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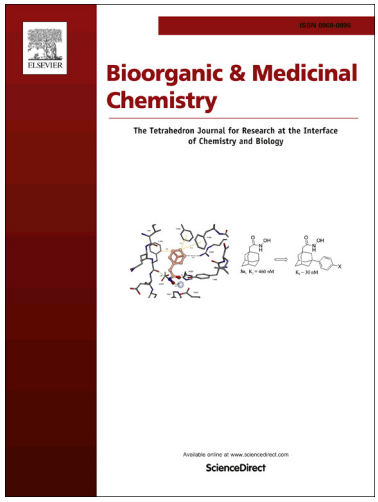

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Debjit Basu, André Richters, Daniel Rauh

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Structure-based design and synthesis of covalent-reversible inhibitors to overcome drug resistance in EGFR

*Debjit Basu, André Richters, Daniel Rauh**

Department of Chemistry and Chemical Biology, Technische Universität Dortmund, Otto-Hahn-Straße 6, 44227 Dortmund, Germany

* Corresponding author. Tel.: +49 231 755 7080

E-mail address: daniel.rauh@tu-dortmund.de (D. Rauh)

Abstract

The clinical success of covalent kinase inhibitors in the treatment of EGFR-dependent non-small cell lung cancer (NSCLC) has rejuvenated the appreciation of reactive small molecules. Acquired drug resistance against first-line EGFR inhibitors remains the major bottleneck in NSCLC and is currently addressed by the application of fine-tuned covalent drugs. Here we report the design, synthesis and biochemical evaluation of a novel class of EGFR inhibitors with a covalent yet reversible warhead. A series of WZ4002 analogues, derived from anilinopyrimidine and 3-substituted-2-cyanoacrylamide scaffolds, exhibit strong and selective inhibitory activity against clinically relevant $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$.

1. Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase which plays pivotal roles in cell proliferation, survival, adhesion, migration and differentiation through downstream signaling pathway activation.[1] Dysregulation of EGFR caused by overexpression or mutation is associated with the onset and development of various cancers, including lung cancer, gastrointestinal malignancies, glioblastoma multiform and thus EGFR represents an important drug target for the treatment of tumor diseases.[2-4] In particular, clinically acquired drug resistance against EGFR kinase inhibitors is a major challenge in targeted cancer therapies for non-small cell lung cancer (NSCLC) treatment.[5, 6] For instance, $EGFR^{L858R}$ mutant positive NSCLC patients receiving erlotinib or gefitinib as first-line treatment suffer from a dramatic relapse which is caused by the acquired secondary drug resistance mutation T790M.[7-9] In this specific example, the oncogenic mutant form L858R evoking increased catalytic activity of EGFR undergoes an additional mutation at the gatekeeper position (T790M) of the kinase domain.[10] The latter mutation increases the off-rate of conventional reversible inhibitors rendering them as ineffective.[11] Since the residence time of an inhibitor toward its target is a relevant marker for its efficacy and potentially translates into beneficial *in vivo* efficiency, the development of inhibitors with extended drug-target residence time, amongst other strategies, is a powerful approach to tackling mutation associated drug resistance.[12] Second generation EGFR inhibitors (e.g., afatinib and dacomitinib)[13, 14] are equipped with Michael acceptor systems that take advantage of the covalent modification of a rare cysteine (Cys797) located at the lip of the ATP binding cleft of EGFR.[15, 16] Due to the covalent modification of Cys797, these irreversible inhibitors exhibit a marginal off-rate and maximal drug-target residence time when compared to reversible inhibitors. Such features are thought to be instrumental to overcome T790M associated drug resistance in NSCLC.[10, 17, 18] Despite promising *in vitro* results, second generation inhibitors exhibit insufficient efficacy in patients at clinically achievable concentrations. Thus, on-target

toxicity represents the dose-limiting factor since in addition to the oncogenic mutant variants, the wildtype form of EGFR is likewise addressed which results in severe side effects such as skin rash and diarrhea.[19-21] However, the third generation EGFR inhibitors, e.g. WZ4002, CO-1686 (rociletinib) and AZD9291 combine the advantageous properties of covalent inhibition with an improved selectivity profile since they spare the inhibition of wildtype EGFR. CO-1686 and AZD9291 are at the leading edge of the treatment of acquired drugs resistance in NSCLC. Both compounds exhibit beneficial properties in terms of residence times and toxicity profiles.[22-25] Notably, these compounds induce synergistic binding interactions with the hydrophobic gatekeeper substitutions in T790M and, therefore, retain their inhibitory efficiency towards clinically relevant EGFR mutants. However, solely few compounds possessing such beneficial structural features are under clinical investigation. Since T790M continues to be the predominant drug resistance mutation, there is an urgent medical need to identify novel mutant selective molecules that potentially amalgamate the advantages of both reversible and covalent inhibition yet devoid of the potential disadvantages associated with both strategies.[6, 9]

Within the wide spectrum of kinase inhibitors, covalent-reversible inhibitors (CRIs) represent an innovative class of promising inhibitors that can be optimized in terms of extended drug-target residence times while retaining the advantages of non-covalent inhibition with respect to limited off-target effects.[26] Recent studies by Taunton and co-workers indicate potential advantages of CRIs since the covalent protein modification is exclusively elicited and stable within the properly formed binding site of the target protein. The covalent bond is formed temporally and cleaved reversibly when the ligand binding site is disintegrated upon protein unfolding or proteolysis. Michael acceptor elements of CRIs (Fig. 1C) are as a matter of principle dually activated and thus lead to increased target affinity by covalent modification. In combination with the reversible hinge binding properties, this class of inhibitors provides prolonged pharmacodynamics over classical reversible inhibitors. Although CRIs are to date

exclusively used as tool compounds, they might very well translate into the development of innovative therapeutics to overcome the challenges associated with conventional covalent inhibition strategies, such as potential toxicity and off-target activities.[26-28]

Here, we set out to explore the concept of CRIs for the inhibition of clinically relevant EGFR mutants while sparing wildtype EGFR. In this proof of concept study, we designed, synthesized and biochemically characterized a focused library of CRIs based on the WZ4002 scaffold possessing the ability to address Cys797 in drug resistant mutants of EGFR with a fine-tuned electron-deficient olefin.

2. Materials and methods

2.1. Molecular modeling

We utilized the co-crystal structure of WZ4002 in complex with EGFR (PDB-code: 3IKA) as the template for the *in silico* design of covalent reversible analogues. We altered R_1 and R_2 and superposed the generated derivatives with the principal core of WZ4002. Extensions at R_1 and R_2 were introduced according to their spatial and electronic features to i) promote the reversibility of binding (strong electron withdrawing groups at R_1, CN and CF_3) and ii) for tuning the properties of the Michael acceptor (aliphatic and aromatic groups at R_2). DS Viewer Pro 6.0 (Accelrys) was used for defining structural features (e.g., aromaticity) of the designed derivatives as well as performing energy minimization. The drafted compounds were then superposed with the ligand WZ4002 using PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

2.2. Chemistry

Unless otherwise noted, all reagents and solvents were purchased from Acros, Fluka, Sigma, Aldrich, or Merck and used without further purification. Dry solvents were purchased as anhydrous reagents from commercial suppliers. ^{1}H and ^{13}C NMR spectra were recorded on a

Bruker Avance DRX 400 or Bruker Avance DRX 500 spectrometer at 400 MHz or 500 MHz and 101 MHz or 125 MHz respectively. ^{1}H chemical shifts are reported in δ (ppm) as s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), m (multiplet), and bs (broad singlet) and are referenced to the residual solvent signal: $CDCl_3$ (7.26), DMSO-d_6 (2.50). Coupling constants (J) are expressed in hertz (Hz). ^{13}C spectra are referenced to the residual solvent signal: $CDCl_3$ (77.0) or DMSO-d_6 (39.0). All final compounds were purified to >95% purity, as determined by high-performance liquid chromatography (HPLC). Purity was measured using Agilent 1200 series HPLC systems with UV detection at 210 nm (system: Agilent Eclipse XDB-C18 4.6 mm × 150 mm, 5 μM, 10-100% CH_3CN in H_2O, with 0.1% TFA, for 15 min at 1.0 mL/min). Analytical TLC was carried out on Merck 60 F245 aluminum-backed silica gel plates. Compounds were purified by column chromatography using Baker silica gel (40-70 μm particle size). Preparative HPLC was conducted on a Varian HPLC system (Pro Star 215) with a VP 250/21 nucleosil C18 PPN column from Macherey-Nagel and monitored by UV at λ = 254 nm.

2.2.1. 2,5-Dichloro-4-(3-nitrophenoxy)pyrimidine (8)

Potassium carbonate (0.75 g, 5.45 mmol) and 2,4,5-trichloropyrimidine (0.50 g, 2.72 mmol) were added to a solution of 3-nitrophenol (0.38 g, 2.72 mmol) in DMF (10 mL). The reaction was heated to 60 °C and stirred for 2 h. Then it was cooled to ambient temperature and filtered through celite. The filtrate was diluted with ethyl acetate (20 mL) and washed with 1M HCl (20 mL), water (10 mL) and brine (10 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated to afford 0.67 g of title compound **8** (2.34 mmol, 86%) as light yellow solid. The product was used without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.89 (s, 1H), 8.30 (t, J = 1.9 Hz, 1H), 8.23 (d, J = 7.9 Hz, 1H), 7.89-

7.78 (m, 2H); ^{13}C NMR (101 MHz, DMSO) δ 164.62, 159.28, 155.95, 151.57, 148.57, 131.27, 128.77, 121.56, 117.33, 116.88.

2.2.2. 1-(3-Methoxy-4-nitrophenyl)-4-methylpiperazine (10)

To a solution of 1-methyl-4-piperazine (1.56 mL, 11.7 mmol) in DMF (20 mL) was added potassium carbonate (2.42 g, 17.5 mmol) and 4-fluoro-2-methoxy-1-nitrobenzene (2.0 g, 11.7 mmol). The resulting mixture was stirred at room temperature for 18 h. The reaction mixture was then diluted with water (50 mL) and extracted with ethyl acetate (3 x 30 mL). The organic extract was washed with brine (20 mL), dried over anhydrous sodium sulfate and evaporated to dryness to obtain an oily crude product. The crude was purified by flash silica gel chromatography using DCM/MeOH (97/3, v/v) as eluent to obtain 2.9 g of the desired product **10** (11.5 mmol, 98%) as yellow oil.^{1}H NMR (400 MHz, DMSO-d_6) δ 7.87 (d, J = 9.4 Hz, 1H), 6.58 (dd, J = 9.4, 2.4 Hz, 1H), 6.52 (d, J = 2.3 Hz, 1H), 3.90 (s, 3H), 3.49-3.37 (m, 4H), 2.46-2.36 (m, 4H), 2.22 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.99, 155.48, 128.17, 128.02, 105.15, 96.93, 56.26, 54.34, 46.31, 45.62.

2.2.3. 2-Methoxy-4-(4-methylpiperazin-1-yl)aniline (11)

Nitro compound **10** (2.9 g, 11.5 mmol) was dissolved in a mixture of THF (12 mL) and MeOH (8 mL) and cooled to 0 °C. Subsequently, zinc powder (3.7 g, 57.7 mmol), followed by ammonium chloride (3.1 g, 57.7 mmol) were added and the reaction mixture was stirred at room temperature for 18 h. The reaction mixture was then filtered over celite and the filtrate was concentrated *in vacuo*. The crude was purified by flash silica gel chromatography using DCM/MeOH (96/4, v/v) as eluent to obtain 1.9 g of the desired product **11** (8.59 mmol, 75%) as dark brown oil.^{1}H NMR (400 MHz, DMSO-d_6) δ 6.52 (d, J = 8.3 Hz, 1H), 6.48 (d, J = 2.1 Hz, 1H), 6.28 (dd, J = 8.3, 2.1 Hz, 1H), 4.08 (bs, 2H), 3.74 (s, 3H), 3.03-2.82 (m, 4H), 2.47-2.36 (m, 4H), 2.21 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 146.99, 143.29, 130.93, 114.20, 108.53, 101.86, 55.21, 54.96, 50.21, 45.81.

