Supporting Information for

Selective Covalent Targeting of Mutated EGFR(T790M) with Chlorofluoroacetamide-Pyrimidines

Mami Sato[1], Hirokazu Fuchida[1], Naoya Shindo[1], Keiko Kuwata[2], Keisuke Tokunaga[1], Guo Xiao-Lin[1], Ryo Inamori[1], Keitaro Hosokawa[1], Kosuke Watari[1], Tomohiro Shibata[1], Naoya Matsunaga[1], Satoru Koyanagi[1], Shigehiro Ohdo[1], Mayumi Ono[1], Akio Ojida[1]*

[1]Graduate School of Pharmaceutical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan

[2] Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Furo-cho, Chikusa, Nagoya, Japan

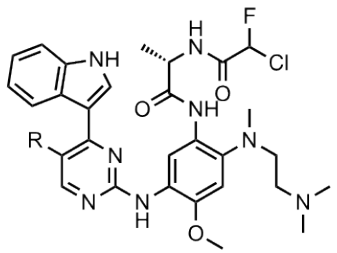


Table S1. Summary of Anti-proliferative activity against EGFR-dependent cell lines (IC_{50}, μM)[a] and cell selectivity index.

compound	**9**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**1**
R	Cl	H	F	Me	ethynyl	CN	OMe	Br	CF_3	(osimertinib)
H1975	0.031 ± 0.003	0.46 ± 0.005	0.33 ± 0.05	0.13 ± 0.01	0.23 ± 0.009	0.15 ± 0.022	0.086 ± 0.01	0.022 ± 0.002	0.015 ± 0.002	0.016 ± 0.003
H292	0.71 ± 0.15	2.71 ± 0.18	1.04 ± 0.038	1.45 ± 0.10	1.20 ± 0.036	1.10 ± 0.031	1.27 ± 0.17	1.15 ± 0.06	1.37 ± 0.018	0.22 ± 0.015
selectivity index	22.9	5.9	3.2	11.2	5.2	7.3	22.9	52.3	91.3	13.9

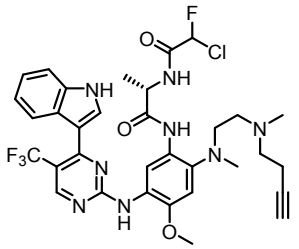
[a] Data represents mean ± standard error of triplicate experiments.

Table S2. Kinase inhibitory activities (IC_{50}, nM) of CFA inhibitor **18** and osimertinib **1**[a, b].

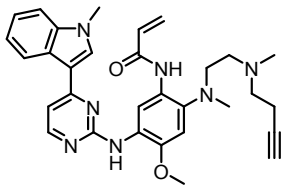
	18	**1** (osimertinib)
EGFR (T790M and L858R)	10.4	1.70
wild-type EGFR	465	6.96
selectivity index	44.7	4.1

[a] Data were obtained by off-chip mobility shift assay conducted at Carna Bioscience (Kobe, Japan). [b] Kinase activity was measured in the presence of ATP at the K_m value concentration (1.9 and 2.7 μM for the mutated EGFR and wild-type EGFR, respectively).

Table S3. Anti-proliferative activity against EGFR-dependent cell lines (IC_{50}, μM)[a] of alkyne probes **21** and **22**.



21



22

	21	**22**
H1975	0.051 ± 0.017	0.072 ± 0.024
H292	0.82 ± 0.19	2.23 ± 0.16

[a] Data represent mean ± standard error of triplicate experiments.

Table S4. Summary of high-occupancy protein targets of **18** and osimertinib (**1**).[a, b]

Gene name	Function	Cysteines
PSMD14	26S proteasome non-ATPase regulatory subunit 14	C120, C238, C299
PSMB5	Proteasome subunit beta type-5	C111, C122, C161
CTSC	Dipeptidyl peptidase 1	C24, C30, C54, C118, C146, C255, C258, C291, C298, C321, C331, C337, C355, C448
TXNDC17	Disulfide reductase	**C43**, **C46**[c], C64, C69, C110
TEX264	Reticulophagy receptor protein	C68, C92, C94, C165, C182
SCARB1	Lipid recepor protein	C3, C484, C511, C518, C530[d]
XPO1	RNA-binding protein	C34, C99, C119, C164, C199, C209, C267, C327, C369, C498, C528, C585, C595, C623, C699, C723,C829, C859, C920, C1070
CTSL	Thiol protease	C11, C135, C138, C169, C178, C211, C269, C322
HMOX2	Heme oxygenase	C127, C265, C282
RPL12	26S ribosomal RNA binding protein	C17, C141, C162
IFI30	Lysosomal thiol reductase	**C72**, **C75**[e], C117, C124, C132, C148, C162, C178, C226, C237, C248
SELENOT	Thioredoxin reductase-like protein	**C45**, **C49**[f], C129, C143

[a] ERBB2 was omitted from Table due to its well-known function and high number of cysteine residues. [b] Cysteine residues known to have function are highlighted in red. [c] Jeong, W. *et al.* Identification and Characterization of TRP14, a Thioredoxin-related Protein of 14 kDa. *J. Biol. Chem.* **2004**, *279*, 3142-3150. [d] Transmembrane and extracellular domains were excluded. [d] Transmembrane helices were excluded. [e] VanHeeke, G. e*t al. N*-terminal cysteine of human asparagine synthetase is essential for glutamine-dependent activity. *J. Biol. Chem*. **1989**, *264*, 19475-19477. [f] Dikiy, A. *et al*. SelT, SelW, SelH, and Rdx12: genomics and molecular insights into the functions of selenoproteins of a novel thioredoxin-like family. *Biochemistry*. **2007**, *46*, 6871-6882.

Table S5. Summary of SILAC ratio for EGFR in competitive SILAC experiments between H1975 cells (EGFR L858R/T790M) and H292 cells (EGFR wild-type) using probe **21** or **22**.

	$\log_2$(ratio) H1975/H292	median	SE
CFA probe **21** H1975 (Light) vs H292 (Heavy)	2.73	2.7	3.8
	2.38		
	4.30		
CFA probe **21** H1975 (Heavy) vs H292 (Light)	3.90	3.8	1.3
	3.26		
	3.76		
Michael acceptor probe **22** H1975 (Light) vs H292 (Heavy)	0.10	0.1	0.1
	0.33		
	0.02		
Michael acceptor probe **22** H1975 (Heavy) vs H292 (Light)	-0.22	-0.2	0.0
	-0.23		
	-0.03		

Table S6. Quantitative data of western blot analysis in EGFR pull-down assay upon treatment of H1975 cells (EGFR L858R/T790M) and H292 cells (EGFR wild-type) with probe **21** or **22**.

		21	**22**
H1975	elution	1.983	2.714
	input	1.000	0.8254
	elution / input	1.98	3.29
H292	elution	0.3055	2.113
	input	1.231	1.097
	elution / input	0.248	1.92
Ratio	(H1975 / H292)	8.0	1.7

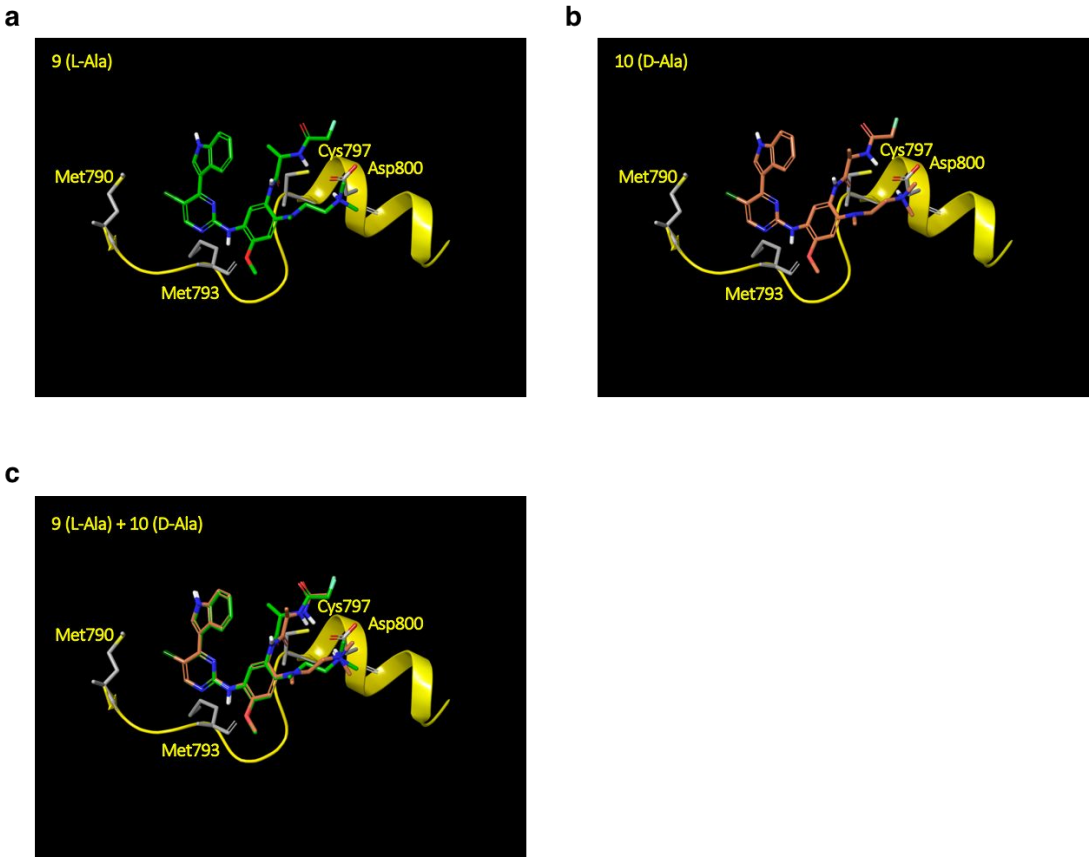


Figure S1. Modeled binding modes of the CFA-pyrimidine derivatives **9** and **10** with EGFR (T790M). In the docking study, protein structure was generated from the crystal structure (pdb code 3UG2)[1] by the protein preparation wizard in Maestro 9.8 (Schrodinger). All crystallographic water molecules were deleted. The active site was defined by manually selecting the amino acids in and around the ATP pocket. Glide was used for the protein-ligand docking in XP protocol. A 3D structure of each ligand as an initial input was generated using LigPrep/Epik in Maestro 9.8. Docked binding modes were ranked using Docking Score and manually inspected for retention of the key interactions with the hinge region residue (Met793). Binding modes were deprioritized if the conformation of the docked ligand was considered unsatisfactory.7 Yoshikawa, S. *et al*. Structural basis for the altered drug sensitivities of non-small cell lung cancer-associated mutants of human epidermal growth factor receptor. *Oncogene*, **2013**, *32*, 27–38.

