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Brief Article

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Structure-Based Design of 5-Methylpyrimidopyridone Derivatives as New Wild-Type Sparing Inhibitors of the Epidermal Growth Factor Receptor Triple Mutant ($EGFR^{L858R/T790M/C797S}$)

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KEYWORDS: Epidermal Growth Factor Receptor (EGFR), C797S mutation, resistance, Non-small cell lung cancer (NSCLC)

ABSTRACT: Tertiary $EGFR^{C797S}$ mutation induced resistance against osimertinib (**1**) is an emerging "unmet clinical need" for non-small cell lung cancer (NSCLC) patients. A series of 5-methylpyrimidopyridone derivatives were designed and synthesized as new selective $EGFR^{L858R/T790M/C797S}$ inhibitors. A representative compound, **8r-B**, exhibited an IC_{50} value of 27.5 nM against the $EGFR^{L858R/T790M/C797S}$ mutant, while being a significantly less potent for $EGFR^{WT}$ (IC50 > 1.0 μM). Co-crystallographic structure determination and computational investigation were conducted to elucidate its target selectivity.

INTRODUCTION

The epidermal growth factor receptor (EGFR) has been well validated as a therapeutic target for anti-cancer drug discovery. [1] Three generations of EGFR inhibitors, e.g., reversible gefitinib [2] and erlotinib, [3] irreversible afatinib [4] and dacomitinib, [5, 6] and the wild-type sparing osimertinib (**1**, AZD9291), [7] have been approved by the US FDA and have achieved significant clinical benefit for NSCLC patients with EGFR activating mutations (i.e., L858R and delE746-A750) and/or secondary threonine^{790} to methionine^{790} (T790M) mutation, respectively. In particular, drug **1** has become the first globally accessible 3rd-generation EGFR inhibitor, having been recently approved as a first-line treatment for metastatic NSCLC patients, [8, 9] representing one of the most advanced developments in NSCLC therapy.

Mode of Action (MOA) investigation revealed that the acrylamide moiety in drug **1** could undergo a Michael addition reaction with the side chain of the Cys^{797} residue under physiological conditions to increase the target residence time and restore inhibitory activity against the $EGFR^{T790M}$ mutants. [10, 11, 12] However, a tertiary Cys^{797} to Ser^{797} (C797S) point mutation becomes a leading mechanism of clinically acquired resistance in ~40% of drug **1** treated NSCLC patients, by disturbing the corresponding covalent bond formation.[13] Accordingly, development of selective $EGFR^{C797S}$ inhibitors is considered as a new potential strategy to overcome acquired resistance against the 3rd-generation EGFR inhibitors. [14, 15, 16]

Recently, an allosteric inhibitor EAI045 (**2a**, Figure **1**) was discovered that selectively exhibits low nM IC_{50} potency against the $EGFR^{C797S}$ mutant. However, combination with an EGFR antibody, e.g. cetuximab, was required for the compound to demonstrate *in vivo* therapeutic efficacy, because of asymmetric dimerization of the EGFR receptor. [17] Brigatinib (**3**), and compounds **4** and **5** were also reported to display strong ATP-competitive inhibition against $EGFR^{C797S}$. Similar to the allosteric inhibitor **2a**, drug **3** also required combination

with cetuximab to display *in vivo* efficacy in xenograft mouse models with EGFR^{C797S} mutations. [18] Compound **5** represents one of the most selective ATP-competitive inhibitors of EGFR^{C797S} reported, however no *in vitro* or *in vivo* efficacy data has been disclosed to date. [19, 20] Most recently, JBJ-04-125-02 (**2b**) was disclosed as a new allosteric inhibitor of the EGFR$^{L858R/T790M/C797S}$ mutant with promising *in vitro* and *in vivo* efficacy. However, the molecule is inactive against the clinically important EGFR^{Del-19} variants (e.g., EGFR$^{delE746-A750}$, EGFR$^{delE746-A750/T790M}$ and EGFR$^{delE746-A750/T790M/C797S}$) [21]. We have also identified a pyrimidopyrimidinone derivative, JND3229 (**6**), as a new reversible EGFR^{C797S} mutant inhibitor with *in vitro* and i*n vivo* single-agent efficacy, but its relatively low EGFR$^{L858R/T790M/C797S}$ target specificity over the wild-type kinase may raise some concern about potential EGFR mechanism-related toxicity. [22]

In this paper, we describe the design and synthesis of a series of 5-methylpyrimidopyridone derivatives as new wild-type sparing EGFR$^{L858R/T790M/C797S}$ inhibitors. X-ray co-crystallographic structure determination and computational investigation were employed to rationalize the target selectivity of the molecules.

