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Insight into the Inhibition of Drug-Resistant Mutants of the Receptor Tyrosine Kinase EGFR

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Abstract: *Targeting acquired drug resistance represents the major challenge in the treatment of EGFR-driven non-small-cell lung cancer (NSCLC). Herein, we describe the structure-based design, synthesis, and biological evaluation of a novel class of covalent EGFR inhibitors that exhibit excellent inhibition of EGFR-mutant drug-resistant cells. Protein X-ray crystallography combined with detailed kinetic studies led to a deeper understanding of the mode of inhibition of EGFR-T790M and provided insight into the key principles for effective inhibition of the recently discovered tertiary mutation at EGFR-C797S.*

Targeted approaches have revolutionized the treatment of cancer patients, but are plagued by the emergence of drug resistance. The first-generation EGFR inhibitors gefitinib and erlotinib achieved objective response rates of 50–80 % in patients suffering from EGFR-dependent non-small-cell lung cancer (NSCLC).[1] Whereas the median progression-free survival was doubled, more than 80 % of patients still suffered from tumor progression within 18 months of treatment. Biopsies of resistant tumors revealed a point mutation of the gatekeeper residue (T790M).[2] Replacement of the threonine with methionine leads to steric repulsion of the first- and second-generation inhibitors (afatinib and dacomitinib) and results in a slightly different binding geometry, which accounts for the loss of inhibitory activity both in vitro and in vivo.[3] Third-generation EGFR inhibitors (WZ4002, EGF816 (nazartinib), AZD9291 (osimertinib), and CO-1686 (rociletinib)) were designed to avoid steric interference with Met790. These inhibitors bind in a covalent fashion and alkylate EGFR at Cys797 at the lip of the ATP-binding site (Figure 1 a).[4]

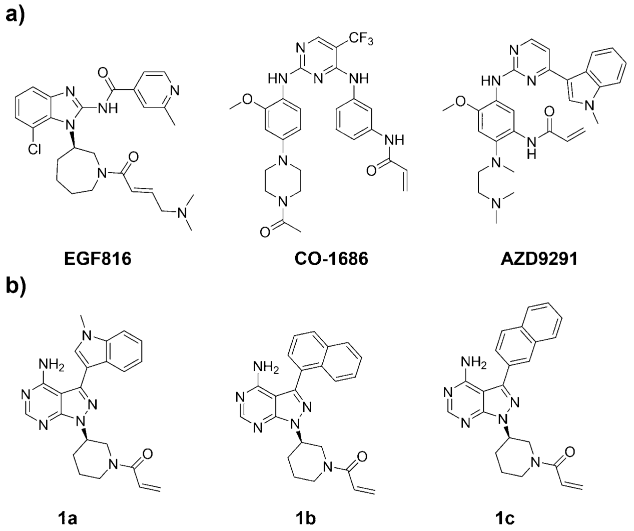

Figure 1. a) Third-generation EGFR inhibitors. b) A novel series of covalent inhibitors based on the pyrazolopyrimidine framework.

However, the effective treatment of patients that harbor the EGFR-T790M drug resistance mutation is limited by the emergence of new drug resistances to the tyrosine kinase inhibitor therapy.[5] C797S is a recently discovered resistance mutation in the kinase domain of EGFR. This mutation prevents covalent bond formation with third-generation inhibitors and thereby reduces their efficacy, thus demonstrating that there is an urgent demand for a new class of EGFR inhibitors that effectively inhibits both drug-resistant mutant variants of EGFR (L858R/T790M and L858R/T790M/C797S).

