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# Discovery of JND3229 as a New EGFR$^{C797S}$ Mutant Inhibitor with In Vivo Monodrug Efficacy

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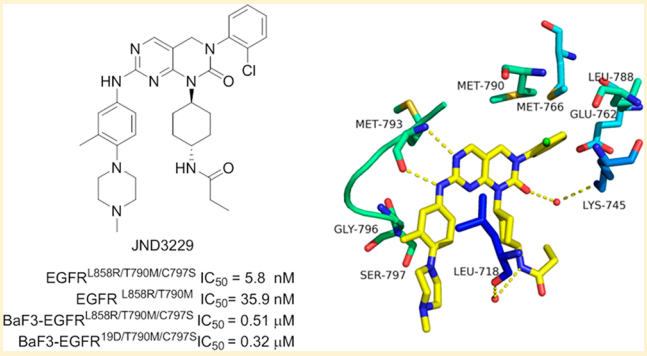
*Supporting Information*

**ABSTRACT:** EGFR$^{C797S}$ mutation inducing resistance against third generation EGFR inhibitor drugs is an emerging "unmet clinical need" for nonsmall cell lung cancer patients. The pyrimidopyrimidinone derivative JND3229 was identified as a new highly potent EGFR$^{C797S}$ inhibitor with single digit nM potency. It also exhibited good in vitro and in vivo monodrug anticancer efficacy in a xenograft mouse model of BaF3/EGFR$^{19D/T790M/C797S}$ cells. A high-resolution X-ray crystallographic structure was also determined to elucidate the interactions between JND3229 and EGFR$^{T790M/C797S}$. Our study provides an important structural and chemical basis for future development of new generation EGFR$^{C797S}$ inhibitors as anticancer drugs.




**KEYWORDS:** *EGFR$^{C797S}$, Clinical resistance, Fourth-generation inhibitors, Monodrug efficacy*

The epidermal growth factor receptor (EGFR, ErbB1, HER1) is one of the most validated molecular targets for anticancer drug discovery.[1] Three generations of EGFR inhibitors, e.g., gefitinib,[2] erlotinib,[3] afatinib,[4] and osimertinib (AZD9291),[5] have been approved by US FDA and achieved significantly clinical benefit in nonsmall cell lung cancer (NSCLC) patients. Particularly, the wild-type sparing third generation EGFR threonine$^{790}$ to methionine$^{790}$ mutant (T790M) inhibitor osimertinib demonstrates ∼75% overall response rate (ORR) in EGFR$^{T790M}$ mutation-positive NSCLC patients and was approved as the first-line treatment for metastatic NSCLC patients in 2018,[6,7] representing one of the most advanced progresses in human cancer therapy.

However, a tertiary Cys797 to Ser797 (C797S) point mutation, disturbing covalent bond formation with the irreversible third generation EGFR inhibitors, becomes a leading mechanism of clinically acquired resistance in ∼40% of patients treated with osimertinib.[8−10] An allosteric inhibitor, EAI045 (**1**, Figure 1),[11] has been discovered to exhibit low nM $IC_{50}$ values against EGFR$^{T790M}$ and EGFR$^{C797S}$ mutants. However, the combination with an EGFR antibody cetuximab was required for EAI045 to demonstrate in vivo therapeutic efficacy because of asymmetric dimerization of the receptor.[11] (2-Hydroxy)phenyl-4-substituted quinazoline derivatives (**2a** and **2b**),[12] trisubstituted imidazole (**3**),[13−15] and a pan kinase inhibitor Gö6976 (**4**)[16] were also discovered to display strong inhibition against EGFR$^{C797S}$ kinase, but neither cell-based activity nor in vivo efficacy of these molecules was disclosed. Encouragingly, brigatinib (**5**),[17] an FDA-approved ALK kinase inhibitor, was recently reported to strongly suppress the kinase activity of EGFR$^{C797S}$ and inhibit the proliferation of NSCLC cancer cells harboring the EGFR$^{C797S}$ mutation with $IC_{50}$