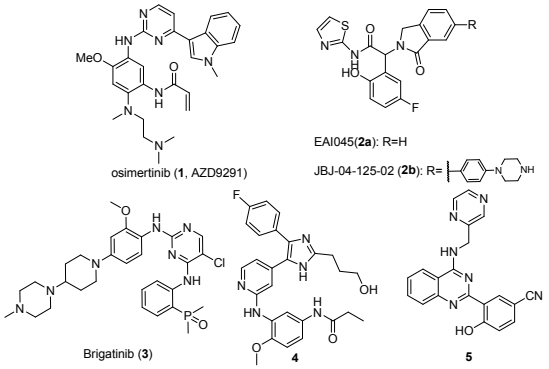


Figure 1. Chemical structures of osimertinib and the reported EGFR^{C797S} inhibitors.

MOLECULAR DESIGN

Structural feature analysis of the **6**-EGFR$^{T790M/C797S}$ complex (PDB: 5ZTO) revealed that the compound bound to the ATP-binding pocket with a similar "U-shaped" configuration to that of our previously reported EGFR^{T790M} selective inhibitor **7** (PDB: 5GMP, Figure **2**). [22, 23] Previous studies also demonstrated that the 5-methyl substituent of compound **7** was directed towards the "gatekeeper" residue Met^{790}, contributing greatly to EGFR^{T790M} selectivity over the wild-type protein. [23] A preliminary superimposition further suggested that the 5-methyl pyrido[2, 3-*d*]pyrimidin-7-one scaffold of the irreversible inhibitor **7** [22, 23] overlapped with the pyrimido[4, 5-*d*]pyrimidine-7-one core of **6** in the EGFR$^{T790M/C797S}$ complex. Based on these observations, a series of 5-methylpyrimidopyridone derivatives (**8**) were designed and synthesized as new potential EGFR$^{L858R/T790M/C797S}$ inhibitors, with the aim of improving the target selectivity of this class of derivatives over wild-type EGFR (Figure **2**).

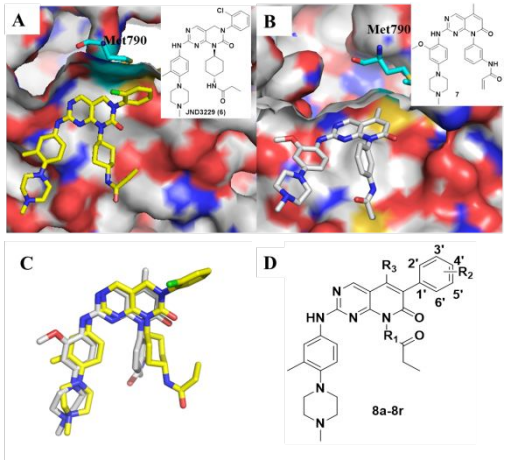
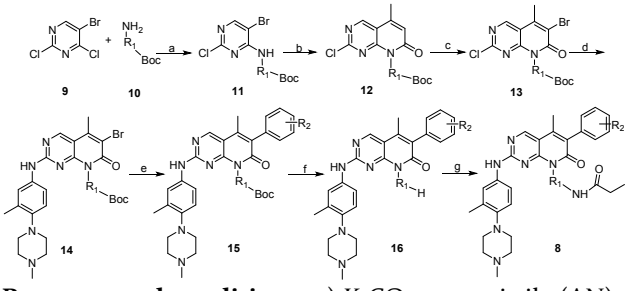


Figure 2. Structure-based design of new EGFR$^{L858R/T790M/C797S}$ inhibitors. A) Chemical structure of **6** and its complex with EGFR$^{T790M/C797S}$ (PDB: 5ZTO); B) Chemical structure of **7** and its complex with EGFR^{T790M} (PDB: 5GMP); C) Superimposition of **7** with inhibitor **6** in EGFR$^{T790M/C797S}$ (PDB: 5GMP); D) Designed new EGFR$^{L858R/T790M/C797S}$ inhibitors.