2.2.4. 5-Chloro-*N*-(2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)-4-(3-nitrophenoxy)pyrimidin-2-amine (12)

To a solution of compound **8** (1.33 g, 4.66 mmol) and compound **11** (1.03 g, 4.66 mmol) in anhydrous 1-butanol (20 mL), trifluoroacetic acid (0.36 mL, 4.66 mmol) was added. The reaction mixture was heated to 100 °C and stirred for 18 h. Subsequently, it was cooled to room temperature and saturated aqueous sodium bicarbonate solution was added drop wise until basic pH was obtained. The volatiles were removed *in vacuo* and the obtained thick slurry was dissolved in DCM (50 mL). The organic layer was washed with water (20 mL), brine (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The crude was purified by flash silica gel chromatography using DCM/MeOH (96:4, v/v) as eluent to afford 1.42 g of the desired product **12** (3.02 mmol, 65%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.37 (s, 1H), 8.27 (s, 1H), 8.18-8.14 (m, 2H), 7.77-7.71 (m, 2H), 7.09 (d, J = 6.8 Hz, 1H), 6.48 (d, J = 2.2 Hz, 1H), 6.14 (bs, 1H), 3.70 (s, 3H), 3.09-3.00 (m, 4H), 2.48-2.40 (m, 4H), 2.22 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.12, 158.42, 158.15, 156.19, 152.10, 148.26, 147.39, 130.81, 128.85, 120.52, 119.86, 119.07, 117.71, 105.96, 103.59, 99.77, 55.33, 54.55, 48.39, 45.66.

2.2.5. 4-(3-Aminophenoxy)-5-chloro-*N*-(2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)pyrimidin-2-amine (13)

Nitro compound **12** (1.40 g, 2.9 mmol) was dissolved in a mixture of THF (12 mL) and MeOH (8 mL) and cooled to 0 °C. Subsequently, zinc powder (0.97 g, 14.9 mmol), followed by ammonium chloride (0.79 g, 14.9 mmol) were added and the reaction mixture was stirred at room temperature for 18 h. The reaction mixture was then filtered over celite and the filtrate was concentrated *in vacuo*. The crude was purified by flash silica gel chromatography using DCM/MeOH (97/3, v/v) as eluent to obtain 1.19 g of the title compound **13** (2.7 mmol, 93%) as white solid.^{1}H NMR (500 MHz, DMSO-d_6) δ 8.35 (s, 1H), 8.05 (s, 1H), 7.42 (d,

J = 8.8 Hz, 1H), 7.12 (t, *J* = 8.0 Hz, 1H), 6.59 (d, *J* = 2.5 Hz, 1H), 6.54 (dd, *J* = 8.1, 1.3 Hz, 1H), 6.42 (t, *J* = 2.2 Hz, 1H), 6.37 (ddd, *J* = 8.0, 2.2, 0.7 Hz, 1H), 6.33 (d, *J* = 8.1 Hz, 1H), 5.32 (bs, 2H), 3.80 (s, 3H), 3.16-3.05 (m, 4H), 2.52-2.46 (m, 4H), 2.28 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.76, 158.30, 157.59, 153.02, 150.22, 148.24, 140.01, 136.07, 129.59, 122.87, 119.74, 111.17, 108.45, 106.65, 104.13, 99.89, 55.52, 54.56, 48.57, 45.63; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{22}H_{26}O_2N_6Cl$, 441.18003; found, 441.17968.

2.2.6. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)phenyl)acetamide (14)

Compound **13** (0.10 g, 0.23 mmol) was dissolved in anhydrous DCM (4 mL) and cooled to 0 °C. Subsequently, acetic anhydride (27 µL, 0.27 mmol) and DIPEA (110 µL, 0.91 mmol) were added and the resulting reaction mixture was stirred at room temperature for 4 h. The reaction mixture was then diluted with DCM (10 mL) and washed with saturated aqueous sodium bicarbonate solution (10 mL), saturated aqueous ammonium chloride solution (10 mL) and water (10 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated *in vacuo*. The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.031 g of compound **14** (0.064 mmol, 28%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22 (s, 1H), 7.60 (bs, 1H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.46-7.34 (m, 3H), 7.31 (s, 1H), 6.97 (d, *J* = 7.2 Hz, 1H), 6.45 (d, *J* = 2.2 Hz, 1H), 6.21 (bs, 1H), 3.82 (s, 3H), 3.16- 3.07 (m, 4H), 2.59- 2.62 (m, 4H), 2.38 (s, 3H), 2.17 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.22, 157.83, 157.58, 140.49, 140.24, 139.22, 129.93, 121.96, 121.89, 119.40, 118.05, 116.98, 113.73, 113.70, 111.36, 108.14, 100.44, 55.76, 55.25, 50.06, 46.12, 24.86; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{24}H_{28}O_3N_6Cl$, 483.19059; found, 483.19045.

2.2.7. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy) -phenyl)-2-cyanoacetamide (15a)

Compound **13** (0.58 g, 1.31 mmol) and cyanoacetic acid (0.17 g, 1.97 mmol) were dissolved in anhydrous DCM (10 mL) and cooled to 0 °C. Subsequently, EDC.HCl (0.38 g, 1.97 mmol), HOBt (0.27 g, 1.97 mmol) and DIPEA (0.68 mL, 3.95 mmol) were added. The reaction mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with DCM (20 mL) and washed with saturated aqueous sodium bicarbonate solution (10 mL), saturated aqueous ammonium chloride solution (10 mL) and water (10 mL). The organic layer was dried over anhydrous sodium sulfate and the volatiles were removed under reduced pressure. The crude product was purified by flash silica gel chromatography to obtain 0.60 g of compound **15a** (1.18 mmol, 90%) as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.53 (s, 1H), 8.34 (s, 1H), 8.10 (s, 1H), 7.49 (s, 1H), 7.47-7.37 (m, 2H), 7.23 (d, J = 8.5 Hz, 1H), 7.04-6.93 (m, 1H), 6.53 (d, J = 2.2 Hz, 1H), 6.20 (bs, 1H), 3.93 (s, 2H), 3.74 (s, 3H), 3.13-3.01 (m, 4H), 2.52-2.44 (m, 4H), 2.26 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 164.02, 163.62, 161.26, 158.31, 157.98, 155.53, 152.21, 139.54, 130.19, 129.95, 119.54, 117.28, 116.37, 115.72, 112.60, 106.44, 103.97, 99.89, 55.52, 54.47, 48.42, 45.50, 26.79; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{25}H_{27}O_3N_7Cl$, 508.18584; found, 508.18593.

General procedure I for the preparation of compounds **5a**-**5i**: To a solution of an aldehyde of choice in anhydrous ethanol was added intermediate **15a** and piperidine. The reaction mixture was stirred at 80 °C for 18 h and then allowed to cool to room temperature. Excess of solvent was removed *in vacuo* and the remaining residue was dissolved in ethyl acetate (10 mL). The organic layer was washed with water (10 mL), dried over anhydrous sodium sulfate and evaporated to give the crude product as pale yellow oil. The crude was purified by flash silica gel chromatography to yield the desired compounds.

2.2.8. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-2-cyano-3-(4-fluorophenyl)acrylamide (5a)

Compound **5a** was prepared as described in **general procedure I** using 4-fluorobenzaldehyde (0.018 g, 0.14 mmol), **15a** (0.050 g, 0.10 mmol), piperidine (1.5 µL, 0.014 mmol) and anhydrous ethanol (2 mL). The crude was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.018 g of **5a** (0.003 mmol, 30%) as yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36 (s, 1H), 8.23 (s, 1H), 8.08 (s, 1H), 8.00 (dd, J = 8.5, 5.3 Hz, 2H), 7.62 (s, 2H), 7.56 (d, J = 8.1 Hz, 1H), 7.47 (t, J = 8.1 Hz, 1H), 7.43 (bs, 1H), 7.20 (t, J = 8.5 Hz, 2H), 7.07 (d, J = 8.0 Hz, 1H), 6.44 (d, J = 1.9 Hz, 1H), 6.22 (bs, 1H), 3.80 (s, 3H), 3.15-3.07 (m, 4H), 2.62-2.52 (m, 4H), 2.34 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 187.12, 166.48, 164.48, 158.09, 157.94, 157.60, 153.11, 152.77, 148.84, 147.26, 138.15, 133.52, 133.44, 130.03, 128.15, 121.79, 119.40, 117.58, 116.97, 116.79, 114.66, 108.02, 103.56, 100.31, 55.72, 55.19, 50.04, 46.16; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{32}H_{30}O_3N_7ClF$, 614.20772; found, 614.20877.

2.2.9. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-2-cyano-3-(4-(methylthio)phenyl)acrylamide (5b)

Compound **5b** was prepared as described in **general procedure I** using **15a** (0.050 g, 0.10 mmol), 4-(methylthio)benzaldehyde (0.022 g, 0.15 mmol), piperidine (1 µL, 0.01 mmol) and anhydrous ethanol (2 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.034 g of **5b** (0.053 mmol, 53%) as a pale-yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.32 (s, 1H), 8.23 (s, 1H), 8.05 (s, 1H), 7.89 (d, J = 8.5 Hz, 2H), 7.62 (t, J = 1.9 Hz, 2H), 7.56 (d, J = 8.1 Hz, 1H), 7.46 (t, J = 8.1 Hz, 1H), 7.43 (s, 1H), 7.30 (d, J = 8.5 Hz, 2H), 7.06 (dd, J = 8.1, 1.5 Hz, 1H), 6.44 (d, J = 2.3 Hz, 1H), 6.23 (s, 1H), 3.81 (s, 3H), 3.21- 3.02 (m, 4H), 2.61- 2.58 (m, 4H), 2.54 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.78, 158.80, 158.20, 157.89, 153.67, 153.36, 149.14, 147.45, 141.45, 138.55, 131.61, 130.27, 128.19, 125.93,

122.10, 119.74, 119.49, 117.76, 117.68, 116.17, 114.89, 108.42, 102.21, 100.63, 55.98, 55.46, 50.24, 46.35, 15.07; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{33}H_{33}O_3N_7ClS$, 642.20486; found, 642.20579.

2.2.10. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-2-cyano-3-(4-nitrophenyl)acrylamide (5c)

Compound **5c** was prepared as described in **general procedure I** using **15a** (0.060 g, 0.12 mmol), 4-nitrobenzaldehyde (0.017 g, 0.12 mmol), piperidine (1.2 µL, 0.01 mmol) and anhydrous ethanol (2 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98.5/1.5, v/v) as eluent to obtain 0.065 g of **5c** (0.10 mmol, 84%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (s, 1H), 8.48-8.31 (m, 4H), 8.17 (d, J = 8.1 Hz, 2H), 8.13 (s, 1H), 7.71-7.57 (m, 2H), 7.49 (t, J = 7.8 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 7.08 (d, J = 7.7 Hz, 1H), 6.53 (s, 1H), 6.22 (s, 1H), 3.73 (s, 3H), 3.12-2.98 (m, 4H), 2.50-2.34 (s, 4H), 2.20 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.61, 160.03, 158.04, 152.15, 148.92, 148.74, 139.31, 137.87, 131.07, 129.95, 124.68, 124.32, 123.68, 119.52, 117.92, 117.65, 117.61, 115.44, 113.82, 110.90, 106.41, 103.88, 99.87, 55.54, 54.57, 48.54, 45.68; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{32}H_{30}O_5N_8Cl$, 641.20222; found, 641.20295.