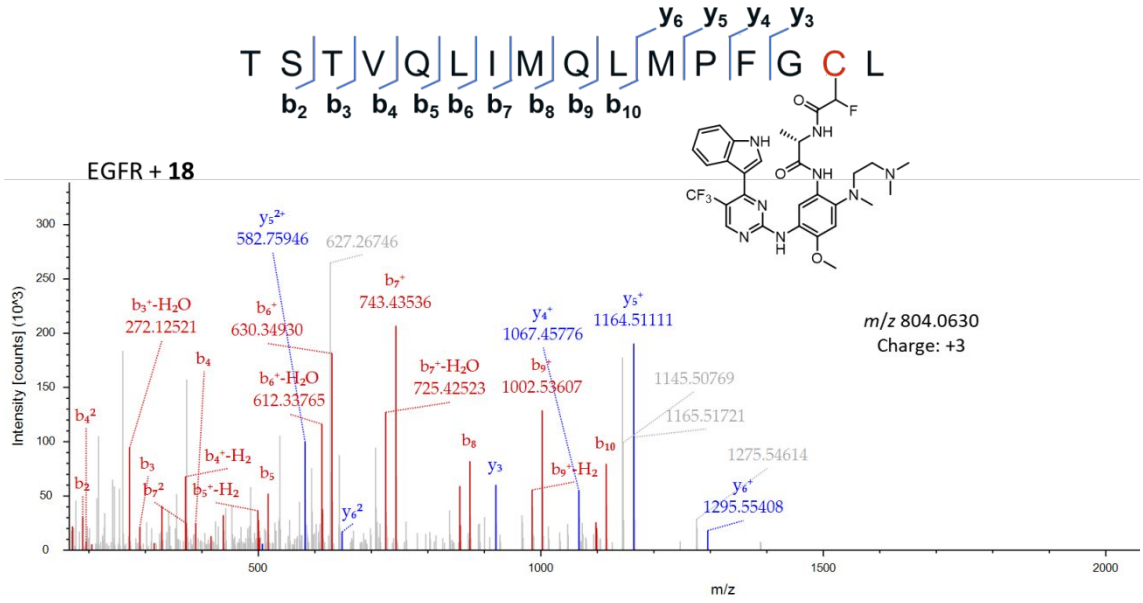


Figure S2. MS/MS analysis of reactive site of inhibitor **18** on recombinant EGFR L858R/T790M kinase domain. The kinase domain labeled with **18** was digested with trypsin/chymotrypsin and subjected to LC-MS/MS analysis. The data represents MS/MS spectrum of **18**-modified peptide fragment containing Cys797 (highlighted in red).

Figure S3. Raw images of western blot analysis. Cropped gel data are shown in **Figure 3**.

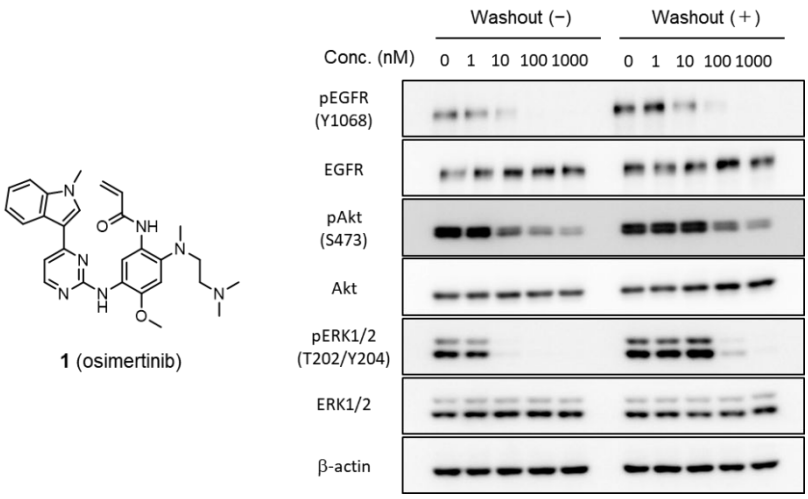


Figure S4. Western blot analysis of inhibition activity of **1** against phosphorylation of EGFR (L858R/T790M) and the related signaling proteins in H1975 cells.

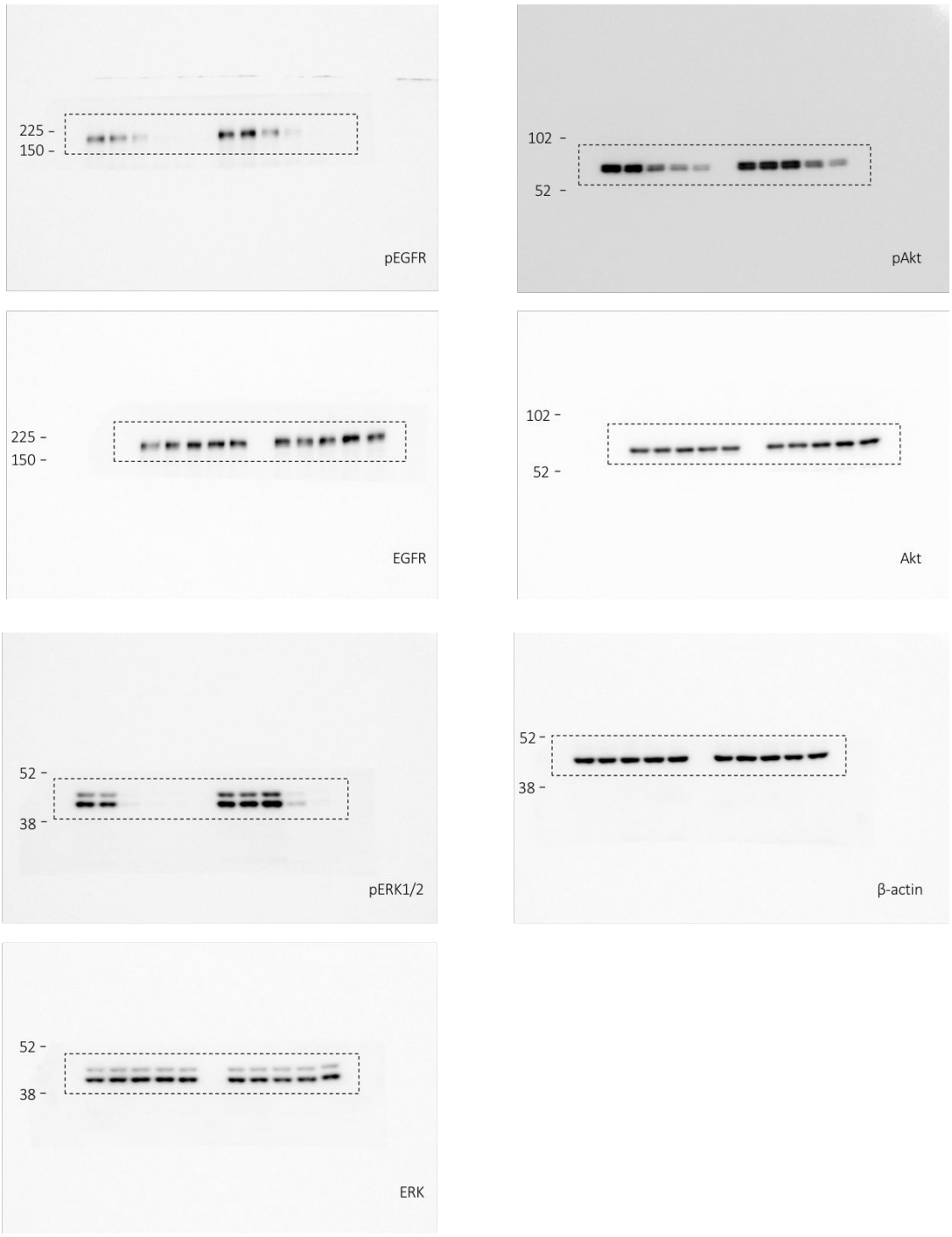


Figure S5. Raw images of western blot analysis. Cropped gel data are shown in **Figure S4**.

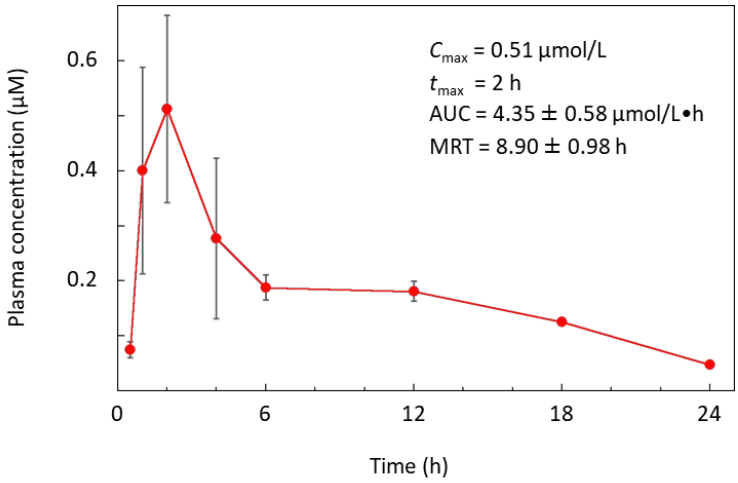


Figure S6. Mouse (BALB/c) plasma concentration profiles of CFA-pyrimidine **18** after a single oral administration (25 mg/kg). Each plot represents the mean ± standard error (n = 5). MRT: median residence time.

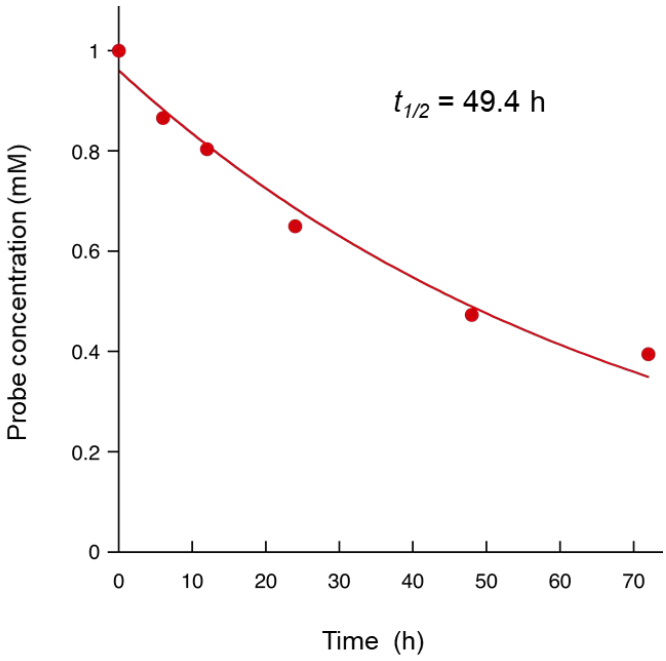


Figure S7. Time-trace of the reaction of CFA-pyrimidine **18** with glutathione. Concentration of the unreacted **18** was determined by HPLC analysis (UV absorbance at 250 nm). The plot was analyzed based on first-order reaction kinetics to yield half-reaction time ($t_{1/2}$, h). Conditions: [**18**] = 1 mM, [glutathione] = 10 mM, 100 mM phosphate buffer (pH 7.4) containing 20% acetonitrile, 37 °C. Benzoic acid was used as an internal standard.

