intravenous injection (i.v.) or p.o. administration. Gefitinib was used as a control. As shown in Fig. 7, the mean plasma concentration–time curves of WS-157 and gefitinib groups were similar after i.v. administration. Based on non-compartment model analysis, the pharmacokinetic parameters were calculated and summarized in Table 5. Parameters, such as CL, $t_{1/2}$ and AUC were analogous between WS-157 and gefitinib after i.v. treatment, but mean residence time (MRT) of WS-157 was shorter than that of gefitinib. In this study, the $C_{\text{max}}$ of WS-157 was slightly higher and earlier than that of gefitinib, which was consistent with the

### Table 3 Permeability coefficients of the compounds.

| Test article | Direction | $P_{\text{app}}$ (10^{-6} cm/s) Mean ± RSD | $P_{\text{app}}$ (B–A)/$P_{\text{app}}$ (A–B) Mean ± RSD |
|--------------|-----------|------------------------------------------|--------------------------------------------------|
| Metoprolol   | A–B       | 40.04 ± 0.05                             | 0.77                                             |
|              | B–A       | 30.92 ± 0.03                             |                                                  |
| Atenolol     | A–B       | 0.66 ± 0.01                              | 2.31                                             |
|              | B–A       | 1.53 ± 0.01                              |                                                  |
| Erythromycin | A–B       | 0.09 ± 0.03                              | 240.3                                            |
|              | B–A       | 22.18 ± 0.02                             |                                                  |
| WS-157       | A–B       | 9.13 ± 0.02                              | 2.51                                             |
|              | B–A       | 22.91 ± 0.02                             |                                                  |
| Gefitinib    | A–B       | 8.37 ± 0.01                              | 1.85                                             |
|              | B–A       | 15.52 ± 0.02                             |                                                  |
better intestinal absorption of WS-157 in Caco-2 cell permeability assay. The oral bioavailability of WS-157 and gefitinib were 86.2 ± 19.7% and 66.5 ± 22.8%, respectively, which had no statistically significant difference.

3. Discussion

EGFR has emerged as a key target for anticancer therapeutics. Tyrosine kinase inhibitors, such as erlotinib, gefitinib, icotinib, and lapatinib, are recommended as first-line treatments for NSCLC patients. By screening our diverse compound collection against EGFR, we identified some promising hit compounds, of which WS-157 was fully characterized as a potent and selective EGFR-TKI with excellent potency in vivo and in vitro. Compared with gefitinib, WS-157 showed better inhibitory activities against EGFR, EGFR(1746-1750) and EGFR(L858R), but was less potent against EGFR(T790M). This was further confirmed by the effects of WS-157 on the biochemical pathways in cells. EGFR is stimulated by a number of autocrine growth factors, including EGF and PI3K/AKT is one of major downstream effectors following EGFR activation. WS-157 dose-dependently inhibited EGF-induced p-EGFR and p-AKT in EGFR wt cells, but was ineffective in EGFR(T790M/L858R) mutation NCI-H1975 cells. We also explored the inhibitory effect of WS-157 on EGFR, showing that the inhibition of WS-157 on EGFR activation was gradually recovered after the removal of WS-157, and the activity was completely recovered after 12 h. Thus, our findings suggest that WS-157 is a selective and reversible EGFR TKI.

WS-157 displayed excellent antiproliferative activities against a panel of human cancer cell lines and the IC50 values of this compound were similar to those of gefitinib. Particularly, WS-157 significantly inhibited the proliferation of PC-9 and A431 cells (IC50 < 1.0 μmol/L). In addition, WS-157 had excellent inhibitory activities on cell proliferation and migration. Furthermore, the in vivo antitumor activity of WS-157 was also carried out in xenograft models bearing A431 cells, a recognized model for the testing of biological effects on EGFR signaling. WS-157 inhibited the growth of A431 xenografts in a dose-dependent manner, and complete inhibition was observed in animals receiving a daily i.g. doses of 40 mg/kg WS-157. Interestingly, we observed that WS-157 showed slightly better anti-tumor activity compared to gefitinib at the same dose (10 mg/kg/day). However, weight loss observed during the administration of WS-157 was not influenced. Therefore, our data demonstrate that WS-157 exhibits promising anti-tumor activity and the slightly greater weight losses observed in the present study might reflect the greater duration of dosing and more chemo-sensitive nature of the nude mice used.