2.2.11. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-2-cyano-3-(3-nitrophenyl)acrylamide (5d)

Compound **5d** was prepared as described in **general procedure I** using **15a** (0.060 g, 0.12 mmol), 3-nitrobenzaldehyde (0.017 g, 0.12 mmol), piperidine (1.2 µL, 0.01 mmol) and anhydrous ethanol (2 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98.5/1.5, v/v) as eluent to obtain 0.064 g of **5d** (0.1 mmol, 83%) as yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.69 (s, 1H), 8.80 (s, 1H), 8.49-8.36 (m, 2H), 8.37-8.30 (m, 2H), 8.13 (s, 1H), 7.90 (t, J = 8.0 Hz, 1H), 7.71-7.58 (m, 2H), 7.49 (t, J = 8.1 Hz, 1H), 7.25 (d, J = 8.7 Hz, 1H), 7.08 (d, J = 7.8 Hz, 1H), 6.53 (s, 1H), 6.20 (s, 1H),

3.74 (s, 3H), 3.13-2.99 (m, 4H), 2.50-2.38 (m, 4H), 2.20 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.65, 160.06, 158.31, 158.09, 152.14, 148.82, 148.43, 148.10, 139.33, 135.80, 133.32, 131.03, 129.95, 126.54, 124.36, 119.54, 117.92, 117.59, 115.47, 113.83, 113.70, 109.87, 109.83, 106.40, 103.97, 99.85, 55.54, 54.56, 48.52, 45.65; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{32}H_{30}O_5N_8Cl$, 641.20222; found, 641.20323.

2.2.12. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-2-cyano-3-(4-(dimethylamino)phenyl)acrylamide (5e)

Compound **5e** was prepared as described in **general procedure I** using **15a** (0.050 g, 0.10 mmol), 4-(dimethylamino)benzaldehyde (0.022 g, 0.15 mmol), piperidine (1.5 µL, 0.01 mmol) and anhydrous ethanol (2 mL). The precipitate was filtered and washed with cold ethanol (10 mL) to obtain 0.045 g of **5e** (0.07 mmol, 70%) as bright yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (d, J = 2.4 Hz, 2H), 7.99 (s, 1H), 7.91 (d, J = 9.0 Hz, 2H), 7.64 (s, 2H), 7.54 (d, J = 8.2 Hz, 1H), 7.43 (t, J = 8.1 Hz, 2H), 7.02 (dd, J = 8.1, 1.4 Hz, 1H), 6.70 (d, J = 9.0 Hz, 2H), 6.44 (d, J = 2.2 Hz, 1H), 6.24 (s, 1H), 3.81 (s, 3H), 3.11- 3.08 (m, 10H), 2.61-2.49 (m, 4H), 2.32 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.58, 160.21, 157.86, 157.69, 153.72, 153.67, 153.11, 148.87, 139.82, 138.88, 133.98, 129.89, 121.75, 119.68, 119.57, 119.04, 118.62, 117.25, 114.38, 111.66, 108.14, 103.87, 100.27, 95.05, 55.75, 55.28, 50.10, 46.23, 40.15; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{34}H_{36}O_3N_8Cl$, 639.25934; found, 639.26022.

2.2.13. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-2-cyano-3-(4-cyanophenyl)acrylamide (5f)

Compound **5f** was prepared as described in **general procedure I** using **15a** (0.060 g, 0.12 mmol), 4-cyanobenzaldehyde (0.015 g, 0.12 mmol), piperidine (2.0 µL, 0.02 mmol) and anhydrous ethanol (2 mL). The crude product was purified by flash silica gel chromatography

using DCM/MeOH (98.5/1.5, v/v) as eluent to obtain 0.064 g of **5f** (0.1 mmol, 86%) as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.71 (s, 1H), 8.37 (s, 2H), 8.22-7.98 (m, 5H), 7.75-7.55 (m, 2H), 7.49 (t, J = 7.7 Hz, 1H), 7.26 (d, J = 8.2 Hz, 1H), 7.08 (d, J = 7.6 Hz, 1H), 6.54 (s, 1H), 6.21 (s, 1H), 3.74 (s, 3H), 3.13-2.98 (m, 4H), 2.41-2.37 (m, 4H), 2.21 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.69, 160.06, 158.29, 158.10, 152.19, 151.09, 149.22, 148.47, 139.32, 136.14, 133.11, 130.45, 129.95, 119.53, 118.23, 117.95, 117.62, 115.48, 114.04, 113.84, 110.33, 106.42, 103.97, 100.14, 99.87, 55.55, 54.57, 48.54, 45.68; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{33}H_{30}O_3N_8Cl$, 621.21239; found, 621.21303.

2.2.14. *(E)-N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-2-cyano-3-(pyridin-4-yl)acrylamide (5g)

Compound **5g** was prepared as described in **general procedure I** using **15a** (0.060 g, 0.12 mmol), 4-pyridinecarboxaldehyde (16 µL, 0.17 mmol), piperidine (1.2 µL, 0.01 mmol) and anhydrous ethanol (2 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (96/4, v/v) as eluent to obtain 0.058 g of **5g** (0.097 mmol, 81%) as pale yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.84 (d, J = 5.3 Hz, 2H), 8.37 (s, 1H), 8.33 (s, 1H), 8.11 (s, 1H), 7.83 (d, J = 5.1 Hz, 2H), 7.70-7.61 (m, 2H), 7.50 (t, J = 8.1 Hz, 1H), 7.28 (d, J = 8.6 Hz, 1H), 7.08 (d, J = 7.9 Hz, 1H), 6.54 (s, 1H), 6.22 (bs, 1H), 3.75 (s, 3H), 3.15-3.00 (m, 4H), 2.51-2.39 (m, 4H), 2.24 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.52, 160.61, 158.91, 152.97, 151.66, 149.51, 140.08, 139.75, 130.73, 123.77, 123.44, 122.93, 120.46, 118.79, 118.43, 115.90, 114.69, 112.68, 110.58, 107.32, 104.89, 104.78, 100.78, 56.43, 55.35, 49.33, 46.39; $[M+H]^+$ calcd. for $C_{31}H_{30}O_3N_8Cl$, 597.21239; found, 597.21285.

2.2.15. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-2-cyano-3-(furan-3-yl)acrylamide (5h)

Compound **5h** was prepared as described in **general procedure I** using **15a** (0.048 g, 0.09 mmol), 3-furaldehyde (15 µL, 0.19 mmol), piperidine (1.0 µL, 0.01 mmol) and anhydrous ethanol (2 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) to obtain 0.048 g of **5h** (0.08 mmol, 89%) as brown solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (s, 1H), 8.26 (s, 1H), 8.08 (s, 1H), 7.96 (s, 1H), 7.70-7.61 (m, 2H), 7.61-7.53 (m, 2H), 7.49 (t, J = 8.1 Hz, 1H) 7.45 (bs, 1H), 7.25 (s, 1H), 7.09 (d, J = 8.0 Hz, 1H), 6.47 (d, J = 2.3 Hz, 1H), 6.24 (bs, 1H), 3.84 (s, 3H), 3.19-3.09 (m, 4H), 2.66-2.55 (m, 4H), 2.38 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.64, 160.43, 158.08, 152.07, 150.86, 146.27, 144.77, 142.49, 139.53, 137.59, 130.09, 129.81, 124.53, 120.43, 119.58, 117.61, 116.32, 115.62, 113.85, 107.54, 106.40, 105.02, 103.98, 99.91, 55.50, 54.42, 48.32, 45.46; $[M+H]^+$ calcd. for $C_{30}H_{29}O_4N_7Cl$, 586.19641; found, 586.19564.

2.2.16. *N*-(3-((5-chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-2-cyano-3-(1*H*-imidazol-5-yl)acrylamide (5i)

Compound **5i** was prepared as described in **general procedure I** using **15a** (0.050 g, 0.10 mmol), 4-formylimidazole (0.014 g, 0.15 mmol), piperidine (1.0 µL, 0.01 mmol) and anhydrous ethanol (2 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (97/3, v/v) to obtain 0.044 g of **5i** (0.075 mmol, 75% yield) as brown solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.84 (bs, 1H), 10.31 (s, 1H), 8.35 (s, 1H), 8.18 (s, 1H), 8.14 (s, 1H), 8.05 (s, 1H), 7.99 (s, 1H), 7.65 (d, J = 6.7 Hz, 2H), 7.43 (t, J = 8.4 Hz, 1H), 7.25 (d, J = 8.8 Hz, 1H), 7.01 (d, J = 7.4 Hz, 1H), 6.52 (s, 1H), 6.20 (s, 1H), 3.73 (s, 3H), 3.09-2.99 (m, 4H), 2.45-2.36 (m, 4H), 2.19 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.48, 162.07, 159.20, 158.89, 152.85, 140.61, 139.41, 139.38, 130.56, 120.31, 118.38, 118.11, 117.89, 117.48, 116.69, 114.63, 112.80, 111.37, 107.17, 104.73, 101.75, 100.68,

100.64, 56.35, 55.43, 49.40, 46.59; $[M+H]^+$ calcd. for $C_{30}H_{29}O_4N_7Cl$, 586.20764; found, 586.20699.

General procedure II for the preparation of compounds **5j**-**5m**: In a sealed tube an aldehyde of choice and piperidine were added to a solution of **15a** in acetic acid. The solution was heated to 80 °C for 0.5 h, cooled to ambient temperature and then poured into ice-water. The aqueous layer was extracted with DCM (3 x 10 mL) and the combined extract was further washed with saturated aqueous sodium bicarbonate solution (10 mL) and water (10 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by flash silica gel chromatography to obtain the desired products as a mixture of *cis/trans* isomers.

2.2.17. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-2-cyano-3-cyclopropylacrylamide (5j)

Compound **5j** was prepared as described in **general procedure II** using **15a** (0.050 g, 0.10 mmol), cyclopropanecarbaldehyde (7.3 µL, 0.10 mmol), piperidine (1 µL, 0.01 mmol) and acetic acid (1 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.046 g of **5j** (0.082 mmol, 82%) as a mixture of *cis/trans* isomers. Solely the spectroscopic data of the major isomer are reported. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (s, 1H), 7.83 (s, 1H), 7.62 (bs, 1H), 7.56 (s, 1H), 7.51 (d, J = 7.7 Hz, 1H), 7.44 (t, J = 7.8 Hz, 2H), 7.14 (d, J = 11.5 Hz, 1H), 7.04 (d, J = 7.6 Hz, 1H), 6.44 (s, 1H), 6.21 (bs, 1H), 3.81 (s, 3H), 3.-3.14 (m, 4H), 2.77-2.65 (m, 4H), 2.44 (s, 3H), 2.14-2.04 (m, 1H), 1.32-1.34 (m, 2H), 1.06-0.98 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.74, 164.50, 157.87, 157.57, 153.12, 148.88, 147.82, 146.86, 138.28, 129.98, 122.19, 119.43, 119.14, 117.58, 115.89, 114.65, 108.44, 106.77, 105.73, 100.55, 55.78, 54.96, 49.72, 45.75, 29.79, 16.42, 11.77; $[M+H]^+$ calcd. for $C_{29}H_{31}O_3N_7Cl$, 560.21714; found, 560.21751.

2.2.18. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-2-cyano-4-methylpent-2-enamide (5k)

Compound **5k** was prepared as described in **general procedure II** using **15a** (0.060 g, 0.12 mmol), isobutyraldehyde (21 µL, 0.24 mmol), piperidine (1 µL, 0.01 mmol) and acetic acid (1 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) to obtain 0.032 g of **5k** (0.057 mmol, 48%) as a mixture of *cis/trans* isomers. Solely the spectroscopic data of the major isomer are reported. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (s, 1H), 7.81 (s, 1H), 7.61 (d, J = 10.5 Hz, 2H), 7.55 (s, 1H), 7.52 (d, J = 8.5 Hz, 1H), 7.45 (t, J = 8.1 Hz, 1H), 7.42 (bs, 1H), 7.06 (d, J = 9.1 Hz, 1H), 6.46 (d, J = 2.1 Hz, 1H), 6.21 (bs, 1H), 3.82 (s, 3H), 3.13-3.11 (m, 4H), 3.00 (qd, J = 13.3, 6.6 Hz, 1H), 2.64-2.53 (m, 4H), 2.36 (s, 3H), 1.19 (d, J = 6.6 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.31, 164.51, 157.95, 157.64, 157.46, 153.13, 147.21, 138.09, 135.85, 130.03, 121.87, 119.41, 118.19, 118.17, 117.59, 114.97, 114.65, 108.53, 108.10, 100.35, 55.75, 55.23, 50.03, 46.13, 29.84, 21.58; $[M+H]^+$ calcd. for $C_{29}H_{33}O_3N_7Cl$, 562.23279; found, 562.23313.