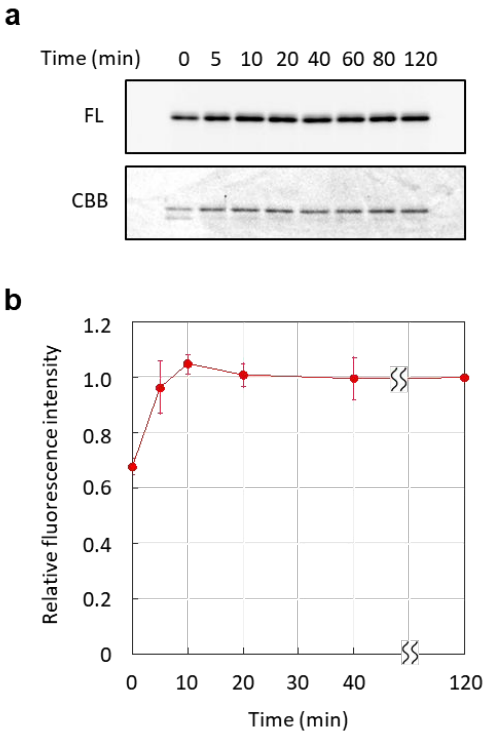
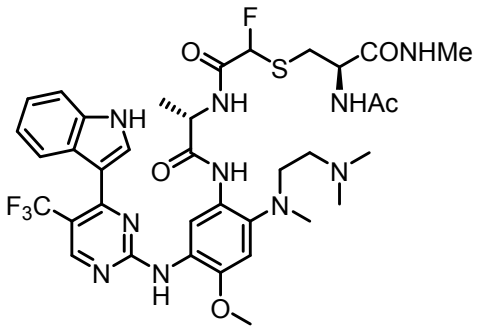


Figure S8. Reactivity profiles of probe **22** with kinase domain of mutated EGFR (L858R/T790M). (a) In-gel fluorescence (FL) and Coomassie Brilliant Blue (CBB). (b) Time plot of the relative fluorescence intensity (0.5%; mean ± s.d. obtained from three independent experiments). The fluorescence intensity at 120 min was set to arbitrary value of 1.0.



23

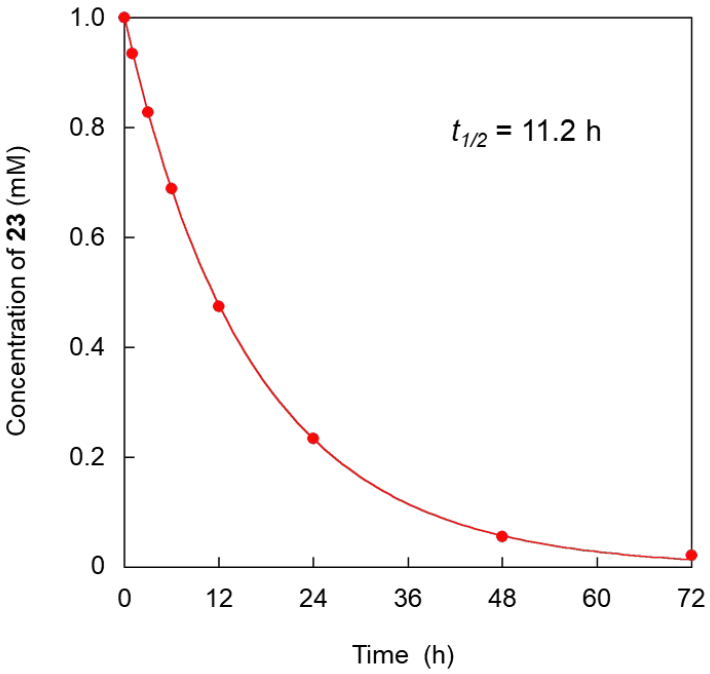


Figure S9. Time-trace of the hydrolytic degradation of CFA-pyrimidine **18**–*N*-acetylcysteine adduct **23**. Concentration of **23** was determined by HPLC analysis (UV absorbance at 250 nm). The plot was analyzed based on first-order reaction kinetics to yield half-reaction time ($t_{1/2}$, h). Conditions: [**23**] = 1 mM in 100 mM phosphate buffer (pH 7.4) at 37 °C. 1-Naphthoic acid was used as an internal standard.

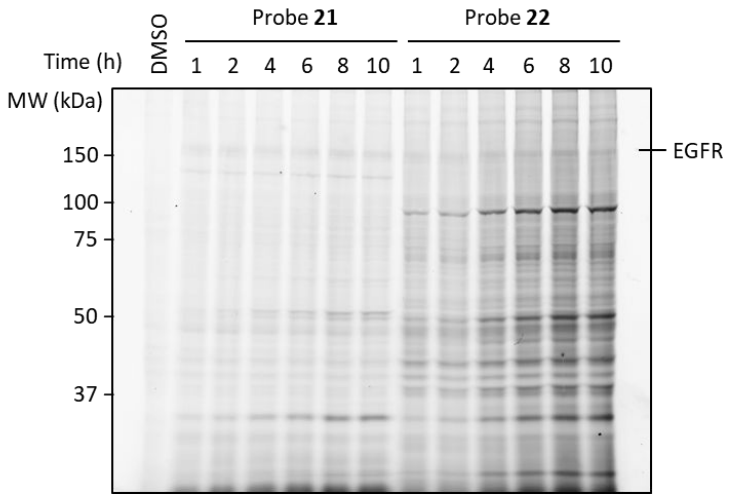


Figure S10. Time-dependent reactivity profile of CFA probe **21** and Michael acceptor probe **22** in H1975 cells ([probe] = 1 μM, 1–10 h, 37 °C).

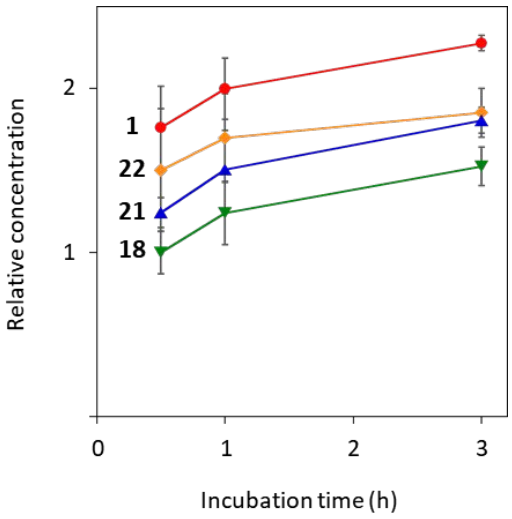


Figure S11. Time trace plot of the relative concentrations of compounds **1**, **18**, **21**, and **22** in H1975 cell lysates. H1975 cells were incubated with RPMI containing 1 μM of each compound for indicated time. The collected cell lysates were analyzed by HPLC (UV absorbance at 350 nm). Data show relative concentration of each compound normalized to the concentration of CFA-pyrimidine **18** at 0.5 h (set to arbitrary value of 1.0). Each plot represents the mean ± standard deviation of triplicate experiments.

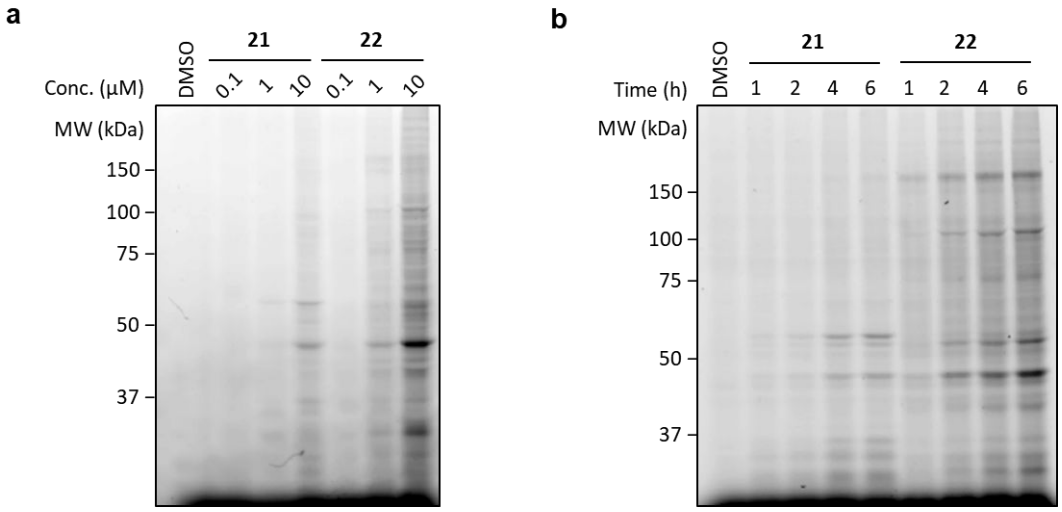


Figure S12. Reactivity profiles of CFA probe **21** and Michael acceptor probe **22** in H292 cells. (a) Concentration-dependent reactivity profiles of **21** and **22** ([probe] = 0.1–10 μM, 2 h, 37 °C). (b) Time-dependent reactivity profiles of **21** and **22** ([probe] = 1 μM, 1–6 h, 37 °C).

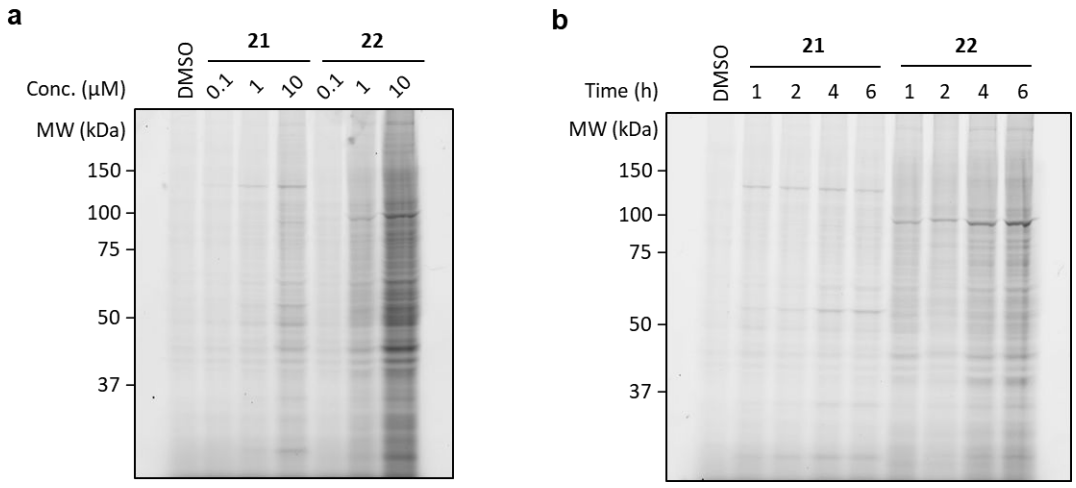


Figure S13. Reactivity profiles of CFA probe **21** and Michael acceptor probe **22** in HEK293 cells. (a) Concentration-dependent reactivity profiles of **21** and **22** ([probe] = 0.1–10 μM, 2 h, 37 °C). (b) Time-dependent reactivity profiles of **21** and **22** ([probe] = 1 μM, 1–6 h, 37 °C).

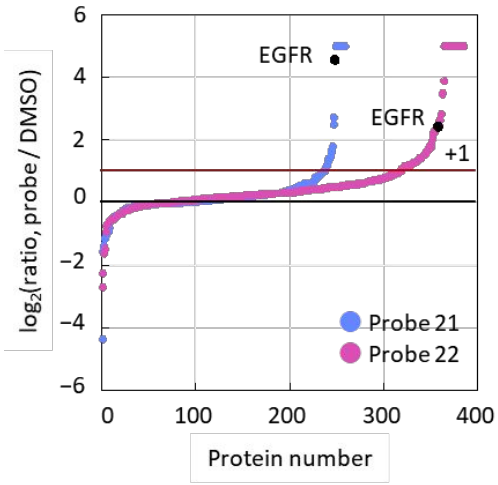


Figure S14. Plot of SILAC ratio values of proteins in probe / DMSO experiment (probe in Heavy / DMSO in Light). H1975 cells were treated with **21** or **22** (5 μM, 2 h, 37 °C). Results are plotted as $\log_2$ of the median SILAC ratios obtained from triplicate mass spectrometry (MS) analyses of a single streptavidin-enriched sample.