CHEMISTRY

The designed molecules **8a-8r** were readily synthesized by using a protocol outlined in Scheme 1. Briefly, commercially available 5-bromo-2, 4-dichloropyrimidine **9** was reacted with protected aliphatic amines **10** to obtain **11**. Compounds **11** were condensed with *trans*-but-2-enoic acid under Heck coupling conditions [24] in the presence of bis(benzonitrile)palladium(II) dichloride, before being treated with acetic anhydride to produce intermediates **12**. Selective bromination of intermediates **12** with liquid bromine in acetic acid gave **13**. Compounds **13** were coupled with 3-methyl-4-(4-methylpiperazin-1-yl)aniline to produce intermediates **14**. Intermediates **14** underwent Suzuki coupling with a range of phenylboronic acids to give **15** which were deprotected under acidic conditions to yield the substituted amines **16**. With **16** in hand, designed molecules **8a**, **8c-8r** were easily prepared by a standard acylation reaction. Alternatively, synthetic procedures for **8b** and **8l** are provided in the Supporting Information.

Scheme 1. Synthesis of compounds **8a**, **8c-8r**.



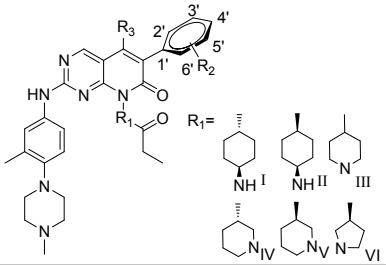
Reagents and conditions: a) K_2CO_3, acetonitrile (AN), 0 °C to r.t., 80-95%; b) (i) (*E*)-but-2-enoic acid, bis(benzonitrile) palladium(II) dichloride, DIPEA, tri(o-tolyl)phosphine, THF, 80 °C, argon, 16 hrs, (ii) acetic anhydride, 90 °C, 24 hrs, 40-50%; c) Br_2, AcONa, AcOH,

50 °C, 30 hrs, 60-75%; d) 3-methyl-4-(4-methylpiperazin-1-yl) aniline, TFA, 2-butanol, 110 °C, 18 hrs, 50-65%; e) $[(C_6H_5)_3P]_2PdCl_2$, Na_2CO_3, substituted phenylboronic acid, 1, 4-dioxane, H_2O, 110 °C, 45-70%; f) TFA, DCM, r.t., 85-90%; g) propionyl chloride, Et_3N, DCM, 0 °C to r.t., 80-90%.

RESULTS AND DISCUSSION

Compound **8a**, in which the original pyrimido[4, 5-*d*]pyrimidine-7-one core of **6** was simply replaced by a 5-methyl pyrido[2, 3-*d*]pyrimidin-7-one moiety, was first designed and synthesized. Its kinase inhibition activities against $EGFR^{WT}$ and $EGFR^{L858R/T790M/C797S}$ were determined by utilizing an enzyme-linked immunosorbent assay (ELISA). [21] Four previously reported $EGFR^{C797S}$ inhibitors, i.e. **2**, **3**, **5** and **6**, were included as the reference compounds. All of the positive control compounds exhibited comparable IC_{50} values to the reported data. [15-19] Disappointingly, compound **8a** displayed obviously decreased potency with IC_{50} values of 180.9 and 519.9 nM against $EGFR^{WT}$ and $EGFR^{L858R/T790M/C797S}$, respectively, which was 27-90 fold less potent than the original lead molecule **6** (Table **1**). Preliminary computational investigation suggested that the 5-methyl group failed to extend to the gatekeeper Met^{790} residue and form a favorable hydrophobic interaction, but caused a steric collision with this residue. Removal of the 5-methyl group (**8b**) indeed improved the inhibitory potency against $EGFR^{L858R/T790M/C797S}$ by a factor of 13.6-fold. However, eliminating the methyl group resulted in a greater potency improvement against $EGFR^{WT}$, becoming detrimental to the target selectivity of the molecule. The resulting compound **8b** exhibited IC_{50} values of 3.8 and 38.1 nM against $EGFR^{WT}$ and $EGFR^{L858R/T790M/C797S}$, respectively. These results implied that introduction of a 5-methyl group could be beneficial for the design of wild-type sparing $EGFR^{L858R/T790M/C797S}$ inhibitors, although it might have a negative impact on the $EGFR^{C797S}$ inhibitory potency.