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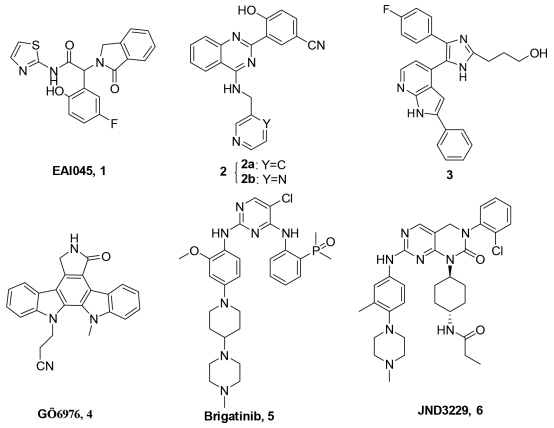
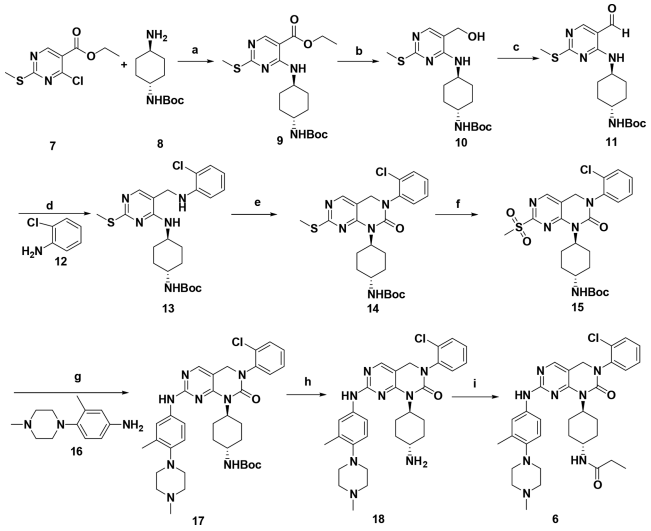



**Figure 1.** Chemical structures of the representative EGFR$^{C797S}$ inhibitors **1**–**6**.

**Scheme 1. Synthesis Protocol for JND3229 (6)**[a]




[a]Reagents and conditions: (a) $K_2CO_3$, DMF, 80 °C, 85%; (b) $LiAlH_4$, THF, −40 to 0 °C, 50%; (c) $MnO_2$, DCM, rt, 92%; (d) AcOH, $NaBH_4$, PhMe, 110 °C to rt, 80%; (e) triphosgene, $Et_3N$, DCM, 0 °C to rt, 94%; (f) m-CPBA, DCM, rt, 88%; (g) $F_3CCOOH$, 2-BuOH, 110 °C; (h) $F_3CCOOH$, DCM, rt, 50% (for two steps); (i) propanoic acid, HATU, DIPEA, DCM, rt, 90%.

values in nM ranges. Moreover, brigatinib also exhibited significant antitumor efficacy in a xenograft mouse model of PC9 and MGH121-res2 NSCLC cancer cells with EGFR$^{C797S}$ mutations by combinations with EGFR antibody cetuximab or pantitumumab.[17] Nevertheless, it is still highly valuable to discover novel EGFR$^{C797S}$ inhibitors with monodrug efficacy for overcoming the required resistance against third generation EGFR inhibitors.[18−20] Herein, we report the identification of JND3229 (**6**, Figure 1) as a new reversible EGFR$^{C797S}$ mutant inhibitor demonstrating both in vitro and in vivo monodrug efficacy against the proliferation of EGFR$^{C797S}$ mutated cancer cells.

For new EGFR$^{C797S}$ inhibitors to be discovered, a random screening was initially conducted by utilizing our in-house kinase inhibitor library containing ∼3000 compounds.[21−26] The effort helped us to identify JND3229 (Figure 1), a pyrimidopyrimidinone derivative, as a new EGFR$^{C797S}$ inhibitor. The molecule was readily synthesized by using a