In our study, the Papp (A–B) value of WS-157 was higher than that of gefitinib, which indicated that the intestinal absorption of WS-157 was better than that of gefitinib. In addition, the Papp (B–A)/Papp (A–B) values of WS-157 and gefitinib were both higher than 1.5, suggesting that WS-157 and gefitinib might be the substrates of the efflux transporters. Several receptor kinase inhibitors were found to interact with the ATP binding site of major ABC transporters, including gefitinib, vandetanib, peltinib and

![Figure 6](image6.png)

**Figure 6** Metabolic stability. Metabolic stability of the WS-157 (1 μmol/L) and gefitinib (1 μmol/L) in liver microsomes of human, rat, mouse, dog and monkey. The results are expressed as the % of remaining substrate concentration ratio of the compounds after different time of incubation.

| Species | WS-157 t1/2 (min) | Gefitinib t1/2 (min) |
|---------|------------------|---------------------|
| Human   | 23.64            | 22.51               |
| Rat     | 11.78            | 21.16               |
| Mouse   | 7.01             | 8.38                |
| Dog     | 33.24            | 34.48               |
| Monkey  | 5.01             | 4.66                |

**Table 4** The t1/2 of WS-157 and gefitinib in liver microsomes of different species.

![Figure 7](image7.png)

**Figure 7** Mean plasma concentration–time profile. (A) WS-157 and gefitinib after i.v. administration of 10 mg/kg, respectively (mean ± SD, n = 3). (B) WS-157 and gefitinib after p.o. administration of 40 mg/kg, respectively (mean ± SD, n = 3).
neratinib\textsuperscript{31–35}. WS-157 was speculated to modulate the function of ABC transporters.

It was indicated that the metabolic stability of WS-157 and gefitinib was similar in liver microsome of human, mouse, dog and monkey in our study. However, WS-157 was less stable than gefitinib in rat liver microsome. To further investigate the pharmacokinetics of gefitinib and WS-157 \textit{in vivo}, we determined the concentration of WS-157 and gefitinib in plasma of rats after treatment. For intravenous administration, the pharmacokinetic features of WS-157 and gefitinib were similar. However, for oral administration, WS-157 was well absorbed and showed good oral bioavailability (\( F = 86.2 \pm 19\% \)), and there was a tendency of increase of the values of \( C_{\text{max}} \), AUC, and \( F \) in WS-157 group compared with gefitinib. The higher \( C_{\text{max}} \) and earlier \( T_{\text{max}} \) in WS-157 treatment group suggested better absorption than gefitinib, which is in accordance with the Caco-2 cell permeability assay. These data indicated that WS-157 had a favorable pharmacokinetic property and deserved further investigation.

4. Conclusions

In summary, WS-157 was identified from our in-house diverse compound library, which was validated to be a highly potent and selective EGFR-TKI. WS-157 showed excellent inhibitory activity against EGFR (IC\(_{50}\) = 0.81 nmol/L), EGFR\textsuperscript{[L858R]} (IC\(_{50}\) = 1.2 nmol/L) and EGFR\textsuperscript{[L858R]} (IC\(_{50}\) = 1.1 nmol/L) and possessed high selectivity over other nine kinases. WS-157 displayed excellent antiproliferative activities against several human cancer cell lines, and exhibited comparable capability of reducing colony formation and wound healing with gefitinib. We found that WS-157 upon oral administration showed better anti-tumor activity in A431 bearing xenograft mouse models compared to gefitinib. In addition, WS-157 showed better intestinal absorption than gefitinib and had favorable pharmacokinetic properties. These studies indicated that WS-157 had strong antitumor activity \textit{in vitro} and \textit{in vivo}, and could be used for the development of anti-lung cancer agent targeting EGFR.

5. Experimental

5.1. Chemicals and antibodies

Gefitinib were purchased from Selleck (Shanghai, China). The HPLC purity of WS-157 was >98%. All of the above compounds were dissolved in DMSO at 10 mmol/L (\textit{used in vitro}) as a stock solution and stored at \(-20^\circ\text{C}\). RIPA were purchased from Beyotime (Nantong, China). EGFR (Cell Signaling Technology, 4223S, Boston, USA), p-EGFR (Cell Signaling Technology, 4277S), AKT (Cell Signaling Technology, 4223S), p-AKT (Cell Signaling Technology, 9728S) and anti-GAPDH antibody were purchased from Cell Signaling Technology. HRP-conjugated donkey anti-goat IgG was obtained from The Golden Bridge.