Table 1. *In Vitro* Kinase Inhibition of **8a–8r** [a]



Cpds	R_1	R_2	R_3	Kinase Inhibition (IC_{50}, nM)		Cpds	R_1	R_2	R_3	Kinase Inhibition (IC_{50}, nM)	
				$EGFR^{WT}$	$EGFR^{TM}$					$EGFR^{WT}$	$EGFR^{TM}$
8a	I	2'or 6'-Cl (mixture)	Me	180.9±80.8	519.9±83.4	**8l-A**	V	2'-Br	Me	>1000	498.1±126.0
8b	I	2'or 6'-Cl	H	3.8±2.1	38.1±10.1	**8l-B**	V	6'-Br	Me	>1000	56.1±9.5
8c	II	2'or 6'-Cl (mixture)	Me	176.6±80.6	35.1±6.8	**8m-A**	V	2'-Me	Me	>1000	587.6±111.1
8c-A	II	2'-Cl	Me	>1000	518.8±78.1	**8m-B**	V	6'-Me	Me	>1000	227.9±9.5
8c-B	II	6'-Cl	Me	168.8±63.4	27.7±10.3	**8n**	V	2'or 6'-OMe (mixture)	Me	>1000	>1000
8d	III	2'or 6'-Cl (mixture)	Me	280.5±39.5	866.9±146.4	**8o**	V	2'or 6'-OH (mixture)	Me	>1000	>1000
8e-A	IV	2'-Cl	Me	>1000	>1000	**8p-A**	V	2'-Cl, 5'-F	Me	>1000	207.0±135.0
8e-B	IV	6'-Cl	Me	>1000	>1000	**8p-B**	V	3'-F, 6'-Cl	Me	>1000	37.1±1.2
8f-A	V	2'-Cl	Me	>1000	>1000	**8q-A**	V	2'-Cl,4'-F	Me	>1000	224.1±6.7
8f-B	V	6'-Cl	Me	>1000	65.1±22.7	**8q-B**	V	4'-F, 6'-Cl	Me	>1000	88.6±13.3
8g-A	VI	2'-Cl	Me	>1000	>1000	**8r-A**	V	2'-Cl, 3'-F	Me	>1000	>1000
8g-B	VI	6'-Cl	Me	>1000	81.7±23.3	**8r-B**	V	5'-F, 6'-Cl	Me	>1000	27.5±11.6
8h	V	3'-Cl	Me	>1000	>1000	**2**	-	-	-	>1000	73.1±12.9
8i	V	4'-Cl	Me	>1000	>1000	**3**	-	-	-	76.6±20.2	3.0±1.4
8j	V	H	Me	>1000	>1000	**5**	-	-	-	>1000	38.7±16.6
8k	V	2'or 6'-F	Me	>1000	>1000	**6**				6.8±3.8	5.8±2.5

[a] $EGFR^{L858R/T790M/C797S}$ ($EGFR^{TM}$) and $EGFR^{WT}$ kinase inhibition was were tested by ELISA assay. The data are mean values from at least three independent experiments.