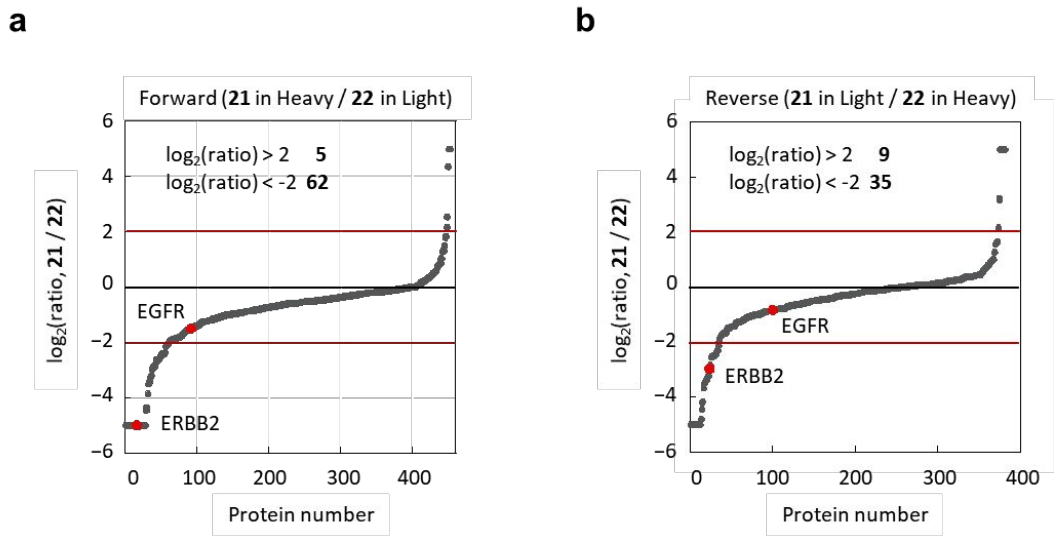


Figure S15. Plot of SILAC ratio values (**21** / **22**) of proteins in probe/probe competitive experiments: (a) forward experiment (**21** in Heavy / **22** in Light) and (b) reverse experiment (**21** in Light / **22** in Heavy). H1975 cells were treated with CFA probe **21** (5 μM, 2 h, 37 °C, Light) or Michael acceptor-type probe **22** (5 μM, 2 h, 37 °C, Heavy). Results are plotted as $\log_2$ of the median SILAC ratios obtained from triplicate MS analyses of a single streptavidin-enriched sample.

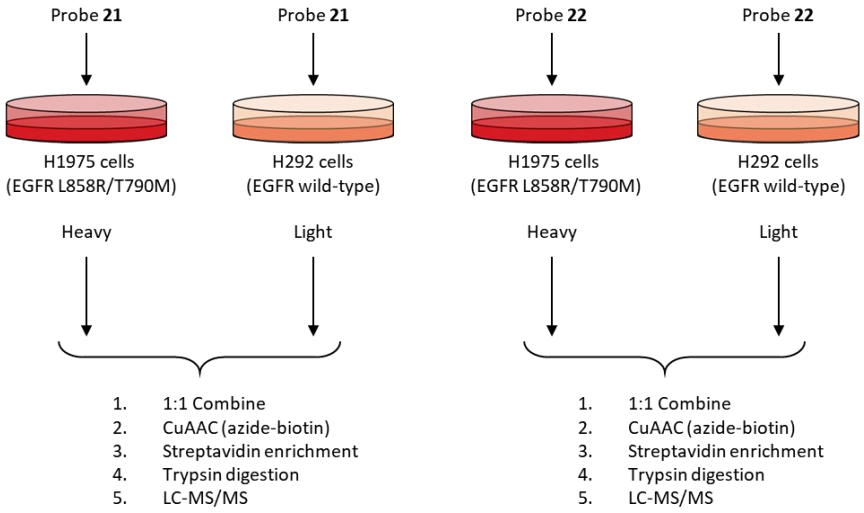


Figure S16. Chemoproteomic workflow to evaluate H1975 (EGFR L858R/T790M) /H292 (EGFR wild-type) selective index ([probe] = 1 μM, 1 h, 37 °C).

METHODS

Cell culture.

H1975 and H292 cells were purchased from the American Type Culture Collection (ATCC, USA). HEK293 cells were kindly provided by Dr. Fujita, Kyushu University (Fukuoka, Japan). Cells were grown at 37 °C under a humidified 5% CO_2 atmosphere in a culture medium containing high-glucose DMEM (Sigma-Aldrich) for HEK293 cells, or RPMI 1640 (Gibco) for H1975 and H292 cells. All media were supplemented with 10% FBS (HyClone), penicillin (50 IU/ml) and streptomycin (50 μg/ml). For SILAC experiments, SILAC RPMI 1640 supplemented with 10% dialyzed FBS (Gibco), penicillin (50 IU/ml) and streptomycin (50 μg/ml) was used. For the isotopically heavy cell samples, 100 mg/mL of both [$^{13}C_6$, $^{15}N_4$]L-arginine-HCl and [$^{13}C_6$, $^{15}N_2$]L-lysine-HCl (Wako) was added to the culture medium. For the isotopically light cell samples, 100 mg/mL of both L-arginine-HCl and L-lysine-HCl (Sigma-Aldrich) was added to the culture medium. Cells were passaged at least six times in isotope-containing medium before in-cell protein labeling and SILAC experiment.

Cell proliferation assay (water soluble tetrazolium assay).

Cells were seeded into 96-well plates. The following day, inhibitors were added to the culture medium at the different concentrations. After incubation for 72 h at 37 ˚C, 15 μL of Cell Count Reagent SF (Nacalai tesque) were added to each well, and the cells were further incubated for several hours. Absorbance at 450 nm was measured with EnSpire multimode plate reader (Perkin Elmer). Triplicate wells were tested at each inhibitor concentration. The IC_{50} value for the inhibitor was calculated from survival curve of the cells.

In-cell protein labeling and in-gel fluorescence analysis.

H1975 and HEK293 cells were grown to ~80% confluence in 10 mL growth medium in a 100 mm culture dish (Primaria, Corning). The growth medium was aspirated off, and the cells were washed twice with DPBS (10 mL), followed by treatment with alkyne probe in culture medium (10 mL, FBS(−)). After incubation at 37 °C in a CO_2 incubator for the indicated time, the medium was aspirated off. The cells were washed twice with cold DPBS (10 mL), and lysed with cold RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 200 μL) containing protease inhibitor cocktail (Roche). The lysed cells were collected with a plastic scraper, transferred to a separated microfuge tube, and centrifuged (17,730*g*, 10 min, 4 °C). The supernatant was transferred to a microfuge tube and stored at −30 °C. After thawing the supernatant on ice, protein concentration was determined using DC protein assay kit (BioRad)

and adjusted to 4 mg/mL by dilution with DPBS. The solution (42 μL) was subjected to CuAAC reaction with 25 μM rhodamine azide, 1 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich), 100 μM tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]-amine (TBTA, Sigma-Aldrich) and 1 mM $CuSO_4$ (final volume 51 μL). The mixture was incubated at 37 °C for 1 h. After addition of 20 μL 5×SDS-PAGE loading buffer, the mixture was further incubated at 37 °C for 1 h and the sample (15 μL) was analyzed with a 10% Acrylamide SDS-PAGE gel. The in-gel fluorescence imaging was performed using LAS-4000 lumino image analyzer (FUJIFILM) or Typhoon Trio$^+$ imager (GE Healthcare) or Amersham ImageQuant 800 (GE Healthcare).

In-cell protein labeling and enrichment for SILAC.

Sample preparation for SILAC study was performed according to the reported method[S1] with minor modifications. The light and heavy cell lysate samples were prepared as described above for in-cell labeling experiments (Incubation conditions are noted in figure legends). All samples were treated with prewashed streptavidin resin (10 μL, Thermo) for 1 h at 4 °C and normalized to 2 mg/mL protein. The equal amounts of light and heavy samples were combined in a 5 mL microfuge tube (total volume 420 μL) and CuAAC reaction was performed with biotin-PEG_4-azide (200 μM, TCI) for 1 h at 37 °C. The excess reagents were removed by $CHCl_3$-MeOH precipitation. The protein pellet was air-dried and re-suspended in 0.5 mL of buffer solution (50 mM Tris-HCl, 150 mM NaCl, 1% SDS, pH 7.4) using a probe sonicator in a 5 mL microfuge tube. The suspension was diluted with 3 mL of RIPA buffer (Thermo) and dissolved using a probe sonicator. The solution (total volume 3.5 mL) was further diluted with 1.5 mL of RIPA buffer and proteins were enriched over prewashed streptavidin resin (50 μL) overnight at 4 °C on rotator. The resin was sequentially washed with 50 mM Tris-HCl, 150 mM NaCl, 6 M urea, pH 7.4 (5 mL × 3), 50 mM Tris-HCl, 150 mM NaCl, 1% SDS, pH 7.4 (5 mL × 3), and 50 mM Tris-HCl, 500 mM NaCl (5 mL × 3). The resin was transferred to a 1.5 mL microfuge tube (PROKEEP low protein binding tube, Watson) with 50 mM Tris-HCl, 500 mM NaCl (0.5 mL × 2) and the supernatant was removed by centrifugation (2,500*g*, 5 min). The collected resin was added with 4× SDS-PAGE loading buffer (20 μL, Wako), and then heated for 10 min at 95 °C. The supernatant was collected by centrifugation (17,730*g*, 10 min) for in-gel digestion. SDS-PAGE was carried out according to the method described by Laemmli.[S2] IP samples were separated partially (~1 cm) using slab gel. Each lane was excised, and the obtained gel pieces were subjected to in-gel tryptic digestion and subsequent MS analysis.

Mass spectroscopic and chromatographic methods, instrumentations and database searches.