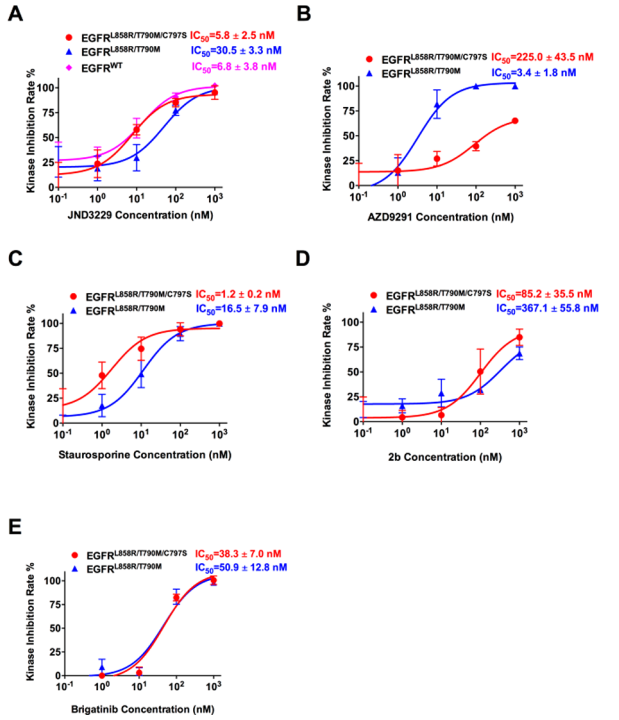



**Figure 2.** JND3229 potently inhibits the kinase activities of EGFR$^{L858R/T790M}$ and EGFR$^{L858R/T790M/C797S}$. The kinase inhibitory efficacy of JND3229 (A), AZD9291 (B), staurosporine (C), inhibitor **2b** (D), and brigatinib (E) against EGFR$^{L858R/T790M}$ (blue mark), EGFR$^{L858R/T790M/C797S}$ (red mark), and EGFR$^{WT}$ (purple mark) were tested by ELISA assay, and the IC$_{50}$ values were calculated based on three independent repeats.

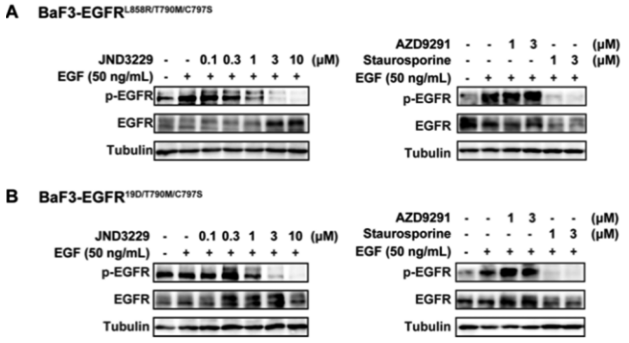



**Figure 3.** JND3229 potently inhibits the phosphorylation of EGFR$^{L858R/T790M/C797S}$ and EGFR$^{19D/T790M/C797S}$ in engineering BaF3 Cells. BaF3 cells that overexpressed EGFR$^{L858R/T790M/C797S}$ (A) or EGFR$^{19D/T790M/C797S}$ (B) were treated with the indicated concentrations of JND3229, AZD9291, and staurosporine for 2 h and stimulated by EGF for 15 min. Cell lysates were harvested for Western blot analysis for EGFR phosphorylation.

protocol outlined in Scheme 1. Briefly, a commercially available ethyl 4-chloro-2-(methylthio)-pyrimidine-5-carboxylate (**7**) was reacted with 1-Boc-1,4-cyclohexanediamine (**8**) followed by reduction, oxidation, and Borch reductive amination and cyclization to yield key pyrimidopyrimidinone **14**. Oxidation of **14** with 3-chloroperbenzoic acid (m-CPBA) yielded the sulfone **15**, which was consequently subjected to nucleophilic deprotection and acylation reaction to give the title compound JND3229.

JND3229 exhibited strong inhibition against the kinase activity of EGFR$^{L858R/T790M/C797S}$ with an IC$_{50}$ value of 5.8 nM



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**Table 1. Antiproliferative Activities of JND3229 against Cells with Different Mutant EGFR**

| cells | EGFR status | antiproliferation $IC_{50}$ ($\mu$M) | | |
|---|---|---|---|---|
| | | JND3229 | AZD9291 | brigatinib |
| BaF3 | L858R/T790M/C797S | 0.51 ± 0.08 | 5.15 ± 1.57 | 0.42 ± 0.08 |
| | 19D/T790M/C797S | 0.32 ± 0.11 | 4.61 ± 2.34 | 0.26 ± 0.02 |
| NCI-H1975 | L858R/T790M | 0.31 ± 0.01 | 0.13 ± 0.04 | 1.09 ± 0.24 |
| A431 | WT | 0.27 ± 0.18 | 1.24 ± 0.37 | |

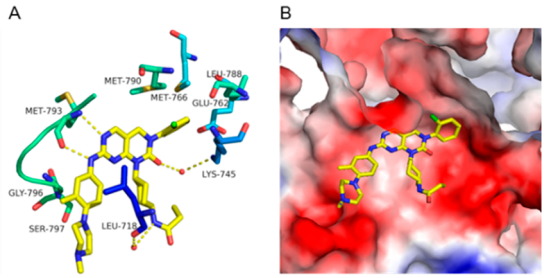


**Figure 4.** X-ray crystal structure of JND3229 with $EGFR^{L858R/T790M/C797S}$ (PDB ID: 5ZTO). (A) EGFR kinase shown in green and blue stick and ribbon representation. JND3229 is shown in yellow stick structure. Hydrogen bonds are indicated by yellow hatched lines to key amino acids. Water is represented as red dots. (B) X-ray crystal structure with the interaction surface.