Our next investigation mainly focused on identification of an optimal R_1-linker group, which could potentially facilitate the 5-methyl substituent to adopt a suitable orientation avoiding steric collision with Met^{790}. The propionamide was kept unchanged because the **6**-EGFR$^{T790M/C797S}$ complex structure (PDB: 5ZTO) suggested that this moiety was solvent-surface accessible and might not make much contribution to interaction with the protein. Interestingly, a replacement of the *trans*-cyclohexanediamine linker in **8a** with a *cis*-cyclohexanediamine moiety (**8c**) resulted in a significant improvement in EGFR$^{L858R/T790M/C797S}$ inhibitory potency. The resulting compound **8c** exhibited an IC_{50} value of 35.1 nM against EGFR$^{L858R/T790M/C797S}$, which is 14.8-fold more potent than the parental compound **8a**. However, the modification barely affected the inhibitory activity against EGFR^{WT}. The steric hindrance between the 5-methyl and 7-carbonyl moieties of the scaffold and the 2'-Cl group of the pendent phenyl ring of **8c** results in hindered rotation of the corresponding C-C bond, inducing an axial chirality in the molecule. Two atropisomers present in the mixture **8c** (i.e. **8c-A** and **8c-B**) were separated by chiral High Performance Liquid Chromatography (HPLC). The absolute stereochemistry of both compounds was determined by using small molecule X-ray crystallographic analysis (Figure **3**). Further biological evaluation revealed that the compounds exhibited IC_{50} values of 518.8 and 27.7 nM against EGFR$^{L858R/T790M/C797S}$, respectively. Thus, **8c-B** represents the preferred configuration to bind with the triple mutant.

Several other aliphatic heterocyclic linkers were also explored (Table 1). Although introduction of 4-piperidine (**8d**) or (*S*)-3-piperidine (**8e-A** & **8e-B**) linkers caused total abolishment of the EGFR$^{L858R/T790M/C797S}$ inhibitory potency, the (*R*)-3-piperidine substituted molecule **8f-B** and the (*R*)-3-pyrrolidine derivative **8g-B** exhibited IC_{50} values of 65.1 and 81.7 nM, respectively, against the EGFR$^{L858R/T790M/C797S}$ mutant. Notably, compounds **8f-B** and **8g-B** were almost equally potent to **8b** and **8c**, but their inhibition of the wild-type kinase was abolished. Similar to the above observation, the alternate stereo-enantiomers **8f-A** and **8g-A** were totally inactive. Consequently, compound **8f-B** was selected as a new starting point for further structural optimization because of its relatively potent EGFR$^{L858R/T790M/C797S}$ inhibition and promising target selectivity.

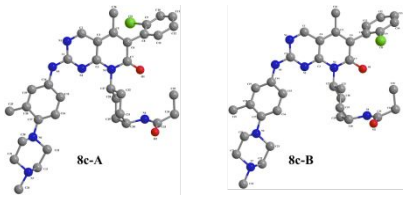


Figure 3. X-ray structures of compound **8c-A** and **8c-B**.

Our previous structural investigation implied that a 2'-chloro group in **6** might be critical to force the pendent phenyl moiety to adopt an appropriate orientation for it to be accommodated in a hydrophobic pocket bounded by Lys^{745}, Glu^{762}, Leu^{788}, Met^{766} and Met^{790} in EGFR^{C797S}. [22] In order to validate the potential contribution of the 2'-chloro group of **8f-B**, compounds **8h** and **8i**, in which the chloro-substituent was moved to the 3'-position or 4'-position, respectively, were design and synthesized. Both **8h** and **8i** were totally inactive with respect to inhibition of EGFR$^{L858R/T790M/C797S}$ and EGFR^{WT}, supporting our hypothesis that a 2'-substituent is optimal for inhibition of the EGFR kinase. Not surprisingly, removal of the critical 2'-chloro group also caused a total abolishment of inhibitory activity against EGFR^{WT} and the C797S mutant (**8j**). Further investigation revealed that this position is highly sensitive to the size and physicochemical properties of the substituted group. When the 2'-chloro substituent was replaced with 2'-fluoro (**8k**), 2'-methoxyl (**8n**), or 2'-hydroxyl (**8o**) groups, all of the resulting molecules were found to be completely inactive against both EGFR$^{L858R/T790M/C797S}$ and EGFR^{WT}. The 2'-methyl analogs (i.e., **8m-A** and **8m-B**) were also notably less active than **8f-B**. Not surprisingly, the 2'-bromo derivative (**8l-B**) exhibited similar EGFR$^{L858R/T790M/C797S}$ inhibitory potency to that of **8f-B**, with an IC_{50} value of 56.1 nM. Interestingly, introduction of a second fluoro-substituent at each available vacant position of the pendent chlorophenyl ring had varying influence on the biological activity of the molecules. For instance, the 4'-F, 6'-Cl disubstituted analogue (**8q-B**) exhibited almost identical potency and selectivity to that of **8f-B**, but the 3'-F, 6'-Cl (**8q-B**) and 5'-F, 6'-Cl (**8r-B**) were 1.8~2.4 fold more potent than the lead molecule. Compounds **8q-B** and **8r-B** exhibited IC_{50} values of 37.1 and 27.5 nM, respectively against the EGFR$^{L858R/T790M/C797S}$ mutant. It was also noteworthy that both compound **8r-B** and **8q-B** were equally potent and selective to the previously reported EGFR^{C797S} inhibitor **5** in a parallel comparison. Different to the allosteric inhibitor **2b**, **8r-B** exhibited similar inhibition against the EGFR$^{del746-750/T790M/C797S}$ mutant with an IC_{50} value of 49.9 nM.