2.2.19. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-2-cyano-4,4-dimethylpent-2-enamide (5l)

Compound **5l** was prepared as described in **general procedure II** using **15a** (0.078 g, 0.15 mmol), trimethylacetalaldehyde (34 µL, 0.30 mmol), piperidine (1.5 µL, 0.01 mmol) and acetic acid (1 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.062 g of **5l** (0.11 mmol, 73%) as a mixture of *cis/trans* isomers. Solely the spectroscopic data of the major isomer are only reported. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (s, 1H), 8.00 (s, 1H), 7.79 (s, 1H), 7.57 (s, 1H), 7.51 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 8.1 Hz, 2H), 7.26 (s, 1H), 7.06 (dd, J = 8.0, 1.2 Hz, 1H), 6.45 (d, J = 2.3 Hz, 1H), 6.21 (s, 1H), 3.82 (d, J = 3.8 Hz, 3H), 3.17-3.07 (m, 4H), 2.55-2.65 (m, 4H), 2.36 (s, 3H), 1.33 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.90, 164.53, 158.03, 157.90,

157.62, 153.12, 148.88, 147.23, 138.19, 129.97, 121.85, 119.32, 117.61, 117.10, 115.99, 114.68, 108.12, 106.46, 100.35, 99.87, 55.75, 55.18, 49.99, 46.07, 35.35, 29.06; $[M+H]^+$ calcd. for $C_{30}H_{35}O_3N_7Cl$, 576.24844; found, 576.24998.

2.2.20. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-2-cyano-3-cyclopentylacrylamide (5m)

Compound **5m** was prepared as described in **general procedure II** using **15a** (0.05 g, 0.10 mmol), cyclopentanecarbaldehyde (21 µL, 0.20 mmol), piperidine (1 µL, 0.01 mmol) and acetic acid (1 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.032 g of **5m** (0.054 mmol, 54%) as a mixture of *cis/trans* isomers. Solely the spectroscopic data of the major isomer are reported. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (s, 1H), 7.86 (s, 1H), 7.68 (d, J = 10.6 Hz, 2H), 7.55 (s, 1H), 7.51 (d, J = 7.8 Hz, 1H), 7.47-7.36 (m, 2H), 7.05 (d, J = 7.8 Hz, 1H), 6.44 (s, 1H), 6.21 (bs, 1H), 3.81 (s, 3H), 3.21-3.03 (m, 5H), 2.69-2.53 (m, 4H), 2.37 (s, 3H), 2.06-1.92 (m, 2H), 1.89-1.63 (m, 4H), 1.57-1.41 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.18, 164.50, 157.97, 157.64, 153.19, 138.07, 132.74, 130.00, 125.01, 121.88, 119.31, 117.58, 115.23, 114.65, 114.58, 108.79, 108.15, 102.95, 102.83, 100.37, 55.76, 55.21, 49.99, 45.91, 42.96, 33.19, 25.96; $[M+H]^+$ calcd. for $C_{31}H_{35}O_3N_7Cl$, 588.24844; found, 588.24740.

General procedure III for the preparation of compounds **17a-c**: To a stirred solution of an aldehyde of choice and trifluoropropionic acid in dry THF was added $TiCl_4$ at room temperature. The reaction mixture was stirred for 0.5 h, followed by drop wise addition of Et_3N. After being stirred for additional 24 h, the reaction was quenched by the addition of water (20 mL), and the obtained mixture was extracted with DCM (3 x 30 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by flash silica gel chromatography to obtain the desired products as a mixture of *cis/trans* isomers.

2.2.21. 3-(4-Fluorophenyl)-2-(trifluoromethyl)acrylic acid (17a)

Compound **17a** was prepared as described in **general procedure III** using 4-flurobenzaldehyde (0.21 g, 1.75 mmol), trifluoropropionic acid (0.15 g, 1.17 mmol), dry THF (2 mL), $TiCl_4$ (1.0 M in DCM, 1.7 mL, 1.75 mmol) and Et_3N (0.82 mL, 5.85 mmol). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.25 g of **17a** (1.07 mmol, 91%) as a mixture of *cis/trans* isomers. Solely the spectroscopic data of the single isomer are reported. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.12 (s, 1H), 7.56-7.45 (m, 2H), 7.28 (t, J = 8.8 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.87, 161.89, 146.41, 131.66 ($J_{C\text{-}F}$ = 6.5 Hz), 128.97, 123.25, 121.07, 115.42 ($J_{C\text{-}F}$ = 21.9 Hz).

2.2.22. 3-(4-(Dimethylamino)phenyl)-2-(trifluoromethyl)acrylic acid (17b)

Compound **17b** was prepared as described in **general procedure III** using 4-dimethylaminobenzaldehyde (0.24 g, 1.75 mmol), trifluoropropionic acid (0.15 g, 1.17 mmol), $TiCl_4$ (1.0 M in DCM, 1.7 mL, 1.75 mmol), Et_3N (0.82 mL, 5.85 mmol) and dry THF (3 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.19 g of **17b** (0.73 mmol, 62%) as a mixture of *cis/trans* isomers. Solely the spectroscopic data of the single isomer are reported. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.93 (s, 1H), 7.42 (d, J = 8.7 Hz, 2H), 7.25 (bs, 1H), 6.75 (d, J = 8.7 Hz, 2H), 3.01 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 189.83, 167.18, 151.26, 150.61, 132.06, 131.29, 120.63, 111.36, 39.73.

2.2.23. 3-(4-Methoxyphenyl)-2-(trifluoromethyl)acrylic acid (17c)

Compound **17c** was prepared as described in **general procedure III** using 4-methoxybenzaldehyde (0.24 g, 1.75 mmol), trifluoropropionic acid (0.15 g, 1.17 mmol), $TiCl_4$ (1.0 M in DCM, 1.7 mL, 1.75 mmol), Et_3N (0.82 mL, 5.85 mmol) and dry THF (3 mL). The crude product was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v)

as eluent to obtain 0.20 g of **17c** (0.08 mmol, 68%) as a mixture of *cis/trans* isomers. Solely the spectroscopic data of the single isomer are reported. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.04 (s, 1H), 7.45 (d, J = 8.7 Hz, 2H), 7.01 (d, J = 8.7 Hz, 2H), 3.80 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.35, 161.13, 147.27, 132.03 ($J_{C\text{-}F}$ = 2.3 Hz), 131.51, 124.40, 114.10, 113.98, 55.36.

General procedure IV for the preparation of compounds **5n-p**: Intermediate **13** and an acrylic acid of choice (**17a**-**c**) were dissolved in anhydrous DMF and cooled to 0 °C. Subsequently, EDC.HCl, HOBt and DIPEA were added and the resulting reaction mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with DCM (20 mL) and washed with saturated aqueous sodium bicarbonate solution (10 mL), saturated aqueous ammonium chloride solution (10 mL) and water (10 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was removed *in vacuo*. The crude was purified by flash silica gel chromatography to obtain the desired compounds.

2.2.24. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-3-(4-fluorophenyl)-2-(trifluoromethyl)acrylamide (5n)

Compound **5n** was prepared as described in **general procedure IV** using **13** (0.020 g, 0.045 mmol), **17a** (0.016 g, 0.068 mmol), anhydrous DMF (2 mL), EDC.HCl (0.013 g, 0.068 mmol), HOBt (0.008 g, 0.054 mmol) and DIPEA (23 µL, 0.136 mmol). The crude was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.006 g of **5n** (0.009 mmol, 20%) as yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.21 (s, 1H), 7.48-7.38 (m, 3H), 7.36-7.23 (m, 5H), 7.08-6.94 (m, 4H), 6.48 (t, J = 2.8 Hz, 1H), 6.17 (bs, 1H), 3.85 (s, 3H), 3.14-3.10 (m, 4H), 2.65 – 2.54 (m, 4H), 2.36 (s, 3H); HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{32}H_{30}O_3N_6ClF_4$, 657.19986; found, 657.20108.

2.2.25. *N*-(3-((5-chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-3-(4-(dimethylamino)phenyl)-2-(trifluoromethyl)acrylamide (5o)

Compound **5o** was prepared as described in **general procedure IV** using **13** (0.020 g, 0.045 mmol), **17b** (0.017 g, 0.068 mmol), EDC.HCl (0.013 g, 0.068 mmol), HOBt (0.008 g, 0.054 mmol), DIPEA (23 µL, 0.136 mmol) and anhydrous DMF (2 mL). The crude was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.005 g of **5o** as yellow solid (0.007 mmol, 16%).^{1}H NMR (500 MHz, $CDCl_3$) δ 8.20 (d, J = 3.9 Hz, 1H), 7.61-7.33 (m, 5H), 7.32-7.29 (m, 1H), 7.16 (d, J = 8.3 Hz, 1H), 7.10 (d, J = 8.1 Hz, 1H), 6.96 (t, J = 6.4 Hz, 2H), 6.61 (d, J = 8.3 Hz, 1H), 6.56 (d, J = 8.3 Hz, 1H), 6.47 (s, 1H), 6.21 (bs, 1H), 3.84 (s, 3H), 3.22-3.14 (m, 4H), 2.91 (s, J = 3.3 Hz, 6H), 2.74-2.63 (m, 4H), 2.42 (s, 3H); HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{34}H_{36}O_3N_7ClF_3$, 682.25148; found, 682.25264.

2.2.26. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-3-(4-methoxyphenyl)-2-(trifluoromethyl)acrylamide (5p)

Compound **5p** was prepared as described in **general procedure IV** using **13** (0.060 g, 0.13 mmol), **17b** (0.067 g, 0.27 mmol), EDC.HCl (0.039 g, 0.20 mmol), HOBt (0.022 g, 0.16 mmol), DIPEA (70 µL, 0.41 mmol) and anhydrous DMF (3 mL). The crude was purified by flash silica gel chromatography using DCM/MeOH (98/2, v/v) as eluent to obtain 0.021 g of **5p** (0.031 mmol, 24%) as yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.20 (d, J = 2.8 Hz, 1H), 7.59-7.34 (m, 5H), 7.26-7.23 (m, 2H), 7.18 (d, J = 8.0 Hz, 1H), 7.08-6.92 (m, 2H), 6.84 (d, J = 7.8 Hz, 1H), 6.79 (d, J = 8.1 Hz, 1H), 6.48 (s, 1H), 6.19 (bs, 1H), 3.85 (s, 3H), 3.75 (s, 3H), 3.20-3.07 (m, 4H), 2.66-2.54 (m, 4H), 2.38 (s, 3H).; HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{33}H_{33}O_4N_6ClF_3$, 669.21984; found, 669.22087.

2.2.27. *(E)*-3-Cyclopropylacrylic acid (20)

Malonic acid (0.29 g, 2.85 mmol), cyclopropanecarbaldehyde (0.10 g, 1.42 mmol) and piperidine (0.14 mL, 1.42 mmol) were dissolved with pyridine (1 mL) and stirred at 80 °C for 2 h. After cooling to ambient temperature the reaction mixture was stirred for further 18 h. The mixture was poured into 2 M hydrochloric acid (10 mL) and then extracted with DCM (30 mL). The organic layer was dried over anhydrous sodium sulfate, concentrated under reduced pressure and the remaining residue was purified by flash silica gel chromatography using ethyl acetate/petroleum ether (1/1, v/v) as eluent to obtain 0.067 g of **20** as off-white solid (0.597 mmol, 42%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.96 (bs, 1H), 6.30 (dd, J = 15.3, 10.2 Hz, 1H), 5.83 (d, J = 15.4 Hz, 1H), 1.72-1.36 (m, 1H), 0.89 (d, J = 5.6 Hz, 2H), 0.63-0.61 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.15, 154.02, 118.72, 14.12, 8.36.