under the conditions of an enzyme-linked immunosorbent assay (ELISA) (Figure 2A). Additionally, the compound also potently suppressed $EGFR^{L858R/T790M}$ and $EGFR^{WT}$ with $IC_{50}$ values of 30.5 and 6.8 nM, respectively (Figure 2A). Similar to the previous observation, osimertinib exhibited ~70-fold less potency against the $EGFR^{C797S}$ mutant compared with its strong inhibition against $EGFR^{L858R/T790M}$ (Figure 2B). The pan-kinase inhibitor staurosporine (Figure 2C), $EGFR^{C797S}$ inhibitor **2b** (Figure 2D), and brigatinib (Figure 2E), used as positive drugs, also displayed strong inhibition against $EGFR^{L858R/T790M/C797S}$ kinase.

The strong kinase inhibition of JND3229 was further validated by investigating its potential suppression on activity of EGFR signals in BaF3 cells stably transfected with $EGFR^{L858R/T790M/C797S}$ and $EGFR^{19D/T790M/C797S}$ (Figure 3). It was shown that JND3229 potently inhibited the phosphorylation of $EGFR^{L858R/T790M/C797S}$ and $EGFR^{19D/T790M/C797S}$ in a dose-dependent manner. Consistent with their kinase activity, staurosporine also demonstrated strong inhibition of $EGFR^{C797S}$ activation in BaF3 cells, but the effect of osimertinib was minor.

The antiproliferative activities of JND3229 were also investigated against a panel of cells with different EGFR status. It was shown that JND3229 potently inhibited the proliferation of BaF3 cells harboring the $EGFR^{L858R/T790M/C797S}$ and $EGFR^{19D/T790M/C797S}$ mutations with $IC_{50}$ values of 0.51 and 0.32 $\mu$M, respectively (Table 1), which are comparable to those of brigatinib. It also potently suppressed the growth of NCI-H1975 NSCLC cells with $EGFR^{T790M}$ mutation with an $IC_{50}$ value of 0.31 $\mu$M. Although osimertinib exhibited strong antiproliferative effect on NCI-H1975 cells with an $IC_{50}$ value of 0.13 $\mu$M, its inhibition against BaF3 cells harboring the $EGFR^{C797S}$ mutation was significantly less potent ($IC_{50}$ > 4 $\mu$M). Consistent with its nonselective inhibition against $EGFR^{WT}$, JND3229 also obviously suppressed the proliferation of A431 cancer cells overexpressing $EGFR^{WT}$ with an $IC_{50}$ value of 0.27 $\mu$M.