X-ray co-crystallographic structures of **8r-B** with EGFR$^{T790M/C797S}$ and EGFR^{WT} were further determined to elucidate detailed interactions and the structural basis of the observed target selectivity (Figure **3**). These confirmed that compound **8r-B** bound at the ATP binding sites of EGFR$^{T790M/C797S}$ and EGFR^{WT} with similar "U-shaped" configurations. The 5-methylpyrimido-pyrimidinone scaffold formed the classical hydrogen bond donor-acceptor interactions with the "hinge" residue Met^{793}, while the 6-(2'-chloro-3'-fluoro)phenyl group occupies a hydrophobic pocket bounded by Lys^{745}, Glu^{762}, Leu^{788}, Met^{766} and Met^{790}. Both the 4-methylpiperazinylphenyl group and the propionyl piperidine were directly accessible to the solvent surface, which would provide feasible positions for future optimization of the physicochemical properties of the compounds. Notably, the 5-methyl group approaches Met^{790} within a distance of 3.5 Å in EGFR$^{T790M/C797S}$ to form a favorable hydrophobic interaction. Alternately, its lipophilic character appears to be incompatible with the hydrophilic Thr^{790} in EGFR^{WT}. In addition, the lipophobic property of Thr^{790} could also weaken the hydrophobic interactions of the 6-(2'-chloro-

3'-fluro)phenyl group with wild-type EGFR. This structural information could provide a rational explanation for the inhibitor's selectivity between EGFRT$^{790M/C797S}$ and EGFR^{WT}.

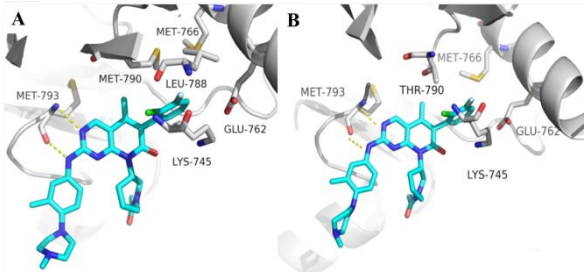


Figure 4. X-ray structures of co-crystals of **8r-B**-EGFR$^{T790M/C797S}$ and **8r-B**-EGFR^{WT} complexes. (A) A 2.943 Å crystallographic structure of **8r-B**-EGFR$^{T790M/C797S}$ complex (PDB ID: 6JRJ). (B) A 2.796 Å crystallographic structure of **8r-B**-EGFRT^{WT} complex (PDB ID: 6JRK).

The binding free energy of compound **8r-B** in EGFR^{WT} and the EGFR$^{T790M/C797S}$ mutant were estimated to be -47.8±2.7 kcal/mol and -57.5±4.0 kcal/mol respectively, by utilizing classical MD simulations and MM-GBSA calculations (Supporting Information). These results suggest that binding of compound **8r-B** to EGFR$^{T790M/C797S}$ is thermodynamically more favorable than binding to EGFR^{WT}, which is consistent with the experimental results. Moreover, contribution of the stabilization energy of each residue to the overall binding affinity of **8r-B** was also evaluated and the difference between the EGFR^{WT} and EGFR$^{T790M/C797S}$ mutant were calculated using the equation $\Delta\Delta G = \Delta G_{WT} - \Delta G_{TMCS}$. The top ten significant residues to enhance the binding affinity are summarized in Figure **5**. The binding energy difference ($\Delta\Delta G = \Delta G_{WT} - \Delta G_{T790M/C797S}$) of compounds **3** and **6** were also calculated to be -1.5 kcal/mol and 2.2 kcal/mol, respectively, which might give a reasonable explanation for their relatively low triple mutant selectivity (Supporting Information).