2.2.28. *(E)*-*N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy) -phenyl)-3-cyclopropylacrylamide (21)

Intermediate **13** (0.124 g, 0.28 mmol) and acrylic acid **20** (0.031 g, 0.28 mmol) were dissolved in anhydrous DCM (4 mL) and cooled to 0 °C. Subsequently, EDC.HCl (0.080 g, 0.41 mmol), HOBt (0.044 g, 0.33 mmol) and DIPEA (0.14 mL, 0.83 mmol) were added and the resulting mixture was stirred for 18 h at room temperature. The reaction mixture was diluted with DCM (20 mL) and washed with saturated aqueous sodium bicarbonate solution (10 mL), saturated aqueous ammonium chloride solution (10 mL) and water (10 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography with DCM/MeOH (98/2, v/v) to obtain 0.053 g of the desired product **21** (0.099 mmol, 35%) as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.09 (s, 1H), 8.34 (s, 1H), 8.10 (s, 1H), 7.61 (s, 1H), 7.55 (d, J = 8.0 Hz, 1H), 7.38 (t, J = 8.1 Hz, 1H), 7.25 (d, J = 8.6 Hz, 1H), 6.91 (d, J = 7.1 Hz, 1H), 6.52 (s, 1H), 6.32 (dd, J = 15.0, 10.0 Hz, 1H), 6.18 (d, J = 15.3 Hz, 2H), 3.73

(s, 3H), 3.11-2.97 (m, 4H), 2.50-2.37 (m, 4H), 2.23 (s, 3H), 1.70-1.56 (m, 1H), 0.91 (d, J = 5.5 Hz, 2H), 0.67-0.57 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.68, 158.27, 158.00, 152.11, 150.08, 148.42, 148.34, 140.70, 129.76, 121.36, 119.55, 116.28, 116.22, 112.66, 112.57, 108.16, 106.39, 103.98, 99.84, 55.52, 54.59, 48.54, 45.70, 14.12, 8.27; HRMS-ESI (m/z): $[M+H]^+$ calcd. for $C_{28}H_{32}O_3N_6Cl$, 535.22189; found, 535.22118.

2.2.29. 2-Cyano-3-cyclopropylacrylic acid (23)

To a solution of cyclopropanecarbaldehyde (0.36 g, 5.25 mmol) in dry toluene (10 mL) was added cyanoacetic acid (0.44 g, 5.25 mmol), piperidine (1.0 mL, 10.5 mmol) and glacial acetic acid (1.2 mL, 20.9 mmol). The reaction mixture was stirred at 80 °C for 2 h and was then cooled to room temperature. Excess solvent was removed *in vacuo* and the remaining residue was dissolved in ethyl acetate (20 mL). The organic layer was washed with aqueous 1 M HCl solution (10 mL), water (10 mL), dried over anhydrous sodium sulfate and evaporated to obtain the crude oily product. Purification by silica gel flash chromatography using DCM/MeOH (98/2, v/v) as eluent yielded 0.35 g of the desired product **23** as white solid (2.55 mmol, 49%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.82 (d, J = 11.3 Hz, 1H), 3.96 (bs, 1H), 1.81 (dd, J = 7.7, 3.7 Hz, 1H), 1.20- 1.04 (m, 2H), 0.86- 0.75 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.38, 163.44, 117.29, 111.10, 14.74, 9.76.

2.2.30. 2-Cyano-3-cyclopropylpropanoic acid (24)

Pd/C (0.05 g, 10 wt. %) was suspended in anhydrous MeOH (4 mL) and to that a solution of acrylic acid **23** (0.25 g, 1.82 mmol) in MeOH (2 mL) was added. The atmosphere of the reaction mixture was exchanged for H_2 using a balloon equipped with a needle adapter and the suspension was stirred at room temperature for 5 h. The crude reaction mixture was diluted with ethyl acetate (10 mL) and filtered through celite. The filtrate was concentrated *in vacuo* to afford 0.25 g compound **24** (1.80 mmol, 99%) as colorless oil which was used without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.75 (bs, 1H), 3.46-3.28 (m,

1H), 1.87-1.54 (m, 2H), 1.46-1.17 (m, 2H), 0.85-0.68 (m, 1H), 0.47-0.33 (m, 1H), 0.19-0.07 (m, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.05, 121.66, 35.66, 9.82, 5.32, 4.84.

2.2.30. *N*-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)-phenyl)-2-cyano-3-cyclopropylpropanamide (25)

Intermediate **13** (0.080 g, 0.18 mmol) and acrylic acid **24** (0.038 g, 0.27 mmol) were dissolved in anhydrous DMF (2 mL) and cooled to 0 °C. Subsequently, EDC.HCl (0.070 g, 0.36 mmol), HOBt (0.030 g, 0.21 mmol) and DIPEA (0.12 mL, 0.72 mmol) were added and the resulting mixture was stirred for 18 h at room temperature. The reaction mixture was diluted with DCM (20 mL) and washed with aqueous sodium bicarbonate solution (10 mL), saturated aqueous ammonium chloride solution (10 mL) and water (10 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was removed *in vacuo*. The crude product was purified by flash silica gel chromatography using DCM/MeOH (97/3, v/v) as eluent to obtain 0.054 g of the desired product **25** (0.096 mmol, 53%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.56 (s, 1H), 8.35 (s, 1H), 8.08 (s, 1H), 7.52-7.50 (m, 1H), 7.49-7.40 (s, 2H), 7.23 (d, J = 8.6 Hz, 1H), 7.02 (d, J = 7.9 Hz, 1H), 6.53 (d, J = 2.3 Hz, 1H), 6.18 (bs, 1H), 3.99-3.87 (m, 1H), 3.74 (s, 3H), 3.11-3.03 (m, 4H), 2.51-2.43 (m, 4H), 2.25 (s, 3H), 1.95-1.77 (m, 2H), 1.37-1.29 (m, 1H), 0.91-0.81 (m, 2H), 0.52-0.51 (m, 1H), 0.24-0.13 (m, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.13, 164.03, 163.62, 157.98, 152.21, 148.24, 139.37, 129.94, 119.52, 118.22, 118.16, 117.44, 116.62, 112.88, 109.89, 106.37, 103.87, 99.85, 55.51, 54.52, 48.47, 45.57, 34.75, 13.59, 8.41, 4.45, 3.91; ESI-MS (*m/z*) calcd. for $C_{28}H_{32}O_3N_6Cl$, 562.0; found 562.2.

2.3. Activity-based assay for IC_{50} determination.

IC_{50} determinations for $EGFR^{WT}$ and its mutants (Invitrogen, Lot# 279551C for $EGFR^{L858R}$ and Invitrogen, Lot# 350247C for $EGFR^{L858R/T790M}$) were performed with the HTRF KinEASE-TK

assay from Cisbio according to the manufacturer's instructions. An artificial substrate peptide was phosphorylated by EGFR. After completion of the reaction, an anti-phosphotyrosine antibody labeled with europium cryptate and streptavidin labeled with the fluorophore XL665 were added. FRET between europium cryptate and XL665 was measured to quantify the phosphorylation of the substrate peptide. ATP concentrations were set at their respective K_m-values (2 µM for $EGFR^{WT}$, 9 µM for $EGFR^{L858R}$ and 4 µM for $EGFR^{L858R/T790M}$) while a substrate concentration of 500 nM, 225 nM and 200 nM, respectively, was used. Kinase and inhibitor were preincubated for 30 min before the reaction was started by addition of ATP and substrate peptide. A Tecan infinite M1000 plate reader was used to measure the fluorescence of the samples at 620 nm (Eu-labeled antibody) and 665 nm (XL665 labeled streptavidin) 60 µs after excitation at 317 nm. The quotient of both intensities for reactions made with eight different inhibitor concentrations was fit to a Hill four-parameter equation to determine IC_{50} values. Each reaction was performed in duplicate, and at least three independent determinations of each IC_{50} were made.

2.4. Protein expression and purification.

2.4.1. cSrc

Protein expression and purification of mutant cSrc (T338M, S345C) was performed as described elsewhere utilizing a construct that includes kinase domain residues 251–533.[29-31]

2.4.2. Construct Design of $EGFR^{WT}$.

DNA encoding residues comprising the kinase domain of human EGFR (uniprot entry P00533, residues 702-1016) were synthesized (GeneArt, life technologies) including an N-terminal His_6-tag, eGFP and a recognition site for HRV 3C protease (LEVLFQGP). The construct was cloned into pIEX/Bac3 expression vector (Merck Millipore), using *NcoI* and *Bsu36I* restriction sites, for deployment in *BacMagic* expression system (Merck Millipore).

Transfection, virus generation and amplification was carried out in *Spodoptera frugiperda* cell line Sf21 following the *BacMagic* protocol.

2.4.3 EGFR^{WT} protein expression and purification.

The protein was expressed in Sf9 cells using the BacMagic system. Following protein expression the cells were harvested (3000 x g, 10 min), resuspended in buffer A (50 mM TRIS-HCl, 500 mM NaCl, 5% glycerol, 1 mM DTT, pH 8) and homogenized by french press. The lysate was cleared by centrifugation at 40.000 x g for 1 h and loaded on a column packed with Ni-NTA Superflow resin (Qiagen). The elution was done with a gradient of buffer B (buffer A + 500 mM imidazole) from 0-250 mM imidazole. HRV 3C cleavage was carried out by dialysis against buffer C (20 mM TRIS-HCl, 150 mM NaCl, 2% glycerol, 1 mM DTT, 1 mM EDTA pH 7.5) overnight at 8 °C. For improved cleavage, 0.01%-0.1% (v/v) Triton X-100 were added to the sample. Subsequently, the NaCl concentration of the preparation was adjusted to 50-80 mM by dialysis or dilution and the protein solution was loaded on a HiTrap-Q-HP column (GE Healthcare). The protein was eluted with buffer D (buffer C + 1 M NaCl) with a gradient of 0-250 mM NaCl. For the final purification step the fractions containing EGFR were combined, concentrated and applied to a HiLoad 16/600 superdex 75 pg column (GE Healthcare) in buffer E (25 mM TRIS, 250 mM NaCl, 1 mM DTT, 10% Glycerol, pH 8). The purified protein was concentrated to 1.74 mg/mL and stored at -80 °C until further use.

2.5. Mass spectrometry experiments.

We used the drug-resistant mutant variant of cSrc (T338M, S345C) for mass spectrometry experiments as a model system for EGFR^{T790M}. We incubated 52 µM of protein with 100 µM of inhibitor in buffer (25 mM TRIS, 250 mM NaCl, 10% glycerol, 1 mM TCEP, pH 8) on ice for 1 h. We analyzed the aliquots by mass spectrometry using a Finnigan LCQ Advantage Max mass spectrometer from Thermo. Deconvolution and visualization of the generated data was performed using MagTran (freeware).

3. Results and discussion

3.1. Structure-based design of covalent reversible inhibitors (CRIs)

The basic idea of our CRI design approach was aimed for the amalgamation of the superior inhibitory properties of the highly $EGFR^{T790M}$ mutant selective WZ4002 scaffold and the potential benefits of covalent-reversible electrophiles. Thus, we designed a series of CRIs by modifying the Michael acceptor of WZ4002 (Fig. 1C), in a way that allows the electron deficient electrophile to reach out for Cys797 of EGFR without disrupting the overall binding mode of the parent inhibitor (Fig. 1D). Designed covalent-reversible WZ4002-analogues (**5**) equipped with various tuned electrophiles were modeled into the ATP binding pocket of $EGFR^{T790M}$ (PDB-code: 3IKA) according to the binding mode of the parent inhibitor. Similar to WZ4002, we expected the CRIs to bind to the active conformation of the kinase, with both the C-helix and the DFG-motif in inward conformation resembling the active state of the kinase domain. The aniline pyrimidine core forms bidentate hydrogen bond interactions with the backbone amide of hinge residue Met793 and the chlorine substituent on the pyrimidine core directs towards the gatekeeper.[23] Our optimization efforts were directed towards the extension of the WZ4002 core at the β-carbon of the Michael acceptor with various aromatic (**5a**-**i**) and aliphatic (**5j**-**n**) substituents (Table 1). We speculated that small groups would have potential advantages over bulkier substituents due to potential steric repulsions with protein surface around Arg841. We contemplated that the introduction of polar motifs (e.g., pyridyl) may lead to beneficial charged interactions with the side chain of Asn800 of EGFR.[32] We predicted that an additional interaction in this area would further strengthen the ligand's affinity towards the gatekeeper mutant.