5.2. The synthetic routes for the synthesis of compound WS-157

Compound 1 (1g, 3.13 mmol) was dissolved in POCl\(_3\) (10 mL), which was then stirred at 90 \(^\circ\text{C}\) for 5–6 h. The mixture was subjected to evaporation of the solvent to generate the unstable compound 2. Compound 2 (200 mg, 592.06 \text{\(\mu\)}mol) then reacted with 3-ethylnylanilne (69.36 mg, 592.06 \text{\(\mu\)}mol) in the presence of triethylamine (179.74 mg, 1.78 mmol) in ethanol at room temperature for 3 h, generating the target compound WS-157. White solid, yield: 84%. \(^1\text{H}\) NMR (400 MHz, DMSO-\(d_6\), 811.18 (s, 1H), 8.86 (s, 1H), 8.64 (s, 1H), 7.93 (s, 1H), 7.85 (d, \(J = 8.1\) Hz, 1H), 7.49 (t, \(J = 8.1\) Hz, 1H), 7.43 (s, 1H), 7.40 (d, \(J = 7.7\) Hz, 1H), 4.29 (t, \(J = 6.0\) Hz, 2H), 2.35 (m, 2H). \(^13\text{C}\) NMR (100 MHz, DMSO-\(d_6\), 135.77, 129.21, 129.01, 127.59, 125.34, 121.90, 107.33, 105.62, 99.88, 82.97, 81.25, 67.31, 63.18, 56.50, 53.50, 51.11, 22.74. HR-MS (ESI): \text{m/z} \text{Calcd. for C}_{24}\text{H}_{27}\text{N}_{4}\text{O}_{3} [\text{M}+\text{H}]^+ \text{ 419.2083}; \text{Found 419.2062.}

5.3. Kinase inhibition assay

The kinases and control compounds used in the experiments are shown in Table 1. The kinase assays were performed in 384-well plates. In briefly, 10 mmol/L tested compounds was diluted 4 times with DMSO to 10 concentrations. Then, 2.5 nmol/L of the kinase peptide\textsuperscript{22} (5-FAM-EEPLYWSFPAKKKCSV) was added to the reaction buffer (50 mmol/L HEPES, 0.01% Brij-35, 50 mmol/L MgCl\(_2\)) to initiate the reaction. After incubate at room temperature for 30 min, the reactions were stopped by adding 25 \(\mu\)L stop buffer (100 mmol/L HEPES, 0.015% Brij-35, 50 mmol/L EDTA). The reaction mixture was analyzed with Caliper EZ Reader (Waltham, MA, USA), and the conversion values were converted to inhibition.

5.4. Cell lines and culture

Human NSCLC NCI–H1975 cell lines were provided by the group of Ding Jian, the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (China). Human NSCLC A549,
Colony formation assay was performed as described previously.24. All cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min. Crystal violet staining was used to stain the cells for 30 min at 37 °C. After that, the staining was washed away with PBS until the colonies were clear enough.

5.8. Wound healing assay

Cells were seeded in 24-well plates to form confluent monolayers. The cell surface was scratched using a 10 μL pipet tip, and then cultured in fresh medium containing 1% FBS and different concentrations of agents. Bright field images of the randomly selected views along the scraped line were taken at 24 h post-scratch.

5.9. Xenograft study

Athymic BALB/c 4–6 weeks old nude mice were purchased from Hunan slice Jingda experimental animal company and were housed in a specific pathogen-free room with a 12 h light/dark cycle at 25 ± 1 °C. All animal experiments were performed according to the institutional ethical guidelines established by the ethics committee of Zhengzhou University (China). For xenografts, 1.5 × 10⁷ cells of A431 cells were injected subcutaneously at one site of anaesthetized mice. Once the tumors reached 100–200 mm³, mice were randomly divided into four groups. The control groups received physiology saline (0.1 mL/10 g) and the treatment groups received compound WS-157 (10, 40 mg/kg) or gefitinib (10 mg/kg) by intragastric administration (i.g.) per day for a period of 27 days. The tumor size was measured and recorded at 2-day intervals after administration. After day 27, animals were sacrificed by cervical dislocation and the tumors were isolated and weighed, and then the tissue protein was extracted by RIPA buffer (Solarbio). Tumor size was determined by caliper measurements, and the body weight was measured at 2-day intervals to monitor drug toxicity.

5.10. Permeability assay

For transport experiments, Caco-2 cells (passage 35) were seeded at approximately 6.5 × 10⁴ cells/cm² on polycarbonate 24-well Transwells® (Corning Costar Corporation, Cambridge, MA, USA) before and after the drug transport experiments. Transepithelial electrical resistance (TEER) of the monolayers was measured using the Millicell-ERS system (Millipore Corp., Bedford, MA, USA) before and after the drug transport experiments. The TEER values of Caco-2 monolayers were all >400 Ω/cm². We used metoprolol, atenolol and erythromycin as a high permeable control, a low permeable control and an efflux substrate, respectively.