Samples were analyzed by nano-flow reverse phase liquid chromatography followed by tandem MS, using a Q Exactive hybrid mass spectrometer (Thermo). A capillary reverse phase

HPLC-MS/MS system composed of a Dionex U3000 gradient pump equipped with VICI CHEMINERT valve, and Q Exactive equipped with a nano-electrospray ionization (NSI) source (AMR, Japan). Samples were automatically injected using PAL system (CTC analytics, Switzerland) using a peptide L-trap column (Trap and Elute mode, Chemical Evaluation Research Institute, Japan) attached to an injector valve for desalinating and concentrating peptides. After washing the trap with MS-grade water containing 0.1% trifluoroacetic acid and 2% acetonitrile (solvent C), the peptides were loaded into a separation capillary C18 reverse phase column (NTCC-360/100-3-125, 125×0.1 mm, Nikkyo Technos, Japan) by switching the valve. The eluents used were: A, 100% water containing 0.5% acetic acid, and B, 80% acetonitrile containing 0.5% acetic acid. The column was developed at a flow rate of 0.5 μL/min with the concentration gradient of acetonitrile: from 5% B to 40% B in 100 min, then from 40% B to 95% B in 1 min, sustaining 95% B for 3 min, from 95% B to 5% B in 1 min, and finally re-equilibrating with 5% B for 10 min. Xcalibur 3.0.63 system (Thermo) was used to record peptide spectra over the mass range of m/z 350–1800. Repeatedly, MS spectra were recorded followed by 10 data-dependent high energy collisional dissociation (HCD) MS/MS spectra generated from 10 highest intensity precursor ions. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted, and peak lists were generated by Proteome Discoverer 2.2.0.388 (Thermo). Searches were performed by using the SEQUEST (Thermo) against homo sapiens (SwissProt TaxID = 9606) peptide sequence. Searching parameters were set as follows: enzyme selected as used with two maximum missing cleavage sites, a mass tolerance of 10 ppm for peptide tolerance, 0.02 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C), Lys8 (K), Arg10 (R), and variable modification of oxidation (M). Peptide identifications were based on significant Xcorr (high confidence filter). Peptide identification and modification information returned from SEQUEST were manually inspected and filtered to obtain confirmed peptide identification and modification lists of HCD MS/MS.

Mass spectrometry data filtration.

For all SILAC experiments, a single streptavidin-enriched sample was subjected to triplicate MS analyses and proteins were first filtered to those detected with high "Protein FDR Confidence" in all of the triplicate analyses. For probe / DMSO SILAC experiment, detected proteins were filtered to those found in both forward (probe in heavy / DMSO in light) and reverse (probe in light / DMSO in heavy) samples. Hit proteins were defined as those displayed $\log_2$(probe / DMSO ratio) ≥ 1 in both forward and reverse experiments. For probe / probe competitive SILAC experiment, proteins found in both forward (probe in heavy / DMSO in light) and reverse (probe in light / DMSO in heavy) samples were plotted. For competitive SILAC experiment, H1975 cells

were pretreated with inhibitor (**18** and **1**, 10 μM, 2 h) or DMSO, followed by treatment with probe (**21** and **22**, respectively; 5 μM, 2 h). Detected proteins were filtered to those found in both forward (inhibitor + probe in light / DMSO + probe in heavy) and reverse (inhibitor + probe in heavy / DMSO + probe in light) samples. High-occupancy targets of the inhibitors were defined as those displayed $\log_2$((DMSO + probe) / (inhibitor + probe) ratio) ≥ 2 in both forward and reverse experiments.

***In vitro* labeling of kinase domain of EGFR(L858R/T790M).**

Human EGFR kinase domain bearing the L858R and T790M mutations was expressed by baculovirus/insect cell system and purified, as described in the literature.[S3] The pFastBac™1 vector was kindly gifted from Takeda Pharmaceutical Company.

3 μM purified EGFR kinase domain and 2 μM probe were incubated at 37 °C in 100 μL of reaction buffer (25 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 0.01% Tween20, 2% DMSO, pH 7.4). 8 μL of the mixture was sampled at the indicated times and diluted with 32 μL of the reaction buffer containing 2.5 mM *N*-ethylmaleimide (NEM) and 0.1% SDS. The mixture was incubated at 37 °C for 30 min and then stored at 4 °C. CuAAC reaction was performed at 37 °C for 1 h using 25 μM rhodamine azide, 1 mM TCEP (Sigma-Aldrich), 100 μM TBTA (Sigma-Aldrich) and 1 mM $CuSO_4$ (final volume of 48 μL). The mixture was diluted with 20 μL of 5× SDS loading buffer and incubated at 37 °C for 30 min, 20 μL of the mixture was subjected to SDS-PAGE (10% acrylamide gel). In-gel fluorescence analysis was performed using LAS-4000 lumino image analyzer (FUJIFILM).

Western blotting.

Primary antibodies: anti-pEGFR (Y1068) (CST, #3777), anti-EGFR (CST, #4267), anti-pAkt (S473) (CST, #4060), anti-Akt (CST, #9272), anti-pERK1/2 (T202/Y204) (CST, #4370), anti-ERK (CST, #9102), anti-β-actin (Abcam, ab8226); secondary antibodies: anti-rabbit IgG-HRP (GE Healthcare, NA934V), anti-mouse IgG-HRP (GE Healthcare, NA931V). Cells were rinsed with ice-cold DPBS and lysed in Triton X-100 buffer (50 mM HEPES, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, and 10% glycerol containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin, 10 μM leupeptin, and 1 mM sodium orthovanadate). Cell lysates were separated by SDS-PAGE (10% acrylamide gel) and transferred to Immobilon membranes (Merck Millipore), and followed by Western blot analysis described previously.[S4]

Pull down assay.

H1975 and H292 cell lysate samples were prepared as described above for in-cell labeling

experiments (1 μM, 1 h, 37 °C). CuAAC reaction and streptavidine enrichment was performed as described above for SILAC experiments and followed by western blotting analysis. The ratio was determined by following formula ($H1975_{eluution}/H1975_{input}$)/($H292_{elution}/H292_{input}$).

References

S1. Lanning, B. R. *et al*. A road map to evaluate the proteome-wide selectivity of covalent kinase inhibitors. Nat. Chem. Biol. **2014**, *10*, 760–767.

S2. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **1970**, *227*, 680–685.

S3. Sogabe, S. *et al*. Structure-based approach for the discovery of pyrrolo[3,2-d]pyrimidine-based EGFR T790M/L858R mutant inhibitors. *ACS Med. Chem. Lett*. **2012**, *4*, 201–205.

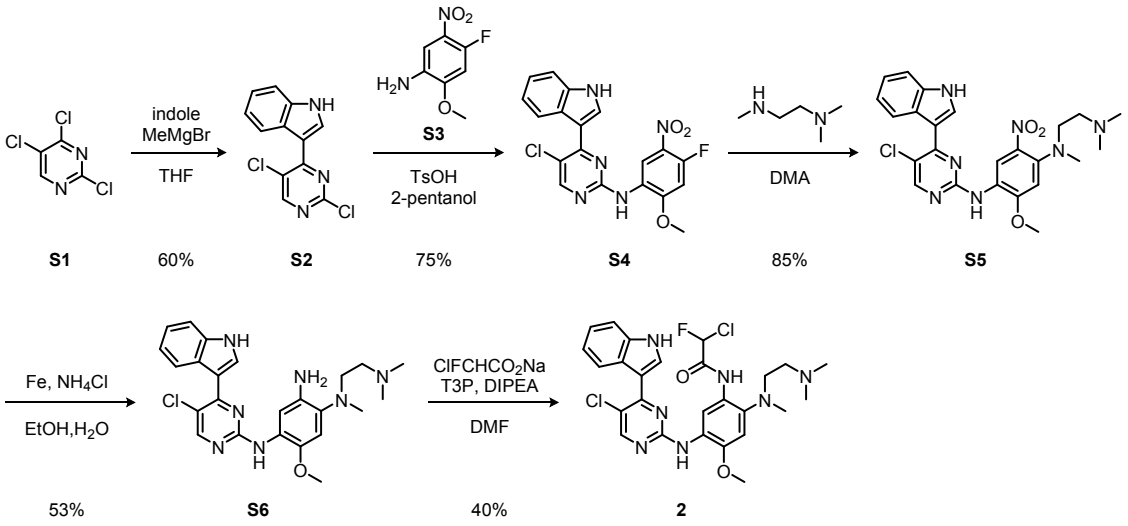
S4. Shibata, T. *et al*. Breast cancer resistance to antiestrogens is enhanced by increased ER degradation and ERBB2 expression. *Cancer Res*. **2017**, *77*, 545–55

Synthetic Procedures

General synthetic methods

Reagents and solvents were obtained from commercial suppliers and used without further purification, unless otherwise stated. Reactions were carried out under a positive atmosphere of nitrogen, unless otherwise stated. Reactions were monitored by thin layer chromatography (TLC) carried out on Merck TLC Silica gel 60 F_{254}, using shortwave UV light as the visualizing agent and phosphomolybdic acid in EtOH and heat as developing agent. Flash column chromatography was performed using Kanto Chemical Silica gel 60 N (spherical, 40-50 μm). ^{1}H NMR spectra were recorded on Varian Unity Plus 400 MHz spectrometer or Bruker Avance III HD 500 MHz spectrometer and were calibrated using residual undeuterated solvent as the internal references ($CDCl_3$: 7.26 ppm; MeOH-d_4: 3.31 ppm, acetone-d_6: 2.05 ppm; DMSO-d_6: 2.50 ppm). The following abbreviations were used to explain NMR peak multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad. Low-resolution and high-resolution mass spectra were recorded on Bruker micrOTOF focus II mass spectrometer using electrospray ionization time-of-flight (ESI-TOF) reflectron experiments. Osimertinib (**1**) was prepared according to the literature[S5].

Preparation of CFA-pyrimidine **2**



3-(2,5-Dichloropyrimidin-4-yl)-1*H*-indole (S2)

To a stirred solution of indole (3.87 g, 33.0 mmol) in dry THF (30 mL) was added MeMgBr (3M in ether, 11.0 mL, 33.0 mmol) dropwise at 0 °C. After stirring at 0 °C for 45 min, to the suspension was added dropwise a solution of 2,4,5-trichloropyrimidine (**S1**) (3.00 g, 16.3 mmol) in dry THF (10 mL). After stirring for 45 min at rt, the mixture was heated to 60 °C and further stirred at the same temperature for 1.5 hr. The mixture was added AcOH (2.00 mL, 35.0 mmol), water (30 mL) and stirred at 60 °C for 15 min. After cooled to rt, the mixture was diluted with hexane. The precipitate was filtrated and washed with hexane to give **S2** (2.50 g, 60%) as a light-yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.25 (s, 1H), 8.73 (d, J = 3.4 Hz, 1H), 8.52-8.50 (m, 1H), 7.56–7.54 (m, 1H), 7.29–7.23 (m, 2H).

5-Chloro-*N*-(4-fluoro-2-methoxy-5-nitrophenyl)-4-(1*H*-indol-3-yl)pyrimidin-2-amine (S4)

S2 (2.19 g, 8.28 mmol), 4-fluoro-2-methoxy-5-nitroaniline (**S3**) (1.61 g, 8.67 mmol) and *p*-toluenesulfonic acid monohydrate (1.81 g, 9.54 mmol) were dissolved in 2-pentanol (60 mL) and the mixture was refluxed overnight. After cooled to rt, the precipitate was filtrated. The residue was dissolved in $CHCl_3$/*i*-PrOH (4:1) and washed successively with sat. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was triturated in hexane to give **S4** (2.58 g, 75%) as a brown solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.94 (s, 1H), 8.76 (s, 1H), 8.65 (d, J = 4.0 Hz, 1H), 8.51 (s, 1H),

8.48 (s, 1H), 8.27 (s, 1H), 7.47 (d, *J* = 4.2 Hz, 1H), 7.40 (d, *J* = 6.6 Hz, 1H), 7.19 (t, *J* = 7.2 Hz, 1H), 6.99 (t, *J* = 7.2 Hz, 1H), 3.96 (s, 3H).