3.2. Synthesis of a focused CRI-library

We synthesized a focused library of EGFR inhibitors (Table 1) utilizing WZ4002 as lead structure by utilizing a generic route to obtain the common intermediate **13** (Scheme 1).[11] Subsequently, we altered the Michael-acceptor by the introduction of various covalent-reversible warheads and further derivatized the *β*-carbon of the resulting 2-substituted acrylamide with suitable aliphatic and aromatic groups to explore the associated structure-activity relationships (SARs). Initially, 3-nitrophenol was regio-selectively attached at the 4-position of 2,4,5-trichloropyrimidine to obtain aryl ether **8** (Scheme 1). In parallel, 5-fluoro-2-nitroanisole was decorated with *N*-methylpiperizine at the 5-position. Following reduction of the nitro group led to compound **11,** which was coupled under acidic condition with aryl ether **8** to obtain the core intermediate **12**. The nitro group of **12** was reduced to obtain the corresponding amine **13**, which was coupled with cyanoacetic acid and 3,3,3-trifluoropropanoic acid to the corresponding amide using standard coupling conditions (EDC, HOBt, DIPEA) to obtain key intermediates **15a** and **15b** respectively. Next, we performed an aldol condensation of compound **15a** with various commercially available aldehydes to generate our designed CRIs. The aromatic aldehydes underwent efficient aldol condensation with compound **15a** using piperidine as base in refluxing ethanol to afford the desired products **5a**-**i** predominantly as *trans*-olefins.[33] The exclusive *E*-configuration was exemplarily assigned for CRI **5g** by NMR experiments (data not shown), which clearly illustrated the NOE cross peak between the amide proton of the cyano acrylamide and the olefin proton (Scheme 1). Equivalent signals were not observed for pyridyl and amide protons thus confirming *E*-configuration for our designed CRIs. Only in few cases a small amount of *cis*-olefin was observed as detected by LC-MS and NMR (data not shown). Tool compound **14** was prepared by treating compound **13** with acetic anhydride and DIPEA.

Although aldol condensations using aromatic aldehydes were successful, analogous reactions using aliphatic aldehydes did not result in the desired products. Constitutive studies revealed

that strong dehydrating reaction conditions are required for the complete formation of the olefin from the respective intermediate. Reaction conditions using various bases such as piperidine, DBU, Et_3N and KH_2PO_4 were applied, but did not translate into the formation of the desired reaction products (Table 2). However, the use of aqueous ammonia or ammonium acetate led to the formation of traces of the aldol products. Ultimately, conditions utilizing catalytic amounts of piperdine and excess of acetic acid in refluxing benzene afforded the desired condensation product in moderate yield (65%) after heating overnight at 80 °C. Further optimizations led to the execution of the aldol condensations in neat acetic acid as solvent with an equivalent amount of piperidine, **15a** and the aliphatic aldehyde of choice to afford the desired condensation products **5j-m** in good yields (90%).

Having successfully synthesized the 3-substituted-2-cyanoacrylamides, we aimed for the synthesis of a novel class of CRIs disclosing a trifluoromethyl- instead of cyano-functionality as electron withdrawing group. The aldol condensation of aromatic aldehydes with compound **15b** to produce the desired products by applying the previously used method was likewise unsuccessful. Hence, **5n-p** were synthesized in a two-step process from amine precursor **13** (Scheme 2). Initially, the 3-aryl-2-trifluoromethylacrylic acids were prepared by aldol condensation of appropriate aromatic aldehydes with 3,3,3-trifluoropropanoic acid using $TiCl_4$ in DCM as solvent. Subsequently, the 3-aryl-2-trifluoromethylacrylic acids were coupled to the amine **13** using EDCI and HOBt in DMF to obtain the desired products **5n-p** in good yields (80-90%).

Tool compound **21** lacking a substituent at the α-carbon was likewise prepared in two steps. Therefore the condensation product **20** of malonic acid and cyclopropane carboxaldehyde was coupled to amine **13** by utilizing EDC/HOBt coupling conditions (Scheme 3). The *trans*-geometry of compound **20** was confirmed by the coupling constant of the olefin protons in ^{1}H NMR. Similarly, cyanoacetic acid was condensed with cyclopropane carboxaldehyde to

obtain compound **23**, which was further subjected to hydrogenation to obtain compound **24**. The latter was coupled with amine **13** to obtain compound the reversible analogue **25** (Scheme 3).

3.3. Activity-based *in vitro* characterization of CRIs and tool compounds against EGFR.

To evaluate the inhibitory potential of our focused library of CRIs towards EGFR, we performed activity-based assays utilizing wildtype EGFR and the corresponding clinically relevant mutant forms L858R as well as L858R/T790M. The inhibitory activity was determined by quantification of the phosphorylation of an artificial substrate by different types of EGFR variants using homogeneous time-resolved FRET measurements. In order to confirm the suitability of the selected assay system, we evaluated the inhibitory activity of WZ4002 towards $EGFR^{WT}$, $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$ and determined IC_{50} values of 0.0156 µM, 0.00042 µM and 0.00019 µM respectively, which is consistent with reported data.[34-36]

The entity of newly designed CRIs was found to be highly selective for the $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$ variants while sparing $EGFR^{WT}$ (Table 1). We observed that the CRIs with cyano as electron withdrawing group at R_1 (**5a-m**) were more potent than the respective trifluoromethyl counterparts (**5n-p**). We speculated that the differences in electronic configurations and therefore strength of electron withdrawing-effects may account for this result. However, steric repulsions of the bulkier trifluoromethyl group with Arg841 of EGFR as compared to the linear cyano moiety might likewise contribute to this observation. Among different 3-aryl-2-cyanoacrylamides, **5b** with an electron donating group at the *para*-position of the aromatic system was equally potent as compared to derivatives which are decorated with electron withdrawing groups (**5c-d** and **5f**). However, *para-N,N*-dimethylamine derivative **5e** was found to be fully inactive among the entity of tested EGFR variants. The

para and *meta*-nitro derivatives **5c** and **5d** were found to evoke similar potency resulting in IC_{50} values of 2.3 µM and 1.8 µM towards $EGFR^{L858R}$ and 1.2 µM and 0.72 µM towards $EGFR^{L858R/T790M}$ respectively. Of note, trifluoromethyl as electron withdrawing element at R_1, inverts the influence of the R_2 electron withdrawing and donating substituents with respect to the inhibitory activity. **5n** ($R_1 = CF_3$) for instance, decorated with an electron withdrawing *p*-fluoro moiety, reveals solely weak inhibition of $EGFR^{L858R/T790M}$ ($IC_{50} = 6.3$ µM) while the respective counterpart **5a** ($R_1 = CN$, $IC_{50} = 0.12$ µM) exhibited 50-fold improved inhibition. On the contrary, an electron donating tertiary amine as in **5e** results in complete loss of inhibitory efficacy towards $EGFR^{L858R/T790M}$ in case of cyano substitution at R_1 while moderate inhibitory effects were observed in case of a trifluoromethyl substitution at R_1 as in **5o** (>10 µM vs. 2.2 µM). Notably, derivatives **5c**, **5d** and **5f** decorated with stronger electron withdrawing groups evoke significantly reduced inhibitory effects (1.2 µM, 0.72 µM and 0.94 µM respectively) which may be dedicated to the sterically demanding features of these substituents as compared to *p*-F and therefore disadvantageous interactions with the protein surface. Heterocycles as introduced in **5g** and **5i,** however, account for the most significant impact with respect to high selectivity towards $EGFR^{L858R/T790M}$ associated with strong inhibitory activity and therefore represent an appropriate compromise between ring size and withdrawing properties which is reflected in the biochemical data. Among different heterocycles, compound **5g** was found to be the most potent CRI in activity-based studies accounting for IC_{50} values of 0.15 µM and 0.037 µM for $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$ respectively. We hypothesize that the superior inhibitory activity results from the beneficial charged interaction of the 4-pyridyl moiety of **5g** with the side chain of Asn800 located at the front lip of the ATP binding pocket of EGFR (Fig. 1D). On the contrary, smaller aliphatic groups illustrate reasonable selectivity for the clinically relevant mutant over constitutively activated and wild type EGFR (e.g., **5j**: 0.083 µM vs. 0.35 µM vs. >10 µM, Table 1). Among

non-functionalized aliphatic substituents at R_2, compound **5j** with a sterically less demanding cyclopropyl group was five to ten-fold more potent as compared to its bulky analogues **5l** and **5m**. In detail, the IC_{50} values of compound **5j** against $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$ were 0.35 μM and 0.083 μM while the corresponding values for the cyclopentyl derivative **5m** were increased to 2.2 μM and 1.2 μM respectively.

The impact of the cyano group at R_1 on the inhibitory effect towards EGFR was investigated by directly comparing the inhibitory activity of CRI **5j** and its covalent counterpart **21** lacking the respective cyano functionality (Table 1). CRI **5j** was ten-fold more potent than its covalent analogue **21** with an IC_{50} of 0.35 μM and 0.083 μM compared to 2.8 μM and 0.83 μM for $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$ respectively. These observations indicate that the cyano group indeed contributes to increased inhibitory potency by rendering the *β*-carbon more susceptible to nucleophilic attack by Cys797.[26] On the contrary, the significant loss in potency of both **5j** and **21** compared to WZ4002, illustrates that substituents at the *β*-carbon of 2-cyanoacrylamide commonly are associated with unfavorable effects on the inhibitory potency towards wildtype EGFR as well as its mutant forms. However, the importance of covalent modulations for gaining potency was demonstrated by comparative analysis of the purely reversible inhibitor **25** evoking 10-fold less potent inhibition with IC_{50} values of 2.0 μM and 0.77 μM for $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$ respectively, compared to the covalent-reversible counterpart **5j** (0.35 μM and 0.083 μM). In addition, the reversible inhibitor **14** likewise displayed a loss in potency with respect to parent compound WZ4002.

3.4. Evaluation of the mode of binding of CRIs towards $cSrc^{T338M/S345C}$ as model system for $EGFR^{L858R/T790M}$ by mass spectrometry

The mode of binding of our CRIs to Cys797 of EGFR was investigated by using methods of mass spectrometry and genetically engineered cSrc mutants as model system to mimic EGFR.[31] We generated $cSrc^{T338M/S345C}$ as homology model to $EGFR^{L858R/T790M}$ and incubated the

protein with the respective compounds for 1 h on ice. Experimental details of construct design, protein expression and purification are depicted in the Material and Methods section. As a proof of concept we exemplarily investigated the ability to covalently modify wildtype cSrc as well as mutant cSrc$^{T338M/S345C}$ by analogues covering reversible (**25**), covalent (**21**) and reversible-covalent properties (**5j**). As expected, the reversible inhibitor **25** did not lead to an increase in molecular weight in case of wildtype Src as well as the cSrc$^{T338M/S345C}$ mutant variant due to the lack of an appropriate electrophile to target Cys345 (Fig. 2). The covalent inhibitor **21** likewise did not covalently modify the wildtype protein, but was demonstrated to account for a 537 Dalton mass increase of 40% of isolated cSrc$^{T338M/S345C}$, which implies for the covalent labelling of the protein with a single inhibitor molecule. These observations were expected and entirely congruent with the concept of a covalent inhibition strategy. Much to our surprise the CRI **5j** showed covalent binding to mutant cSrc with an increased mass of 560 Dalton in our mass spectrometry setup. This, however, is at odds with the concept of CRIs postulating that the covalent bond is reversibly cleaved when the ligand binding site is disintegrated e.g. in case of protein unfolding or protein lysis. Protein unfolding is to be expected under the denaturing conditions (acetonitrile and strong acid) we chose for the mass spectrometry experiments. **5j** was furthermore illustrated to lead to the formation of a sub cohort of covalently modified cSrc^{WT}. We speculate that the fine tuning of the CRIs with electron withdrawing groups potentiates the effectivity of the formation of an undesired covalent adduct by unspecific labelling of cysteines in close proximity to the active site of cSrc such as Cys280 within the glycine-rich loop of cSrc. However, addressing glycine-rich loop cysteines, amongst others in the "kinase cysteinome", by covalent inhibitors represents a further approach to selectively target other kinases than EGFR and was already demonstrated for FGFR1.[37-39]