The assay was conducted in HBSS solution. AP–BL transport assay: 0.8 mL of test compounds (10 μmol/L) was added to the AP side and 0.4 mL of blank HBSS was added to the BL side. BL–AP transport assay: 0.4 mL of test compounds (10 μmol/L) was added to BL side and 0.8 mL of blank HBSS was added to the AP side. The plates were shaken at 37 °C for 90 min. The concentrations of the compounds in donor and receptor sides were determined by LC–MS/MS. The apparent permeability coefficient ($P_{app}$) of the compounds was calculated according to the following Eq. (1):

$$P_{app} = \frac{(VA/(A \times T)) \times ([Drug]_{accepter}/[Drug]_{initial, donor})}{1}$$

where $VA$ is the volume in the acceptor well, $A$ is the surface area of the membrane (0.7 cm²) and $T$ is the total transport time in seconds.

5.11. Metabolic stability

Metabolic stability was evaluated in liver microsomes of five different species, including human, rat, mouse, dog and monkey.
The in vitro metabolic reactions were incubated in PBS buffer (pH = 7.4). 30 μL of 1.5 μmol/L WS-157 or gefitinib and 0.75 mg/mL microsomes solution was added to a new Eppendorf tube. Then, 15 μL of NADPH stock solution (6 mmol/L) was added to the Eppendorf tube to start the reaction maintained at 37 °C. After 0, 5, 15, 30 and 45 min of incubation, the reaction was ended by adding 135 μL of acetoniitrile containing internal standard (IS). The quenched samples were vortexed for 10 min and centrifuged at 4325 × g for 10 min. Then, the supernatant was injected for qualitative LC–MS/MS analysis.

5.12. Pharmacokinetic study

Healthy adult Sprague–Dawley rats (200 ± 20 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shangsha, China) and were cared under standard conditions (12/12 h light/dark cycle, humidity of 55 ± 5%, 22 ± 2 °C) in accordance with the Guidelines for Animal Experimentation of Zhengzhou University (China) and the protocol was approved by the Animal Ethics Committee of the Institution, China.

To assess the pharmacokinetic studies and compare the bioavailability of WS-157 and gefitinib in rat, rats (n = 6 for each compound) were intragastrically or intravenously administered. Approximately 150 μL of whole blood were collected in K2EDTA tube via tail vein under anesthesia and cardiac puncture for terminal bleeding at 0.25, 0.5, 1, 3, 6 and 24 h after intragastric administration of WS-157 or gefitinib (40 mg/kg). Blood samples were put on ice and centrifuged at 2000 × g for 5 min to obtain plasma sample within 15 min. Blood samples were collected at 0.083, 0.25, 0.5, 1, 3, 6 and 24 h after intravenous administration of WS-157 or gefitinib (10 mg/kg) and processed similarly to the per os (p.o.) group. The supernatant plasma was stored at −70 °C until analysis. The rats were humanely euthanized by carbon dioxide 24 h after experiment without pain.

An aliquot of 20 μL plasma sample was added with 400 μL acetonitrile containing 100 ng/mL diclofenac (internal standard, IS). The mixture was vortexed for 10 min and centrifuged at 4325 × g for 10 min. An aliquot of 5 μL supernatant was injected for qualitative LC–MS/MS analysis.

LC–MS/MS analysis of the compounds was conducted by UPLC–MS/MS-22 (Triple QuadrTM 6500) by a Waters BEH C18 (50 mm × 2.1 mm, 1.7 μmol/L). The temperature of column oven was maintained at 60 °C during the separation. The mobile phase was as follows: (A) aqueous phase: 0.025% formic acid and 1 mmol/L NH₄Ac in water; (B) organic phase: 5 mmol/L NH₄Ac in methanol. The gradient elution was at a flow rate of 0.6 mL/min. Compounds were eluted in the following gradient conditions: the initial proportion was 2% B and hold for 0.4 min, and increased to 65% B in 0.3 min, further to 90% in 0.5 min and maintained for 0.6 min, followed by decreasing to 2% B within 0.01 min; the whole gradient time was 2.5 min. The mass spectrometric detection was performed with an electrospray ionization (ESI) source in positive mode. Quantification was acquired by multiple reaction monitoring (MRM) mode of m/z 419.1 → 128.1 at 1.36 min for WS-157, m/z 447.3 → 128.3 at 1.48 min for gefitinib and m/z 296.2 → 214.2 at 1.48 min for IS.

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Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2019.06.010.

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