N^1-(5-Chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)-N^4-(2-(dimethylamino)ethyl)-2-methoxy-N^4-methyl-5-nitrobenzene-1,4-diamine (S5)

To a stirred solution of *N*,*N*,*N*'-trimethylethylenediamine (1.5 mL) and DMA (5 mL) was added **S4** (1.01 g, 2.45 mmol) and refluxed for 1.5 h. After cooled to rt, the mixture was diluted with sat. $NaHCO_3$ and extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography on SiO_2 ($CHCl_3$:MeOH = 5:1) to give **S5** (1.04 g, 85%) as a red amorphous material.

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.85 (s, 1H), 8.49 (s, 1H), 8.38 (s, 1H), 8.23 (d, *J* = 5.0 Hz, 1H), 8.16 (s, 1H), 7.45 (d, *J* = 7.4 Hz, 1H), 6.96 (t, *J* = 7.2 Hz, 1H), 6.83 (s, 1H), 3.88 (s, 3H), 2.86 (s, 3H), 2.15 (s, 6H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{24}H_{27}ClN_7O_3$ 496.19; Found 496.18.

N^4-(5-Chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)-N^1-(2-(dimethylamino)ethyl)-5-methoxy-N^1-methylbenzene-1,2,4-triamine (S6)

S5 (380 mg, 0.767 mmol), iron powder (214 mg, 3.84 mmol) and NH_4Cl (41 mg, 0.767 mmol) were dissolved in EtOH/H_2O (3:1, 40 mL). After refluxed for 2 h, the mixture was concentrated in vacuo and dissolved in $CHCl_3$:MeOH (10:1, 33 mL). After stirred at rt for 1 h, the mixture was filtrated and the filtrate was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography on SiO_2 ($CHCl_3$:MeOH:NH_3 aq = 150:10:1) to give **S6** (190 mg, 53%) as a yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.84 (s, 1H), 8.47 (s, 1H), 8.34 (s, 1H), 8.32 (s, 1H), 8.18 (s, 1H), 7.44 (d, *J* = 3.8 Hz, 1H), 7.15 (t, *J* = 7.6 Hz, 1H), 7.08 (s, 1H), 7.02 (t, *J* = 7.8 Hz, 1H), 6.76 (s, 1H), 4.50 (s, 2H), 3.67 (s, 3H), 2.90 (t, *J* = 6.6 Hz, 2H), 2.64 (s, 3H), 2.36 (t, *J* = 6.6 Hz, 2H), 2.16 (s, 6H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{24}H_{29}ClN_7O$ 466.21; Found 466.21.

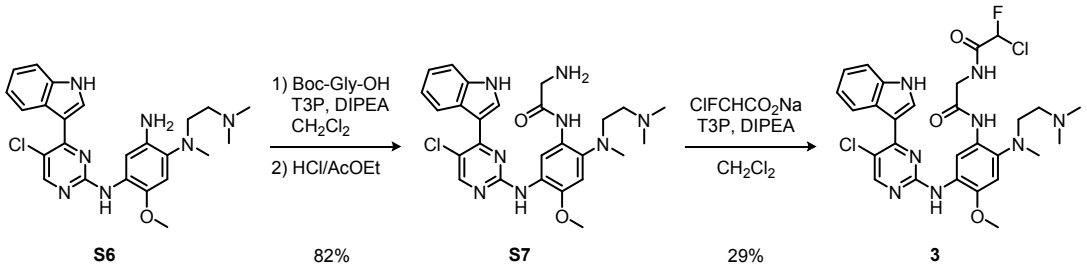
2-Chloro-*N*-(5-((5-chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)-ethyl)(methyl)amino)-4-methoxyphenyl)-2-fluoroacetamide (2)

To a stirred solution of **S6** (32 mg, 0.0693 mmol), DMAP (2.9 mg, 0.0237 mmol) and sodium chlorofluoroacetate (22 mg, 0.164 mmol) in dry DMF (1.5 mL) was added DIPEA (36 µL, 0.208 mmol) and T3P (50 wt.% in AcOEt, 53 µL, 0.130 mmol). After stirred at rt for 6.5 h, T3P (26.0 µL, 0.0637mmol) and sodium chlorofluoroacetate (24 mg, 0.178 mmol) were added and the mixture was stirred at rt for 1 h. The reaction mixture was diluted with sat. $NaHCO_3$ and the water phase was extracted with CH_2Cl_2/*i*-PrOH (4:1). The organic layer was washed with brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by a flash column chromatography on SiO_2 ($CHCl_3$:MeOH = 10:1) and concentrated in vacuo. The residue was triturated in ether to give **2** (16 mg, 40%) as an off-white solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.86 (s, 1H), 10.69 (s, 1H), 8.54 (s, 1H), 8.51 (d, J = 3.0 Hz, 1H), 8.28–8.23 (m, 2H), 7.46 (d, J = 8.0 Hz, 1H), 7.16 (t, J = 8.0 Hz, 1H), 7.13 (s, 1H), 7.00 (t, J = 7.5 Hz, 1H), 6.93 (d, $J_{(H–F)}$ = 49.5 Hz, 1H), 3.79 (s, 3H), 3.19 (s, 3H), 3.01 (t, J = 6.3 Hz, 2H), 2.25 (t, J = 5.9 Hz, 2H), 2.17 (s, 6H).
LRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{26}H_{29}Cl_2FN_7O_2$ 560.17; Found 560.20.

Preparation of CFA-pyrimidines **3**–**10**

Representative procedure: preparation of **3**



S6 82% **S7** 29% **3**

2-Chloro-*N*-(2-((5-((5-chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)-ethyl)(methyl)amino)-4-methoxyphenyl)amino)-2-oxoethyl)-2-fluoroacetamide (3)

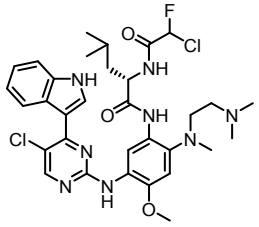
To a stirred solution of **S6** (34.6 mg, 0.0743 mmol) and Boc-Gly-OH (26.8 mg, 0.153 mmol) in dry CH_2Cl_2 (3.0 mL) was added DIPEA (40 µL, 0.230 mmol) and T3P (50 wt.% in AcOEt, 61.0 µL, 0.102 mmol). After stirred for 4 h at ambient temperature, the reaction mixture was diluted with AcOEt and the water phase was extracted twice with AcOEt. The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (3.0 mL) and added 4*N* HCl/AcOEt (2.0 mL). After stirring for 1.5 h, the reaction mixture was basified with sat. $NaHCO_3$ and the water phase was extracted thrice with CH_2Cl_2/*i*-PrOH

(4:1). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo to give crude **S7**, which was used in the next step without further purification.

To a stirred solution of **S7** (31.9 mg, 0.0610 mmol) and sodium chlorofluoroacetate (16.7 mg, 0.124 mmol) in dry CH_2Cl_2 (3.0 mL) was added DIPEA (32.0 µL, 0.184 mmol) and T3P (50 wt. % in AcOEt, 50.0 µL, 0.0832 mmol) at ambient temperature. After stirring for 3 h, the reaction mixture was diluted with AcOEt and sat. $NaHCO_3$. The organic layer was separated and the aqueous phase was extracted thrice with AcOEt. The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH = 7:1 to 5:1) to give **3** (11.0 mg, 29% yield) as a beige solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.86 (s, 1H), 9.61 (brs, 1H), 9.05 (t, J = 6.0 Hz, 1H), 8.51–8.46 (m, 2H), 8.35 (s, 1H), 8.31 (d, J = 5.0 Hz, 1H), 8.18 (brs, 1H), 7.46 (d, J = 8.0 Hz, 1H), 7.18 (t, J = 7.5 Hz, 1H), 7.04 (t, J = 7.5 Hz, 1H), 6.99 (s, 1H), 6.86 (d, $J_{(H–F)}$ = 49.5 Hz, 1H), 4.07 (s, 2H), 3.80 (s, 3H), 2.76 (brs, 2H), 2.66 (s, 6H). Five protons of the trimethylethylenediamine side chain are missing likely due to overlapping to solvent peaks.

HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{28}H_{32}Cl_2FN_8O_3$ 617.1953; Found 617.1959.



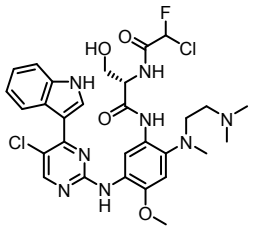
4

(2*S*)-2-(2-Chloro-2-fluoroacetamido)-*N*-(5-((5-chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)-4-methylpentanamide (4)

4 was prepared in a similar manner to **3** using **S6** and Boc-L-Leu-OH.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.83 (s, 1H), 9.83 and 9.82 (s, 1H), 9.00 (t, J = 9.0 Hz, 1H), 8.51 (t, J = 4.0 Hz 2H), 8.34 (t, J = 1.5 Hz, 1H), 8.30 (s, 1H), 8.25 (brs, 1H), 7.44 (d, J = 4.3 Hz, 1H), 7.15–7.12 (m, 1H), 7.06 (s, 1H), 6.98–6.95 (m, 1H), 6.78 (d, $J_{(H–F)}$ = 49.5 Hz, 1H), 4.52–4.48 (m, 1H), 3.76 and 3.75 (s, 3H), 3.01–2.97 (m, 2H), 2.70 (s, 3H), 2.38–2.23 (m, 2H), 2.20 (s, 6H), 1.65–1.53 (m, 3H), 0.93–0.91 (m, 3H), 0.89–0.87 (m, 3H).

LRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{32}H_{40}Cl_2FN_8O_3$ 673.26; Found 673.24.



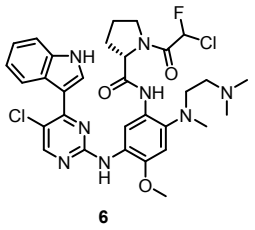
5

(2*S*)-2-(2-Chloro-2-fluoroacetamido)-*N*-(5-((5-chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)-3-hydroxypropanamide (5)

5 was prepared in a similar manner to **3** using **S6** and Boc-L-Ser-OH.

^{1}H NMR (500 MHz, DMSO-d_6, as a mixture of two diastereomers) δ 11.85 (s, 1H), 9.86 and 9.83 (s, 1H), 8.92 and 8.89 (t, J = 8.0 Hz, 1H), 8.59 (s, 1H), 8.50 (d, J = 3.0 Hz, 1H), 8.34 (s, 1H), 8.33 (s, 1H), 8.26 (d, J = 7.5 Hz, 1H), 7.44 (d, J = 8.0 Hz, 1H), 7.14 (t, J = 8.0 Hz, 1H), 7.05 (s, 1H), 6.98 (t, J = 7.5 Hz, 1H), 6.86 and 6.85 (d, $J_{(H–F)}$ = 49.5 Hz, 1H), 5.31 (brs, 1H), 4.53–4.45 (m, 1H), 3.75 (s, 3H), 3.70 (d, J = 5.5 Hz, 2H), 3.03–2.94 (m, 2H), 2.70 and 2.69 (s, 3H), 2.37–2.29 (m, 2H), 2.20 (s, 6H).

LRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{29}H_{34}Cl_2FN_8O_4$ 647.21; Found 647.20.