4. Conclusions

Here we presented the design, synthesis and biochemical characterization of covalent-reversible analogues of WZ4002 as a new class of EGFR inhibitors with chemically tuned electrophiles that possess advantages of both covalent and reversible inhibition strategies. A focused library of CRIs was synthesized and biologically evaluated by activity-based studies. The entity of CRIs was found to be highly mutant selective for $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$ while sparing inhibition of $EGFR^{WT}$. Furthermore, CRIs comprising a cyano functionality (**5a** and **5e**) as an electron withdrawing element were, in general, more potent than their trifluoromethyl decorated counterparts (**5n** and **5o**). Despite this, we observed distinct SARs for the 2-cyanoacrylamide derivatives based on an increased inhibitory impact towards EGFR mutant forms accompanied by the use of small 3-substituted aliphatic groups. Different 3-aryl-2-cyanoacrylamide CRIs with electron-withdrawing and electron-donating groups were found to have similar IC_{50} values. Among 3-substituted heterocycles of 2-cyanoacrylamide CRIs, **5g** decorated with a 4-pyridyl group was found to be the most potent CRI most likely due to a charged interaction of the protonated pyridyl group with Asn800 of EGFR. The mode of binding of our CRIs to EGFR was studied by mass spectrometry utilizing the drug resistant mutant variant $cSrc^{T338M/S345C}$ as a mimic for drug resistant EGFR. This study revealed that both covalent and covalent-reversible derivatives accounted for covalent labeling of $cSrc^{T338M/S345C}$. Comparative analysis of the IC_{50} values of CRI **5j** with its covalent counterpart **21** clearly indicated that an electron withdrawing cyano group is beneficial in terms of gaining inhibitory potency. Despite this, **5j** and its reversible counterpart **25** illustrated the importance of covalent inhibition in order to gain inhibitory impact. However, our studies also revealed that the concept of covalent-reversible inhibition by the application of fine-tuned Michael acceptors likewise translates to the generation of purely covalent modulating inhibitors as we observed covalent labelling of drug resistant $cSrc^{T338M/S345C}$ for **5j**. These observations may influence future endeavors to develop covalent-reversible tool compounds and accordingly novel drugs that make use of a covalent-reversible inhibition strategy.

Acknowledgments

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Abbreviations

CRI (covalent reversible inhibitor); EDC.HCl (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride); HOBt (Hydroxybenzotriazole); DIPEA (N,N-Diisopropylethylamine); EGFR (epidermal growth factor receptor); NOE (nuclear Overhauser effect); NSCLC (non-small cell lung cancer); SAR (Structure-activity relationship)

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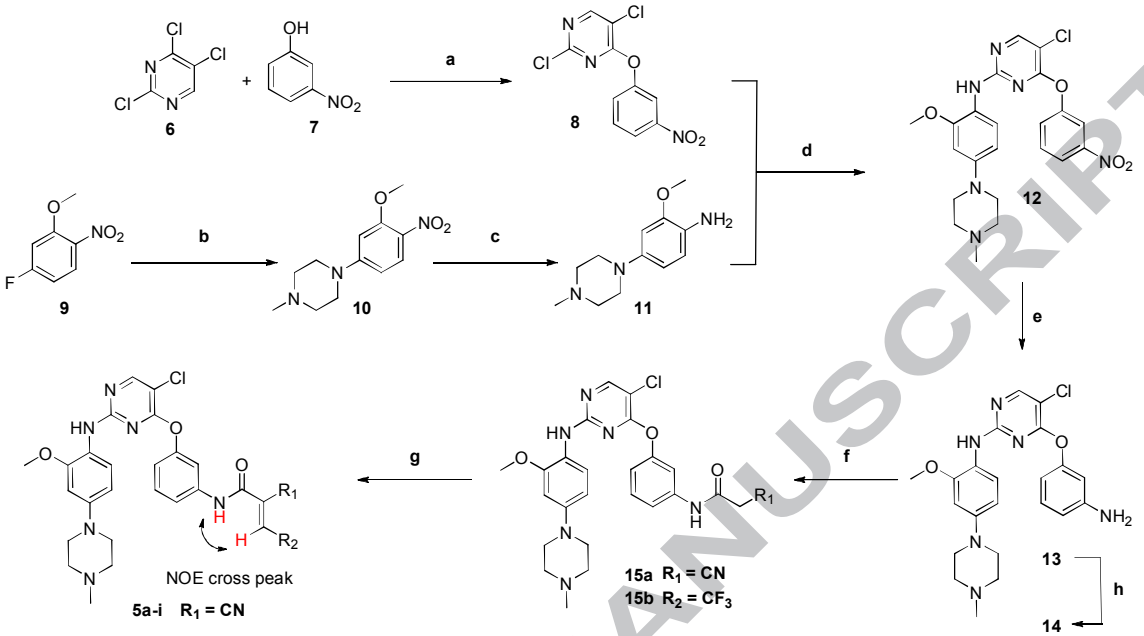
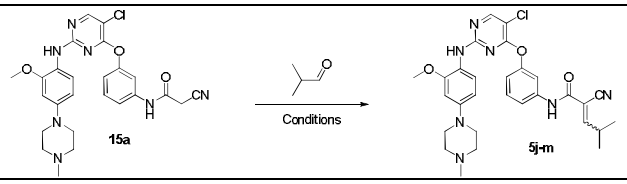
Schemes and Tables

Scheme 1. Synthesis of 3-aryl-2-cyanoacrylamide CRIs



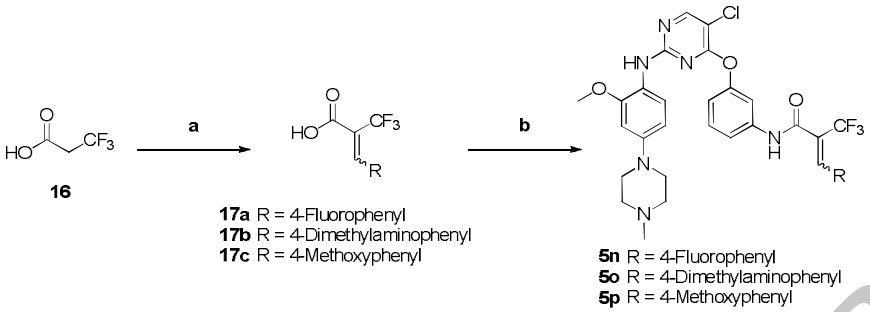
Reagents and conditions: (a) K_2CO_3, DMF, 80 °C, 8 h, 86%; (b) *N*-methylpiperizine, K_2CO_3, DMF, rt, 6 h, 98%; (c) Zn, NH_4Cl, THF, MeOH, rt, 18 h, 74%; (d) TFA, 2-BuOH, 100 °C, 18 h, 65%; (e) Zn, NH_4Cl, MeOH, THF, rt, 18 h, 91%; (f) For R_1 = CN, cyanoacetic acid, EDC, HOBt, *N,N*-diisopropylethylamine, DCM, rt, 18 h, 89%; $R_2 = CF_3$, 3,3,3-trifluoropropanoic acid, EDC, HOBt, *N,N*-diisopropylethylamine, DMF, rt, 18 h, 72%; (g) Compound **15a**, ArCHO, piperidine, EtOH, 80 °C, 8-36 h, 70-82%; (h) Acetic anhydride, Et_3N, DCM, 2 h, 86%.

Table 2. Optimization studies of aldol condensation using aliphatic aldehydes.



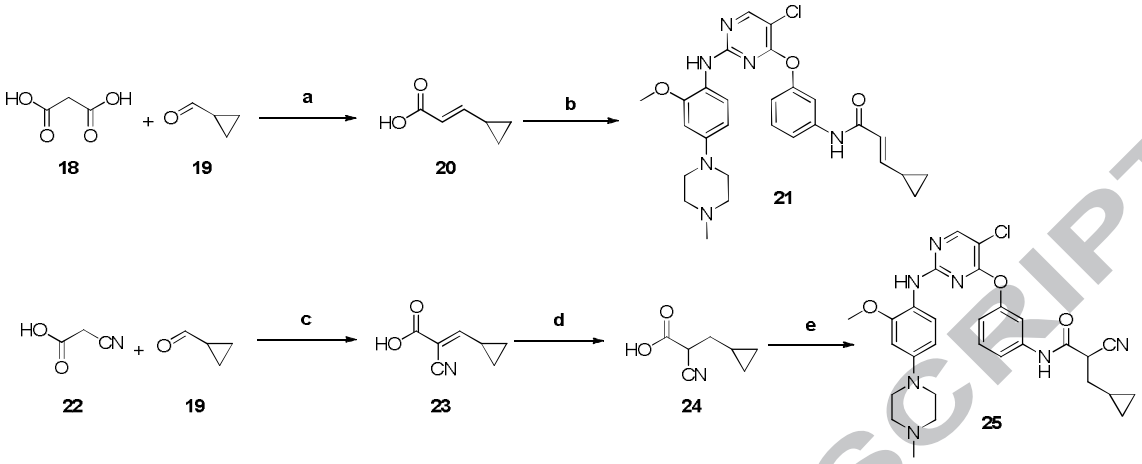
Entry	Conditions	Result/Yield
1	Piperidine, EtOH, rt to reflux	No product formation
2	DBU, THF, rt to reflux	No product formation
3	KH_2PO_4, MeOH, rt to reflux	No product formation
4	KH_2PO_4, benzene, rt to reflux	No product formation
5	Et_3N, DCM, rt to reflux	No product formation
6	Benzene, NH_4OH, reflux	Trace amount of product.
7	Benzene, NH_4OAc, reflux	Trace amount of product.
8	Benzene, AcOH, piperidine, reflux, 18 h	65%
9	AcOH, piperidine, reflux, 0.5 h	90%

Scheme 2. Synthesis of CRIs decorated with trifluoromethyl as electron withdrawing group.



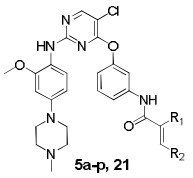
Reagents and conditions: (a) Aromatic aldehyde, $TiCl_4$, DCM, THF, rt, 80-90%; (c) **13**, EDCI, HOBt, DIPEA, DMF, rt, 18 h, 80-90%.

Scheme 3. Synthesis of covalent and reversible tool compounds.

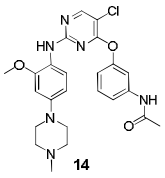


Reagents and conditions: (a) Piperidine, pyridine, 80 °C, 2 h, 60%; (b) **13**, EDC, HOBT, DIPEA, DCM, rt, 18 h, 65%; (c) Piperidine, EtOH, 80 °C, 2 h, 85%; (d) H_2, cat. Pd/C, MeOH, rt, 2 h, quant.; (e) EDC, HOBT, DIPEA, DCM, rt, 18 h, 82%.