Herein, we report the structure-based design, synthesis, and biological evaluation of a new class of covalent pyrazolopyrimidines with high affinity towards the methionine gatekeeper to specifically target and overcome acquired drug resistance in EGFR. X-ray structures of representative compounds of this series in complex with EGFR-T790M together with detailed kinetic studies provided valuable insight into the inhibition of the T790M mutant and further

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revealed the key requirements for targeting the recently discovered drug resistance C797S in EGFR. We focused our efforts on two principles that we expected to be crucial for the development of EGFR inhibitors with sufficient efficacy for the T790M gatekeeper-mutated variant of EGFR. First, we sought to design a covalent inhibitor with an electrophile optimized in terms of geometry and orientation for the alkylation of Cys797. Second, we aimed to avoid steric interference with the gatekeeper residue while exploiting its hydrophobic nature for additional lipophilic interactions to maximize the affinity as well as the selectivity towards the T790M mutant variant of EGFR. Hence, we employed the 4-aminopyrazolopyrimidine core structure as it provides a well-known hinge-binding element and allows for the rapid synthesis of focused libraries. We chose an acrylamide as the Michael acceptor, which was linked to the pyrazolopyrimidine core through a 3-piperidine substituent (Figure 1b), and explored pyrazolopyrimidines with different C3 substituents. To investigate the possible beneficial interplay between lipophilic substituents and Met790 while retaining the crucial direct-hinge interactions of the pyrazolopyrimidine core, we established a generic synthetic route for synthesizing pyrazolopyrimidines **1a**–**1c** (see the Supporting Information, Scheme S1).

We then assessed the inhibitory efficacy of these compounds on the drug-resistant NSCLC cell line H1975, which harbors EGFR-L858R/T790M, as well as on cell line A431, with wild type EGFR (Table 1). The whole series displayed an excellent inhibitory effect on H1975 cells (GI_{50} values of 0.21 μM, 0.49 μM, and 0.14 μM for **1a**, **1b**, and **1c**, respectively) whilst showing a reduced inhibitory impact on EGFR wild type (WT) cells (A431; 26–49 fold), which is consistent with the mutant selectivity observed in the activity-based assay described below. We further analyzed compounds **1a**–**1c** and the third-generation EGFR inhibitor WZ4002 as a reference compound in cellular studies to determine their influence on the phosphorylation of EGFR as well as its downstream signaling in the cell lines A431 and H1975 (Figure 2 and Figure S1). Treatment with **1c** induced a dramatic dose-dependent reduction of EGFR phosphorylation (Y1068) in H1975 cells whilst showing only a marginal effect on pEGFR in the EGFR-WT cancer cell line (A431). This outcome is consistent with the results discussed above, underscoring the remarkable mutant-selective inhibition profile of **1c**.

Moreover, **1c** likewise affected the cellular H1975 EGFR downstream phosphorylation states and decreased the amount of pAkt as well as pMAPK and pS6. We also evaluated the inhibitory activity of the pyrazolopyrimidines **1a**–**1c** in an activity-based assay with respect to WT, activating (L858R), and drug-resistant mutant variants of EGFR (L858R/T790M and L858R/T790M/C797S; Table 2). The three covalent pyrazolopyrimidines exhibited significant potency against the activating (L858R) and drug-resistant (L858R/T790M) mutant variants of EGFR with IC_{50} values in the single-digit nanomolar range. Remarkably, **1c**, which is decorated with 2-napthalene, even showed an inhibitory activity in the subnanomolar range. All of these pyrazolopyrimidines are highly mutant-selective inhibitors as they show reduced activity in EGFR-WT (up to 31 fold), providing an excellent mutant-selectivity profile. Notably, **1c** also displayed significant potency (88 nM) against the second acquired drug resistance L858R/T790M/C797S. This result indicates that covalent inhibition is the driver for the tremendous potency of **1c** in L858R/T790M cells as the point mutation of Cys797 to a serine prevents covalent bond formation. Covalent inhibitors have distinct binding properties owing to their ability to undergo covalent bond formation, and, for complete characterization of these compounds, we went on to investigate their binding kinetics. To explore the rate and efficiency of covalent bond formation of our pyrazolopyrimidine inhibitors **1a**–**1c**, we determined the corresponding K_i and k_{inact} parameters with respect to WT, the activating mutant (L858R), and the drug-resistant mutant variants (L858R/T790M and L858R/T790M/C797S) of EGFR using an activity-based, time-resolved[6] assay (Table 2). Compounds **1a**–**1c** did not display a loss of affinity towards the mutant forms as compared to WT, but rather demonstrated an excellent binding affinity towards the drug-resistant methionine gatekeeper mutant of EGFR (L858R/T790M). Compound **1c**, in particular, exhibited an extremely high affinity with K_i values in the low nanomolar range (1.5 nM), and along with the rate of covalent bond formation, compound **1c** displayed the most favorable inhibition profile