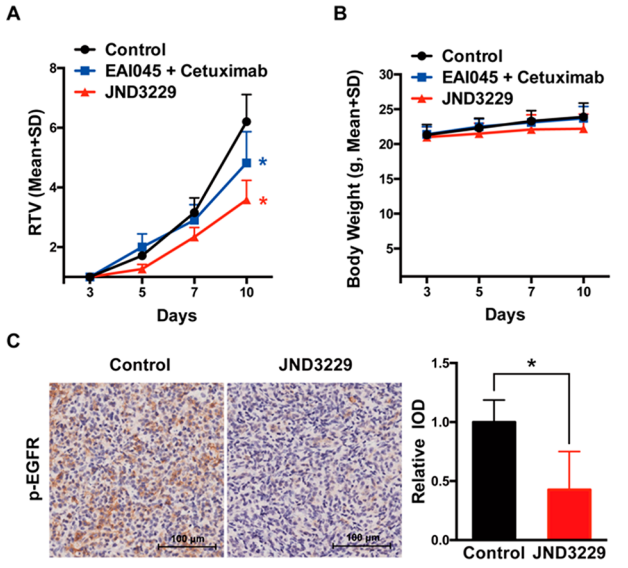


**Figure 5.** JND3229 shows in vivo antitumor efficacy in the $EGFR^{19D/T790M/C797S}$ mouse xenograft model. Mice bearing BaF3-$EGFR^{19D/T790M/C797S}$ tumor xenografts were treated with JND3229 (10 mg/kg in 0.5% HPMC, ip, Bid) or treated with EAI045 (60 mg/kg in 10% NMP/90% PEG300, po, qd) combined with cetuximab (1 mg/kg in 0.9% w/v NaCl/water, ip, qod). Relative tumor volume (RTV) (A) and body weight (B) of $EGFR^{19D/T790M/C797S}$ mouse xenograft model were measured ($n$ = 6) every 2−3 days. The tumor growth inhibition was measured at the final day of the treatment for the drug-treated group versus the control (*$P$ < 0.05 vs control, Student's $t$ test). (C) JND3229 suppresses the expression of p-EGFR in the $EGFR^{19D/T790M/C797S}$ xenograft model. Tumor tissue sections were stained by hematoxylin and eosin (H&E) (blue) and p-EGFR antibody (brown), and the relative integrated optical density (IOD) of p-EGFR labeling is presented by quantitative analysis (*$P$ < 0.05 vs control, Student's $t$ test). IOD, integrated optical density; all values represent mean ± SD or mean ± SEM.

A 2.65 Å resolution X-ray crystallographic structure of JND3229-$EGFR^{T790M/C797S}$ complex was also determined to elucidate detailed interactions between the inhibitor and the kinase (Figure 4 and Table S1). It was shown that JND3229 was accommodated in the ATP binding site of the C797S-mutated EGFR with a reversible "U-shaped" configuration. The pyrido[2,3-$d$]pyrimidine-7-one core formed a bidentate hydrogen bond interaction with the "hinge" residue Met793 of the protein. The 2-chlorophenyl group was directed toward the hydrophobic back pocket composed by Lys745, Glu762, Leu788, Met766, and Met790, and the carbonyl of pyrido-[2,3-$d$]pyrimidine-7-one formed a hydrogen bond with the nitrogen of Lys745 mediated by a water molecule. The propionamide group was located in a solvent-exposing region, and the NH moiety formed a hydrogen bond with Leu718 mediated by another water molecule. The left-hand methyl-substituted phenyl group interacted with Gly796 by van der Waals, and the hydrophilic methyl piperazine moiety was extended directly to the solvent.

The in vivo anticancer efficacy of JND3229 was also examined using a xenograft mouse model. BALB/c mice bearing established BaF3-$EGFR^{19D/T790M/C797S}$ mouse xenograft tumors were treated with JND3229 twice daily at a dose



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of 10 mg/kg by intraperitoneal injection or by vehicle control for 10 days. EAI045 (60 mg/kg, once daily by oral gavage) combination with cetuximab (1 mg/kg, once every other day by intraperitoneal injection) were used as positive control. The data showed that administration of JND3229 caused an obvious suppression of tumor growth with a tumor growth inhibition (TGI) value of 42.2% (Figure 5A), which was more potent than that of EAI045/cetuximab combination (TGI = 22.3%, Figure 5B). In addition, JND3229 was well tolerated, and there is no obvious body weight loss or other obvious toxic sign in the treated animals. Further immunohistochemistry analysis demonstrated that JND3229 treatment significantly decreased the level of phosphorylated EGFR (p-EGFR) in the tumor tissues, confirming in vivo target inhibition of the compound (Figure 5C).

In summary, JND3229 was identified as a new $EGFR^{C797S}$ inhibitor. The compound potently inhibited $EGFR^{C797S}$ mutated kinase with an $IC_{50}$ value of 5.8 nM and strongly suppressed the proliferation of BaF3 cells harboring the $EGFR^{L858R/T790M/C797S}$ and $EGFR^{19D/T790M/C797S}$ mutations with $IC_{50}$ values of 0.51 and 0.32 $\mu$M, respectively. Moreover, the compound also demonstrated in vivo monodrug anticancer efficacy in a xenograft mouse model of BaF3 cells with $EGFR^{19D/T790M/C797S}$ mutation. A high-resolution X-ray crystallographic structure was also determined to elucidate the interactions between JND3229 and $EGFR^{T790M/C797S}$ protein. Although the relatively low target selectivity (Table S2) may raise some concern about the potential off-target toxicity of this molecule, our study might provide a useful structural and chemical basis for further development of the fourth generation $EGFR^{C797S}$ inhibitors to overcome the acquired resistance against osimertinib.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00373.

Chemistry, biological assay, and X-ray crystallography data (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

EGFR, epidermal growth factor receptor; NSCLC, nonsmall cell lung cancer; ORR, overall response rate; C797S, Cys797 to Ser797; m-CPBA, 3-chloroperbenzoic acid; ELISA, enzyme-linked immunosorbent assay; TGI, tumor growth inhibition; RTV, relative tumor volume; IOD, integrated optical density.

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