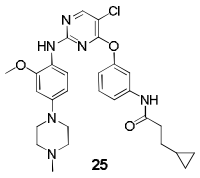
Table 1. IC_{50} determinations of CRIs and analogues on different EGFR variants



5a-p, 21



14



25

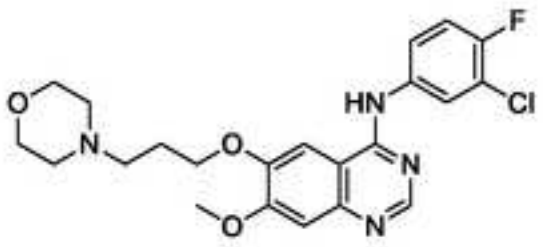
			EGFR IC_{50} [μM]		
Compound	**R_1**	**R_2**	**WT**	**L858R**	**L858R/T790M**
5a	CN		7.0 ± 2.0	0.54 ± 0.46	0.12 ± 0.06
5b	CN		4.8 ± 2.3	0.83 ± 0.65	0.51 ± 0.09
5c	CN		>10	2.3 ± 0.5	1.2 ± 0.1
5d	CN		>10	1.8 ± 0.54	0.72 ± 0.18
5e	CN		>10	>10	>10
5f	CN		>10	1.8 ± 0.7	0.94 ± 0.63
5g	CN		8.7 ± 3.3	0.15 ± 0.03	0.037 ± 0.004
5h	CN		>10	1.3 ± 0.2	0.33 ± 0.14
5i	CN		>10	0.35 ± 0.08	0.081 ± 0.022
5j	CN		7.2 ± 3.1	0.35 ± 0.10	0.083 ± 0.020
5k	CN		>10	1.2 ± 0.1	0.80 ± 0.25
5l	CN		>10	1.3 ± 0.5	0.40 ± 0.03
5m	CN		>10	2.2 ± 1.2	1.2 ± 0.6
5n	CF_3		>10	>10	6.3 ± 2.8
5o	CF_3		>10	6.3 ± 5.7	2.2 ± 0.4
5p	CF_3		>10	>10	8.9 ± 0.5
14	---	---	>10	0.49 ± 0.16	0.18 ± 0.10
21	H		5.6 ± 2.0	2.8 ± 0.4	0.83 ± 0.28
25	---	---	>10	2.0 ± 0.4	0.77 ± 0.06
WZ4002	H	H	0.016 ± 0.006	0.00042 ± 0.00023	0.00019 ± 0.00007

Figure Legends

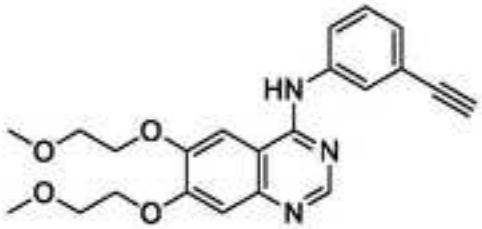
Figure 1. (A) First generation reversible quinazoline-based inhibitors for EGFR. (B) Second generation covalent inhibitors targeting drug resistant $EGFR^{T790M}$. (C) Design of new covalent-reversible inhibitors (CRIs, **5**) for EGFR. (D) CRI **5g** modeled into the ATP-binding cleft of $EGFR^{T790M}$ (modeling based on PDB-code: 3IKA). The 2-aminopyrimidine forms bidentate hydrogen bond interactions with the backbone of hinge residue Met793. The covalent-reversible Michael acceptor is located in close proximity to Cys797, which is therefore feasible for covalent bond formation. Furthermore, the protonated pyridine of the inhibitor is in proximal distance to form charged interactions with the side chain of Asn800.

Figure 2. Comparative illustration of deconvoluted mass spectra of cSrc variants treated with covalent-reversible inhibitor **5j**, covalent inhibitor **21** and the reversible inhibitor **25.** Covalent inhibitor binding was confirmed by mass increase relative to the respective DMSO control.

A

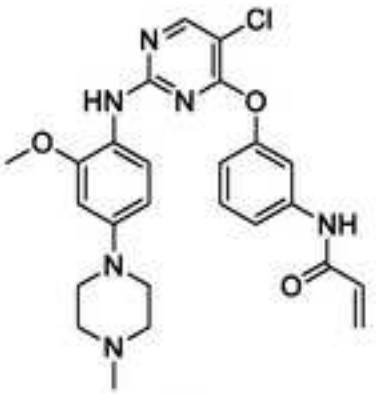


1
(Gefitinib)

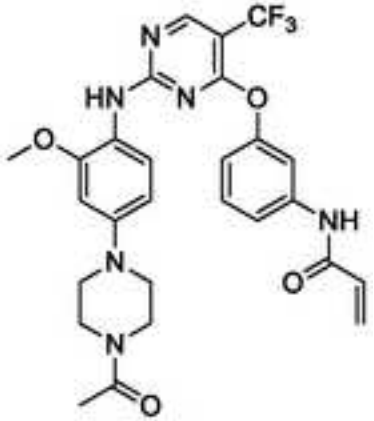


2
(Erlotinib)

B

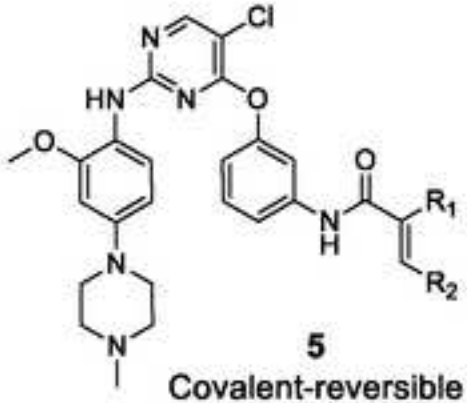


3
(WZ4002)



4
(CO-1686)

C

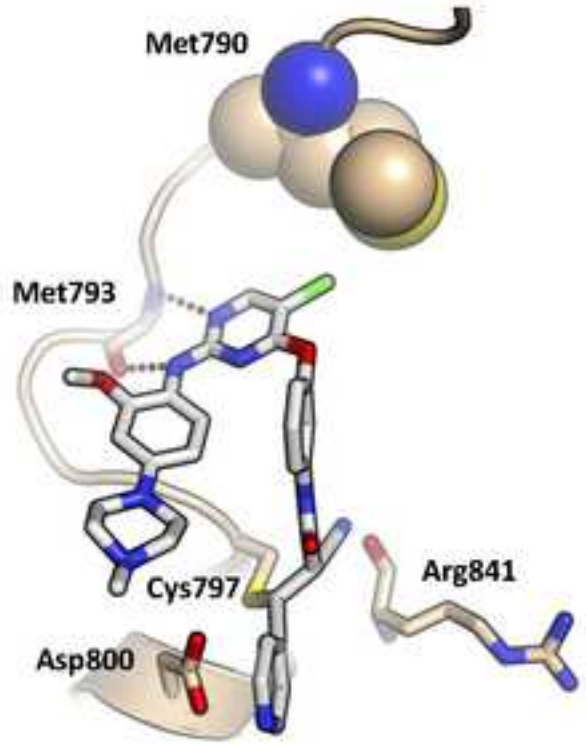


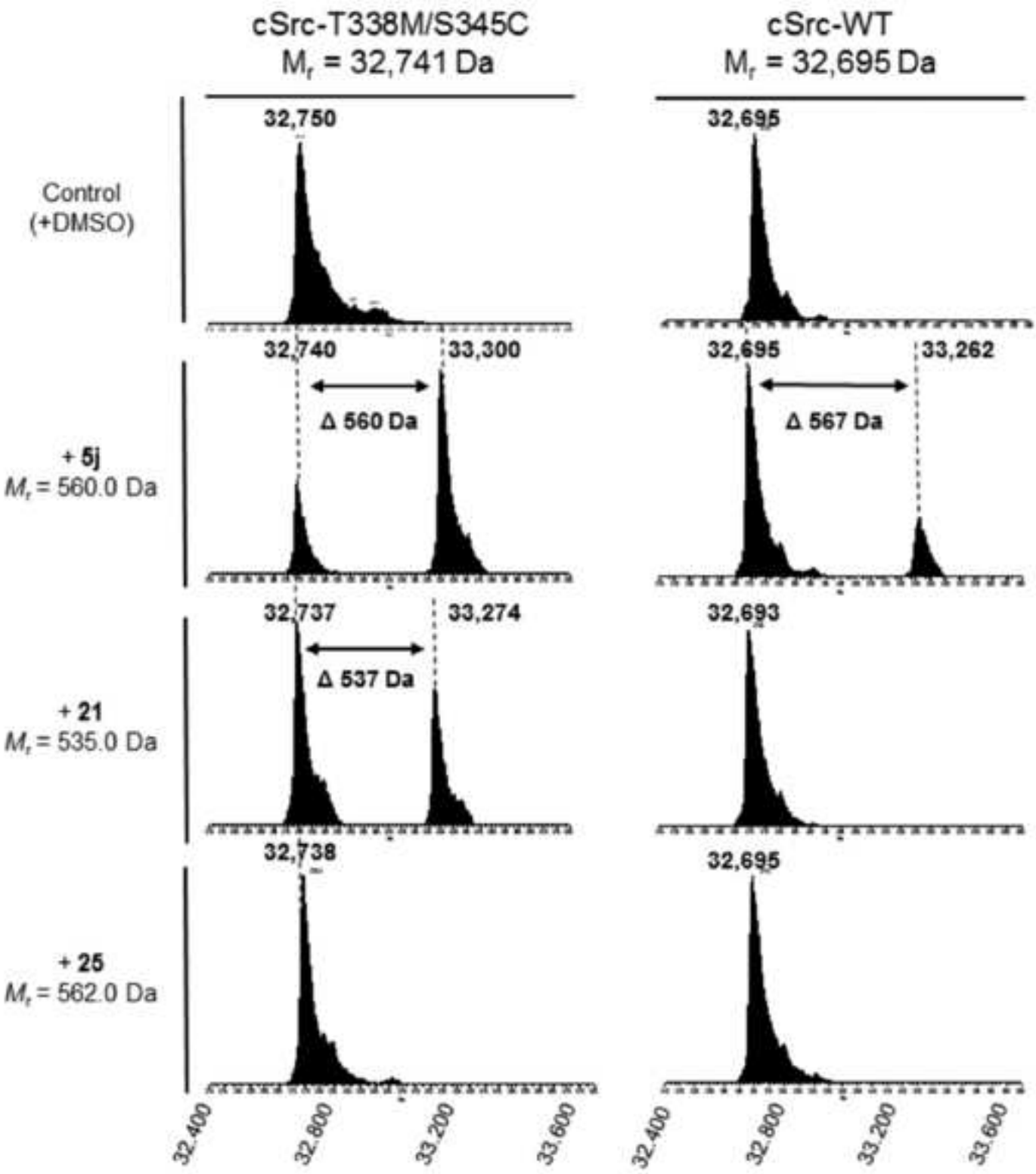
5
Covalent-reversible
inhibitors (CRIs)

R_1 = Electron withdrawing group

R_2 = Aliphatic or aryl group

D




cSrc-T338M/S345C
M$_r$ = 32,741 Da
cSrc-WT
M$_r$ = 32,695 Da
Control
(+DMSO)
32,750
32,695
+ 5j
M$_r$ = 560.0 Da
32,740
33,300
Δ 560 Da
32,695
33,262
Δ 567 Da
+ 21
M$_r$ = 535.0 Da
32,737
33,274
Δ 537 Da
32,693
+ 25
M$_r$ = 562.0 Da
32,738
32,695
32,400
32,800
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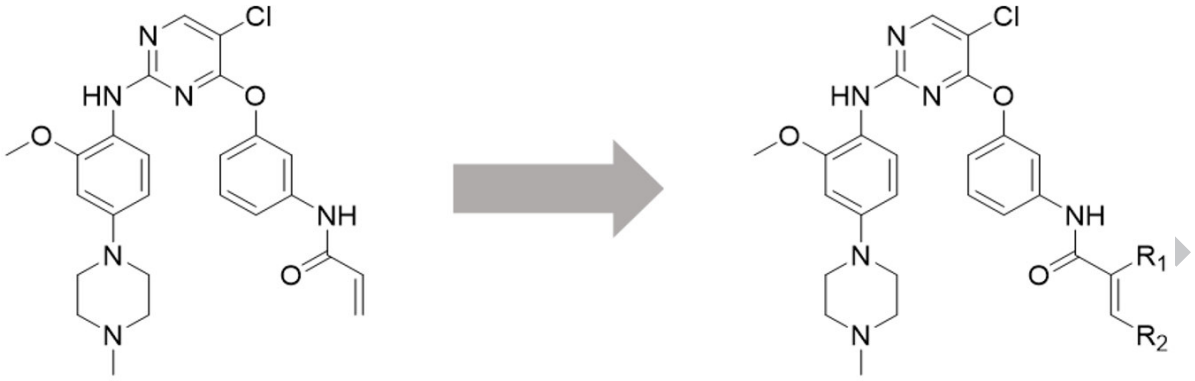
33,600
32,400
32,800
33,200
33,600



Covalent-reversible Inhibitors (CRIs)