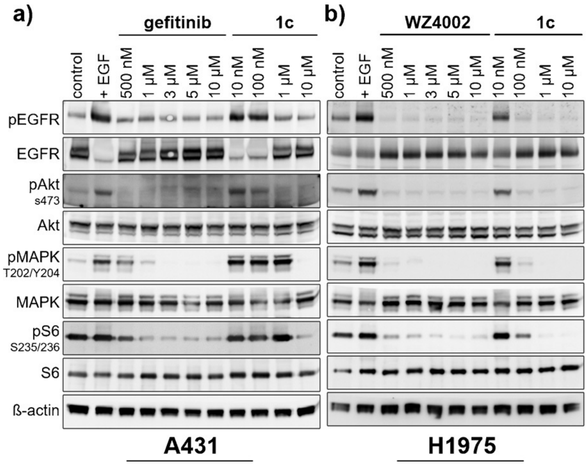

Figure 2. Western blot analysis of **1c** in a) A431 (EGFR-WT) and b) H1975 (L858R/T790M) cancer cell lines. All of the cells except the control were stimulated with EGF (50 ng mL^{-1}). Compound **1c** decreased the phosphorylation level of EGFR and its downstream targets pAkt, pMAPK, and pS6 in H1975 cells, but did not have a significant effect in A431 cells.

Table 1: Inhibition of cellular growth (GI_{50}) with reference compounds and the new pyrazolopyrimidines.

	GI_{50} [μM] for NSCLC cell lines	
Compound	A431 (WT)	H1975 (L858R/T790M)
1a	8.9 ± 5.7	0.21 ± 0.07
1b	24 ± 7.5	0.49 ± 0.32
1c	3.6 ± 1.5	0.14 ± 0.06
EGF816	3.9 ± 1.4	0.27 ± 0.07
CO-1686	1.7 ± 0.12	0.12 ± 0.05
AZD9291	0.67 ± 0.23	0.02 ± 0.01

Table 2: Half-maximal inhibitory concentrations (IC_{50}) and the kinetic parameters K_i, k_{inact}, and k_{inact}/K_i determined for reference compounds and **1a**–**1c** in EGFR-WT, -L858R, -L858R/T790M, and -L858R/T790M/C797S.

Compound	EGFR	IC_{50} [nM]	K_i [nM]	k_{inact} [min^{-1}]	k_{inact}/K_i [µM^{-1} s^{-1}]
1a	WT	35 ± 14	25 ± 7.2	0.11 ± 0.03	0.08 ± 0.02
	L858R	3.8 ± 2.8	19 ± 3.1	0.17 ± 0.08	0.14 ± 0.07
	L858R/T790M	2.5 ± 1.4	16 ± 5.2	0.29 ± 0.02	0.36 ± 0.09
	L858R/T790M/C797S	838 ± 85	1008 ± 372	–	–
1b	WT	58 ± 13	47 ± 5.8	0.13 ± 0.05	0.05 ± 0.04
	L858R	1.0 ± 0.3	44 ± 6.5	0.25 ± 0.06	0.10 ± 0.06
	L858R/T790M	1.9 ± 0.8	58 ± 3.3	0.31 ± 0.04	0.09 ± 0.02
	L858R/T790M/C797S	7900 ± 3700	1068 ± 432	–	–
1c	WT	16 ± 5.6	15 ± 3.2	0.19 ± 0.12	0.21 ± 0.05
	L858R	1.1 ± 0.6	1.6 ± 0.4	0.14 ± 0.01	1.60 ± 0.53
	L858R/T790M	< 1	1.5 ± 0.8	0.17 ± 0.02	3.42 ± 1.44
	L858R/T790M/C797S	88 ± 16	49 ± 14	–	–
EGF816	WT	1.9 ± 0.2	25 ± 7.8	0.31 ± 0.06	0.23 ± 0.13
	L858R	< 1	10 ± 2.7	0.22 ± 0.02	0.38 ± 0.08
	L858R/T790M	< 1	7.7 ± 2.3	0.15 ± 0.04	0.38 ± 0.10
	L858R/T790M/C797S	196 ± 21	433 ± 151	–	–
CO-1686	WT	2 ± 0.5	74 ± 7.1	0.18 ± 0.01	0.04 ± 0.01
	L858R	2 ± 0.2	1.8 ± 0.2	0.18 ± 0.05	1.67 ± 0.32
	L858R/T790M	3 ± 0.6	1.7 ± 0.1	0.29 ± 0.05	2.95 ± 0.66
	L858R/T790M/C797S	69 ± 13	76 ± 40	–	–
AZD9291	WT	2 ± 0.2	14 ± 2.3	0.43 ± 0.11	0.52 ± 0.05
	L858R	1 ± 0.5	1.6 ± 0.3	0.30 ± 0.01	3.24 ± 0.46
	L858R/T790M	< 1	1.5 ± 0.1	0.33 ± 0.06	3.75 ± 0.39
	L858R/T790M/C797S	77 ± 22	25 ± 9.6	–	–