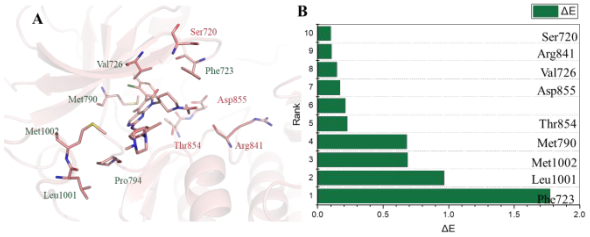


Figure 5 (A) The hydrophobic amino acid residues are marked in dark green, while the polar amino acid residues are marked in red. (B) The top ten hot-spot residues predicited computationally.

Further biological investigation validated that compound **8r-B** potently supressed the activation of EGFR signaling in BaF3 cells stably transfected with EGFR$^{L858R/T790M/C797S}$ (Supporting Information) and inhibited proliferation of this transfected cell line model with an IC_{50} value of 0.662 (±0.045) μM. However, its effect on wild-type EGFR signaling was significantly less obvious (Supporting Information). Investigation also revealed that the compound exhibited moderate aqueous solubility and cellular permeability (Supporting Information).

CONCLUSION

In summary, a series of 5-methylpyrimidopyridone derivatives were designed and synthesized as novel selective wild-type sparing EGFR$^{L858R/T790M/C797S}$ inhibitors. One of the representative compounds, **8r-B** exhibited an IC_{50} value of 27.5 nM against the EGFR$^{L858R/T790M/C797S}$ triple mutant, while being almost totally inactive against the EGFR^{WT} kinase, identifying **8r-B** as one of the most selective 4th generation EGFR inhbitors reported to date. Co-crystallographic structure determination and computational investigation further validated that target selectivity of the inhibitors mainly relied on the hydrophobic nature of the Met^{790} residue in the T790M/C797S mutant protein. Although the relatively low kinome-selectivity and metabolic liability (Supporting Information) of **8r-B** may limit its future development, our study provides a useful lead compound and some fundmental structural information for the rational development of new selective EGFR^{C797S} inhibitor drugs. Further pharmacokinetics-oriented optimization of **8r-B** is on-going and the results will be disclosed in due course.

EXPERIMENTAL SECTION

General Chemistry. Reagents and solvents were obtained from commercial suppliers and used without further purification. Flash chromatography was performed using silica gel (200–300 mesh). All reactions were monitored by TLC, using silica gel plates with fluorescence F254 and UV light visualization. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-400 spectrometer at 400 MHz and Bruker AV-500 spectrometer at 125 MHz, respectively. Coupling constants (*J*) are expressed in hertz (Hz). Chemical shifts (δ) of NMR are reported in parts per million (ppm) units relative to internal control (TMS). The first-order peak patterns are indicated as s (singlet), d (doublet), t (triplet), q (quadruplet). Complex non-first order signals are indicated as m (multiplet). The low- or high- resolution ESI-MS results were recorded on an Agilent 1200 HPLC-MSD mass spectrometer or Applied Biosystems Q-STAR Elite ESILC–MS/MS mass spectrometer, respectively. The purity of compounds was determined by reverse-phase high-performance liquid chromatography (HPLC) analysis confirming to be >95%. Analytical HPLC analyses were conducted using an Agilent 1260 system (G1310B Iso pump and G1365D MWD VL detector) with an YMC-Triart C18 reversed-phase column (250 mm × 4.6 mm, 5 μm) at 254 nm. Elution was MeOH in water, and flow rate was 1.0 mL/min. Preparative HPLC (PHPLC) purifications were performed using Agilent 218 solvent delivery module and an Agilent 325 dual wavelength UV–vis detector with an YMC-Triart C18 reversed-phase column (250 mm × 20 mm, 5 μm) at 254 nm. The LC column was maintained at room temperature.