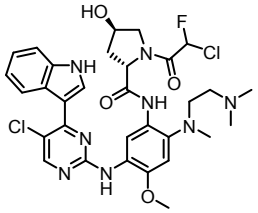
6

(2*S*)-1-(2-Chloro-2-fluoroacetyl)-*N*-(5-((5-chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)pyrrolidine-2-carboxamide (6)

6 was prepared in a similar manner to **3** using **S6** and Boc-L-Pro-OH.

^{1}H NMR (500 MHz, DMSO-d_6, as a mixture of two diastereomers and respective rotamers) δ 11.82 (s, 1H), 9.92 and 9.74 (s, 0.2H), 9.71 and 9.65 (s, 0.8H), 8.53–8.49 (m, 2H), 8.34 (s, 1H), 8.31–8.20 (m, 2H), 7.44 (d, J = 8.0 Hz, 1H), 7.25–7.02 (m, 2.8H), 7.00–6.94 (m, 1H), 6.93 and 6.67 (d, $J_{(H–F)}$ = 49.0 Hz, 0.2H), 4.80–4.78 (m, 0.1H), 4.63–4.61 (m, 0.1H), 4.54–4.50 (m, 0.8H), 3.75 (s, 3H), 3.74–3.68 (m, 0.8H), 3.56–3.45 (m, 1.2H), 3.04–2.97 (m, 2H), 2.71 (s, 3H), 2.36 (brs, 2H), 2.21 (s, 6H), 2.18–2.11 (m, 1.2H), 1.95–1.84 (m, 2.8H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{31}H_{36}Cl_2FN_8O_3$ 657.23; Found 657.22.

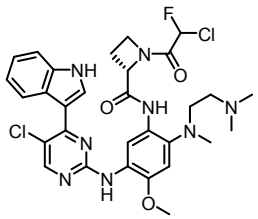


7

(2*S*,4*R*)-1-(2-Chloro-2-fluoroacetyl)-*N*-(5-((5-chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)-4-hydroxypyrrolidine-2-carboxamide (7)

7 was prepared in a similar manner to **3** using **S6** and Boc-L-Hyp-OH.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.85 and 11.84 (s, 1H), 9.84 and 9.82 (brs, 1H), 8.58–8.50 (m, 2H), 8.33–8.12 (m, 2H), 7.44 (d, J = 8.5 Hz, 1H), 7.20–7.10 (m, 2H), 7.02–7.95 (m, 2H), 5.28–5.20 (m, 1H), 4.59–4.54 (m, 1H), 4.42–4.36 (m, 1H), 3.77–3.41 (m, 6H), 3.01–2.97 (m, 2H), 2.73 (s, 3H), 2.37 (brs, 2H), 2.22 (s, 6H), 2.17–2.09 (m, 1H), 1.98–1.88 (m, 1H). Five protons of the trimethylethylenediamine side chain are missing likely due to overlapping to solvent peaks.
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{31}H_{36}Cl_2FN_8O_4$ 673.22; Found 673.19.



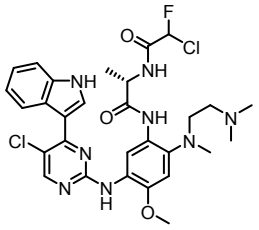
8

(2*S*)-1-(2-Chloro-2-fluoroacetyl)-*N*-(5-((5-chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)azetidine-2-carboxamide (8)

8 was prepared in a similar manner to **3** using **S6** and Boc-L-Aze(2)-OH.

^{1}H NMR (500 MHz, DMSO-d_6, as a mixture of two diastereomers and respective rotamers) δ 11.86 (s, 1H), 10.01 and 9.97 (s, 0.3H), 9.89 and 9.82 (s, 0.7H), 8.57 (s, 1H), 8.51 (d, J = 2.5 Hz, 1H), 8.43–8.23 (m, 3H), 7.44 (d, J = 8.0 Hz, 1H), 7.15 (t, J = 7.5 Hz, 1H), 7.08–6.94 (m, 2.7H), 6.76 and 6.64 (d, $J_{(H-F)}$ = 48.0 Hz, 0.3H), 5.22–5.10 (m, 0.3H), 4.88 (p, J = 5.0 Hz, 0.7H), 4.35–4.25 (m, 0.7H), 4.24–4.14 (m, 0.7H), 3.97–3.89 (m, 0.6H), 3.76 (s, 3H), 2.99 (brs, 2H), 2.79–2.56 (m, 4H), 2.39–2.28 (m, 2.3H), 2.25–2.14 (m, 6.7H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{30}H_{34}Cl_2FN_8O_3$ 643.21; Found 643.21.



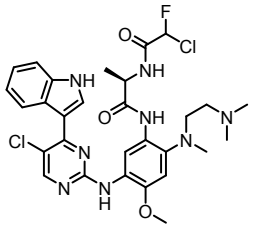
9

(2*S*)-2-(2-Chloro-2-fluoroacetamido)-*N*-(5-((5-chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)-amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)propanamide (9)

9 was prepared in a similar manner to **3** using **S6** and Boc-L-Ala-OH.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.83 and 11.82 (s, 1H), 9.80 and 9.79 (s, 1H), 8.99 (t, *J* = 8.3 Hz, 1H), 8.51(s, 1H), 8.49 (d, *J* = 3.1 Hz, 1H), 8.34 (s, 1H), 8.32 (s, 1H), 8.26 (d, *J* = 7.8 Hz, 1H), 7.44 (d, *J* = 8.1 Hz, 1H), 7.16–7.13 (m, 1H), 7.04 (s, 1H), 6.97 (t, *J* = 7.5 Hz, 1H), 6.78 (d, $J_{(H-F)}$ = 49.5 Hz, 1H), 4.54–4.47 (m, 1H), 3.75 (s, 3H), 2.97 (brs, 2H), 2.70 (s, 3H), 2.36–2.34 (m, 2H), 2.21 (s, 6H), 1.33 (t, *J* = 6.7 Hz, 3H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{29}H_{34}Cl_2FN_8O_3$ 631.21; Found 632.21.



10

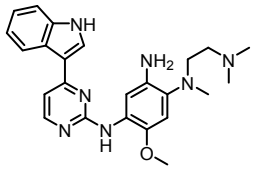
(2*R*)-2-(2-Chloro-2-fluoroacetamido)-*N*-(5-((5-chloro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)-amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)propanamide (9)

10 was prepared in a similar manner to **3** using **S6** and Boc-D-Ala-OH.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.83 and 11.82 (s, 1H), 9.80 and 9.79 (s, 1H), 8.99 (t, *J* = 8.3 Hz, 1H), 8.51(s, 1H), 8.49 (d, *J* = 3.1 Hz, 1H), 8.34 (s, 1H), 8.32 (s, 1H), 8.26 (d, *J* = 7.8 Hz, 1H), 7.44 (d, *J* = 8.1 Hz, 1H), 7.16–7.13 (m, 1H), 7.04 (s, 1H), 6.97 (t, *J* = 7.5 Hz, 1H), 6.78 (d, $J_{(H-F)}$ = 49.5 Hz, 1H), 4.54–4.47 (m, 1H), 3.75 (s, 3H), 2.97 (brs, 2H), 2.70 (s, 3H), 2.36–2.34 (m, 2H), 2.21 (s, 6H), 1.33 (t, *J* = 6.7 Hz, 3H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{29}H_{34}Cl_2FN_8O_3$ 631.21; Found 631.20.

Preparation of starting materials for CFA-pyrimidines **11**–**19**

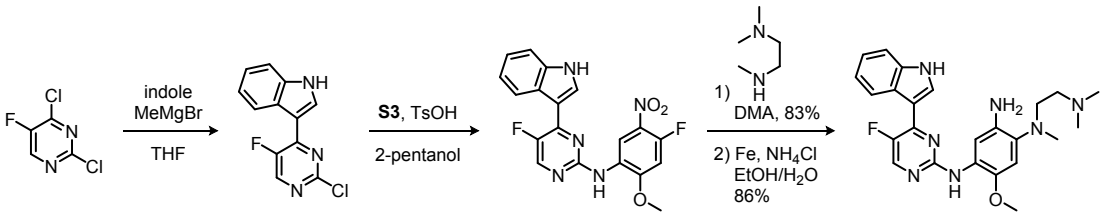


S8

***N*⁴-(4-(1*H*-Indol-3-yl)pyrimidin-2-yl)-*N*¹-(2-(dimethylamino)ethyl)-5-methoxy-*N*¹-methylbenzene-1,2,4-triamine (S8)**

S8 was prepared according to the literature[1].

¹H NMR (400 MHz, DMSO-d_6): δ 11.79 (s, 1H), 8.62 (s, 1H), 8.34–8.23 (m, 3H), 8.04 (s, 1H), 7.44 (d, *J* = 8.4 Hz, 1H), 7.27 (d, *J* = 5.6 Hz, 1H), 7.16 (t, *J* = 7.6 Hz, 1H), 7.09 (s, 1H), 7.06 (t, *J* = 8.0 Hz, 1H), 6.85 (s, 1H), 3.95 (s, 3H), 3.25 (2H, overlapping to water peak), 2.45 (s, 3H), 2.47 (2H, overlapping to DMSO peak), 2.15 (s, 6H).



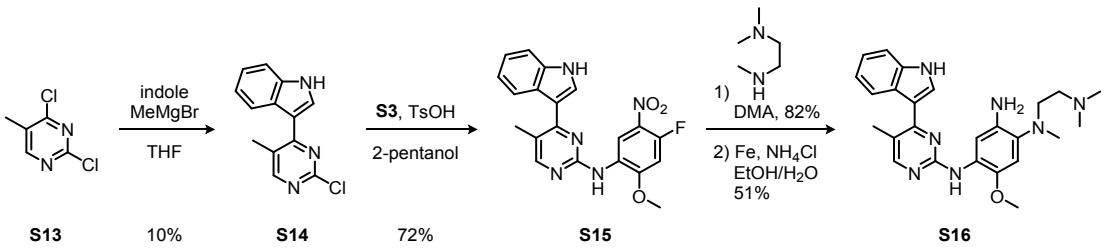
S9 28% **S10** 50% **S11** **S12**

***N*¹-(2-(Dimethylamino)ethyl)-*N*⁴-(5-fluoro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)-5-methoxy-*N*¹-methylbenzene-1,2,4-triamine (S12)**

S12 was prepared in a similar manner to **S6** from **S9**.

¹H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H), 8.47 (d, *J* = 4.2 Hz, 1H), 8.31 (s, 1H), 8.32–8.30 (m, 2H), 8.13 (s, 1H), 7.95 (s, 1H), 7.47 (d, *J* = 4.2 Hz, 1H), 7.21–7.17 (m, 2H), 7.09 (t, *J* = 7.6 Hz, 1H), 6.76 (s, 1H), 4.52 (s, 2H), 3.69 (s, 3H), 2.90 (t, *J* = 6.8 Hz, 2H), 2.64 (s, 3H), 2.37 (t, *J* = 6.8 Hz, 2H), 2.17 (s, 6H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{24}H_{29}FN_7O$ 450.24; Found 450.24.