of that series regarding EGFR L858R/T790M ($k_{inact}/K_i =$ 3.42 µM^{-1}s^{-1}). These results are in line with its strong inhibitory activity. Furthermore, **1c** also displayed an extremely high affinity towards the drug-resistant mutant form of EGFR that lacks Cys797, which is required for covalent bond formation (L858R/T790M/C797S). The K_i value of 49 nM provided an explanation for the potency of **1c** against this mutant. In contrast, the pyrazolopyrimidines **1a** and **1b**, which did not have a significant effect against EGFR L858R/T790M/C797S displayed approximately 20 fold lower affinity ($K_i = 1008$ nM and 1068 nM, respectively) than **1c**. However, **1a** and **1b** exhibited a high rate of covalent bond formation (k_{inact}) for EGFR-L858R/T790M (0.29 min^{-1} and 0.31 min^{-1}, respectively) that compensated for their reduced binding affinity and clarified their mode of action against the T790M mutant of EGFR.

These kinetic studies have disclosed the reason for the high potency of these compounds against EGFR-T790M and clearly demonstrated that a high affinity towards the methionine gatekeeper will be key to target the acquired C797S drug resistance in EGFR. These observations substantiated the value and potential of this novel series of pyrazolopyrimidines. To evaluate the pharmacokinetic characteristics of these compounds, we set out to investigate the cellular absorption of **1a** and **1c** in an artificial membrane permeation assay (PAMPA) and the Caco-2 cell assay, in which the ratio of influx/efflux across Caco-2 cells is determined (Table S1). Compounds **1a** and **1c** displayed high cellular penetration at pH 7.4 (PAMPA, 66 % and 73 % flux, respectively) as well as sufficient cellular absorption (cell penetration over efflux: 1:1 and 4:1 for **1a** and **1c**). For the investigation of the selectivity profiles of our pyrazolopyrimidine series, we implemented SelectScreen® profiling (Life Technologies) of compound **1a** at a concentration of 1 µM against a panel of 100 kinases (including selected mutants; Figure S2). Compound **1a** inhibited the kinase activity of wild type EGFR and its mutant variants (L861Q, L858R, T790M, and L858R/T790M) by more than 90 % while affecting a few off-target sites with > 80 % inhibition for kinases that contain a cysteine residue isostructural to Cys797 in EGFR (BLK, TXK, BMX, BTK, and ErbB-4). Thus **1a** displayed an excellent selectivity profile, particularly with regard to EGFR and its mutant variants.

To confirm the covalent binding mode and to gain deeper insight into the structural interplay within the kinase domain of EGFR, we co-crystallized **1a** and **1b** in complex with the gatekeeper mutant T790M. These crystal structures clearly revealed that both inhibitors form a covalent bond with Cys797 at the front lip of the hinge region (Figure 3). The 3-piperidine-linked acrylamide provided an optimized binding geometry and orientation towards Cys797, and compounds **1a** and **1b** formed two direct hydrogen bonds with the peptide backbone in the hinge region (Gln791 and Met793). The 4-substituted amino moiety formed a hydrogen bond with the backbone carbonyl group of Gln791, while the peptide backbone of Met793 interacted with the pyrazolopyrimidine