***N*-((1*R*,4*R*)-4-(6-(2-chlorophenyl)-5-methyl-2-((3-methyl-4-(4-methylpiperazin-1-yl)phenyl)amino)-7-oxopyrido[2,3-*d*]pyrimidin-8(7*H*)-yl)cyclohexyl) propionamide** (**8a**). Propionyl chloride (25 μL, 0.28 mmol) was added dropwise to a mixture of **16a** which was readily prepared by a protocol described in the Supporting Information (150 mg, 0.26 mmol) and Et_3N (0.11 mL, 0.78 mmol) in dry DCM (10 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and concentrated under reduced pressure. The resulting crude product was purified by silica gel chromatography to afford **8a** as a yellow solid (135 mg, 92%). ^{1}H NMR (400 MHz, DMSO-*d*6) δ 9.87 (s, 1H), 8.89 (s, 1H), 7.60 (d, *J* = 2.3 Hz, 1H), 7.57 – 7.53 (m, 1H), 7.49 (dd, *J* = 8.6, 2.4 Hz, 1H), 7.41 (dd, *J* = 4.6, 2.3 Hz, 2H), 7.31 – 7.24 (m, 1H), 7.02 (d, *J* = 8.7 Hz, 1H), 5.42 (s, 1H), 3.86 (s, 1H), 2.81 (s, 5H), 2.47 (s, 4H), 2.28 (s, 3H), 2.23 (s, 3H), 2.15 (d, *J* = 7.4 Hz, 1H), 2.12 (s, 3H), 1.91 (d, *J* = 11.1 Hz, 2H), 1.55 (d, *J* = 9.1 Hz, 2H), 1.42 (s, 2H), 1.24 (d, *J* = 11.1 Hz, 3H), 1.01 – 0.92 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-*d*6) δ 172.5, 162.8 (2C), 161.9, 159.2, 157.8, 155.2, 135.5, 134.0 (2C), 132.7, 132.5 (2C), 129.9, 129.5 (2C), 127.5, 119.4, 119.1, 106.7, 106.6, 55.7 (2C), 51.8, 46.2, 36.3, 32.7, 31.2, 29.5, 29.4, 29.0, 28.5, 27.1, 18.4, 15.1, 10.5. HRMS (ESI) for $C_{35}H_{42}ClN_7O_2$ $[M+H]^+$, calcd: 628.3089; found, 628.3218. HPLC analysis: MeOH-H_2O (85: 15), 11.86 min, 97.3% purity.

The other designed compounds were synthesized by following a similar procedure (Supporting Information).

ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, *in vitro* ELISA assay, cell proliferation inhibition assay, western blot analysis, experimental data for the X-ray structures of **8c-A** and **8c-B,** computational study, solubility assay, metabolic stability, Caco-2 permeability assay, the kinase selectivity profiling study of **8r-B**, ^{1}H NMR, ^{13}C NMR spectra of compounds **8a-8r** (PDF), HPLC purity analysis of **8a-8r** (PDF) and Molecular Formula Strings (CSV). The material is available free of charge *via* the Internet at http://pubs.acs.org.

Atomic coordinates and experimental data for the co-crystal structures of **8r-B** with $EGFR^{T790M/C797S}$ and $EGFR^{WT}$ will be released upon article publication.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; C797S, Cys797 to Ser797; MOA, mode of action; WT, wild-type; ELISA, enzyme linked immunosorbent assay; AN, acetonitrile; DIPEA, *N, N*-diisopropylethylamine; TFA, trifluoroacetic acid; DCM, dichloromethane; THF, tetrahydrofuran; HPLC, High Performance Liquid Chromatography.

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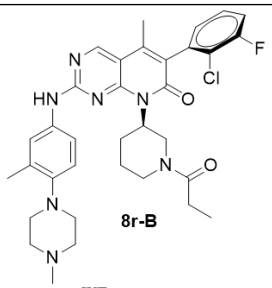
TOC



$EGFR^{WT}$ IC_{50}: >1000 nM

$EGFR^{TM}$ IC_{50}: 27.5 nM