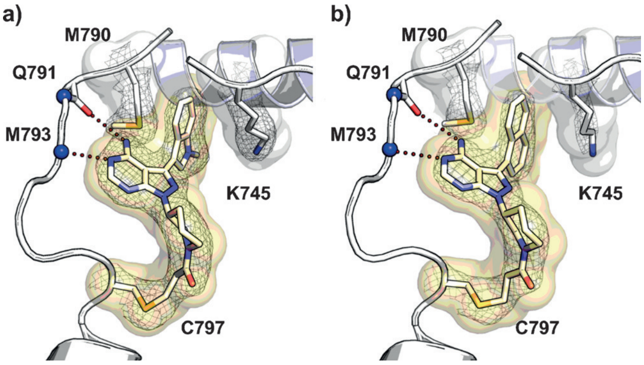

Figure 3. Crystal structures of pyrazolopyrimidines in complex with EGFR-T790M. Diagrams of the experimental electron densities of a) EGFR-T790M/**1a** at 2.5 Å (PDB code: 5J9Z) and b) EGFR-T790M/**1b** at 2.8 Å resolution (PDB code: 5J9Y); $2F_o-F_c$ map contoured at 1σ. Hydrogen-bond interactions of the inhibitors with the hinge region (white) are illustrated by red dotted lines. The structural elements of the N-terminal lobe and the C helix are displayed in white and blue-white, respectively. The lipophilic methylindole (**1a**) and 1-naphthyl (**1b**) moieties at the C3 position of the pyrazolopyrimidine both point towards a hydrophobic back pocket and are sandwiched between the methionine gatekeeper (M790) and the alkyl side chain of K745.

N6 nitrogen atom, which served as a hydrogen-bond donor. Remarkably, the co-crystal structure of compound **1a** revealed a direct association of the 3-substituted methylindole with Met790 and an apparent interaction between this moiety and the alkyl side chain of the conserved, catalytic Lys745. The methylindole moiety was sandwiched between Met790 and Lys745, inducing lipophilic interactions that may explain the increased affinity of these compounds towards the T790M mutant variants. Strikingly, the 1-naphthyl group of compound **1b** displayed an identical orientation towards Met790 and the side chain of Lys745, yielding a favorable lipophilic interaction. These structural observations yield a reasonable explanation for the high potency of this pyrazolopyrimidine series against the T790M mutant and strengthen our conclusion that addressing the hydrophobic space that comprises the methionine gatekeeper with lipophilic motifs may dramatically affect the affinity and mutant selectivity of these inhibitors owing to reduced interactions with the polar Thr790 gatekeeper in wild type EGFR. Overall, lipophilic moieties on C3 of this novel series of pyrazolopyrimidines are indispensable for gaining mutant selectivity as long as the introduced moieties do not interfere with the sterically demanding Met790. Mass-spectrometric analysis of EGFR-T790M treated with **1a** or **1b** further confirmed the covalent binding, which increased the mass of the complex by 401 Da or 398 Da, respectively, versus a DMSO-treated reference kinase, and was consistent with a 1:1 inhibitor/protein ratio in the complex (Figure S3). Tandem mass spectrometry indicated specific single alkylation of Cys797 (Figures S4 and S5).

In summary, we have developed a novel class of covalent and mutant-selective EGFR inhibitors that displayed excellent inhibitory effects on drug-resistant H1975 cells and further demonstrated high potential for targeting the newly acquired C797S drug resistance mutation in EGFR. Further development of these inhibitors should provide a unique opportunity to target all clinically relevant oncogenic mutant variants of EGFR with one and the same drug. X-ray analysis of these inhibitors in complex with EGFR-T790M in combination with kinetic studies of the entire set of clinically relevant EGFR mutants led to deeper insight into the inhibition of drug-resistant EGFR and revealed the key principles for targeting EGFR-C797S. The discovery of this class of inhibitors may stimulate the development of forth-generation EGFR inhibitors targeting acquired T790M and C797S drug resistance, and may lead to a further breakthrough in challenging acquired drug resistance in the treatment of EGFR-driven NSCLC.

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