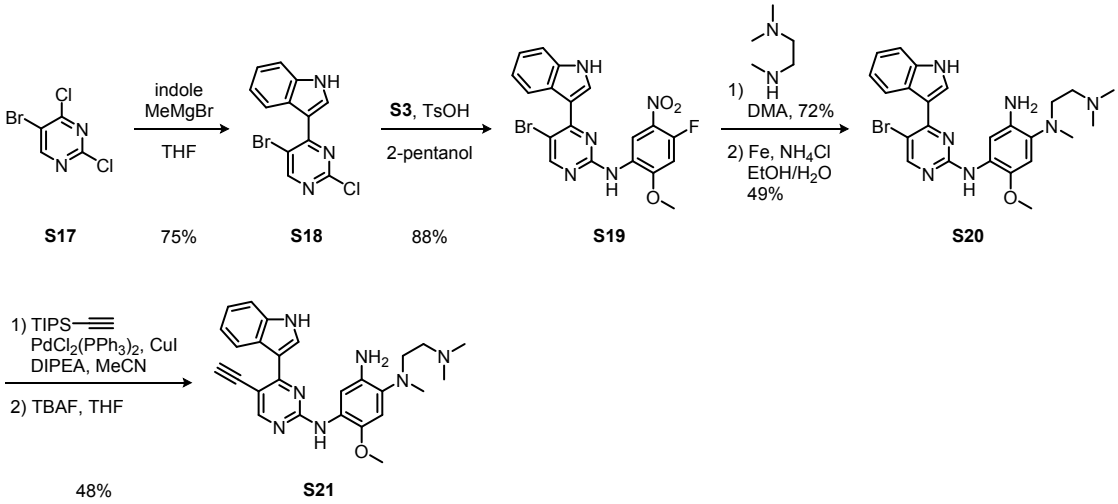


***N*¹-(2-(Dimethylamino)ethyl)-*N*⁴-(5-methyl-4-(1*H*-indol-3-yl)pyrimidin-2-yl)-5-methoxy-*N*¹-methylbenzene-1,2,4-triamine (S16)**

S16 was prepared in a similar manner to **S6** from **S13**.

¹H NMR (400 MHz, DMSO-d_6) δ 11.68 (s, 1H), 8.40 (d, J = 4.0 Hz, 1H), 8.20 (s, 1H), 7.96 (d, J = 1.4 Hz, 1H), 7.60 (s, 1H), 7.50 (s, 1H), 7.44 (d, J = 3.8 Hz, 1H), 7.16 (t, J = 7.4 Hz, 1H), 7.08 (t, J = 7.6 Hz, 1H), 6.74 (s, 1H), 4.43 (s, 2H), 3.73 (s, 3H), 2.86 (t, J = 6.6 Hz, 2H), 2.61 (s, 3H), 2.35–2.32 (m, 5H), 2.16 (s, 6H).

LRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{25}H_{32}N_7O$ 446.27; Found 446.27.



***N*⁴-(5-Bromo-4-(1*H*-indol-3-yl)pyrimidin-2-yl)-*N*¹-(2-(dimethylamino)ethyl)-5-methoxy-*N*¹-methylbenzene-1,2,4-triamine (S20)**

S20 was prepared in a similar manner to **S6** from **S17**.

¹H NMR (400 MHz, DMSO-d_6) δ 11.77 (s, 1H), 8.51 (s, 1H), 8.42 (s, 1H), 8.25 (d, J = 3.8 Hz, 1H), 8.17 (s, 1H), 7.44 (d, J = 3.8 Hz, 1H), 7.14 (t, J = 7.4 Hz, 1H), 7.07 (s, 1H), 7.02 (s, 1H), 6.75 (s,

1H), 4.48 (s, 2H), 3.67 (s, 3H), 2.89 (t, *J* = 6.4 Hz, 2H), 2.63 (s, 3H), 2.36 (t, *J* = 6.6 Hz, 2H), 2.16 (s, 6H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{24}H_{29}BrN_7O$ 510.16; Found 510.16.

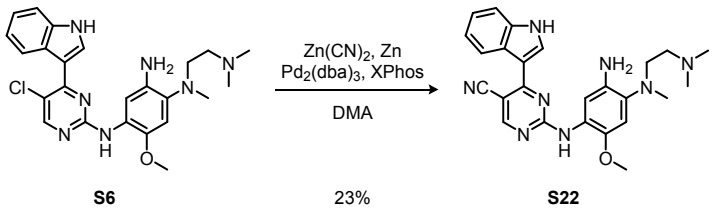
N^1-(2-(Dimethylamino)ethyl)-N^4-(5-ethynyl-4-(1*H*-indol-3-yl)pyrimidin-2-yl)-5-methoxy-N^1-methylbenzene-1,2,4-triamine (S21)

To a stirred solution of **S20** (100 mg, 0.196 mmol), $PdCl_2(PPh_3)_2$ (13.9 mg, 0.0196 mmol, 10 mol%), and CuI (3.4 mg, 0.176 mmol) in degassed MeCN (2 mL) was added DIPEA (34.0 µL, 0.196 mmol) and (triisopropylsilyl)acetylene (75.0 µL, 0.294 mmol) at ambient temperature. The reaction vessel was flushed with nitrogen and the mixture was stirred for 21 h. (Triisopropylsilyl)-acetylene (75.0 µL, 0.294 mmol) was added and the mixture was further stirred for 2 h at 60 °C. After cooling to ambient temperature, the mixture was diluted with CH_2Cl_2/*i*-PrOH (4:1) and filtered through a pad of celite. The filtrate was washed with sat. $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH/NH_3 aq. = 150:10:1) to give 79 mg yellow oil.

To a stirred solution of above oil in MeCN (5 mL) was added CsF (59.0 mg, 0.388 mmol). After stirring for 3.5 h at 50 °C, the reaction mixture was diluted with CH_2Cl_2/*i*-PrOH (4:1) and sat. $NaHCO_3$. The organic layer was separated and the water phase was extracted twice with CH_2Cl_2/*i*-PrOH (4:1). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH/NH_3 aq. = 150:10:1) to give **S21** (38.0 mg, 48% yield) as yellow oil.

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.71 (s, 1H), 8.77 (d, *J* = 1.6 Hz, 1H), 8.39–8.36 (m, 2H), 8.30 (s, 1H), 7.43 (d, *J* = 4.0 Hz, 1H), 7.14 (t, *J* = 7.0 Hz, 1H), 7.04–7.00 (m, 2H), 6.76 (s, 1H), 4.59 (s, 1H), 4.53 (brs, 2H), 3.66 (s, 3H), 2.91 (t, *J* = 6.4 Hz, 2H), 2.65 (s, 3H), 2.38 (t, *J* = 6.8 Hz, 2H), 2.17 (s, 6H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{26}H_{30}N_7O$ 456.25; Found 456.25.

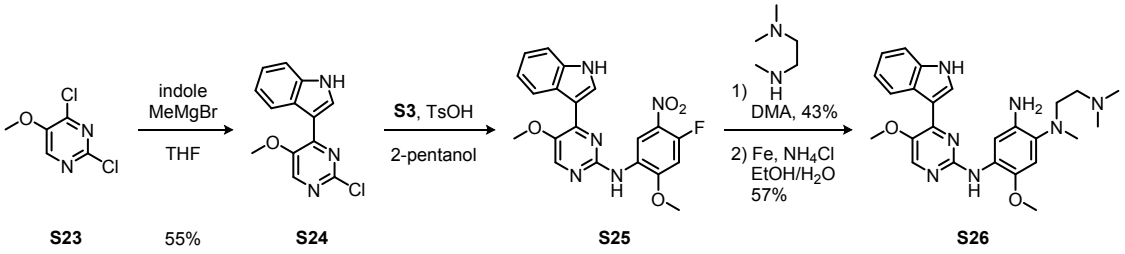


S6 23% **S22**

2-((5-Amino-4-((2-(dimethylamino)ethyl)(methyl)amino)-2-methoxyphenyl)amino)-4-(1*H*-indol-3-yl)pyrimidine-5-carbonitrile (S22)

To a stirred solution of **S6** (50.0 mg, 0.107 mmol), $Zn(CN)_2$ (7.6 mg, 0.0647 mmol) and zinc powder (0.8 mg, 0.0122 mmol) in dry DMA (2.0 mL) was added $Pd_2(dba)_3$ (9.8 mg, 0.0107 mmol) and XPhos (10.2 mg, 0.0214 mmol). After stirring for 4 h at 90 °C, the reaction mixture was diluted with CH_2Cl_2/*i*-PrOH (4:1) and sat. $NaHCO_3$. The organic layer was separated and the water phase was extracted twice with CH_2Cl_2/*i*-PrOH (4:1). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH/NH_3 aq. = 200:10:1) to give **S22** (11.2 mg, 23% yield) as yellow solid.

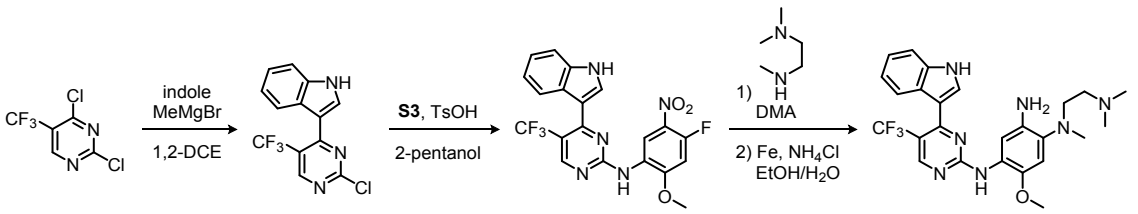
^{1}H NMR (400 MHz, DMSO-d_6) δ 11.94 (s, 1H), 9.13 (s, 1H), 8.63 (s. 1H), 8.50 (s, 1H), 8.30 (s, 1H), 7.46 (d, *J* = 4.0 Hz, 1H), 7.17 (brs, 1H), 7.00 (brs, 1H), 6.78 (s, 1H), 6.19 (s, 1H), 4.60 (brs, 2H), 3.63 (s, 3H), 2.92 (t, *J* = 6.8 Hz, 2H), 2.67 (s, 3H), 2.39 (t, *J* = 6.8 Hz, 2H), 2.17 (s, 6H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{25}H_{29}N_8O$ 457.25; Found 457.25.



***N*^{4}-(4-(1*H*-Indol-3-yl)-5-methoxypyrimidin-2-yl)-*N*^{1}-(2-(dimethylamino)ethyl)-5-methoxy-*N*^{1}-methylbenzene-1,2,4-triamine (S26)**

S26 was prepared in a similar manner to **S6** from **S23**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.68 (s, 1H), 8.61 (d, *J* = 4.2 Hz, 1H), 8.32 (d, *J* = 1.2 Hz, 1H), 8.16 (s, 1H), 7.52 (s, 1H), 7.45-7.43 (m, 2H), 7.16 (t, *J* = 8.0 Hz, 1H), 7.09 (t, *J* = 7.6 Hz, 1H), 6.75 (s, 1H), 4.51 (brs, 2H), 3.99 (s, 3H), 3.73 (s, 3H), 2.88 (t, *J* = 6.6 Hz, 2H), 2.62 (s, 3H), 2.35 (t, *J* = 6.8 Hz, 2H), 2.16 (s, 6H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{25}H_{32}N_7O_2$ 462.26; Found 462.27.



3-[2-Chloro-5-(trifluoromethyl)pyrimidin-4-yl]-1*H*-indole (S28)

To a stirred solution of indole (543 mg, 4.64 mmol) in dry 1,2-dichloroethane (6.0 mL) was added methylmagnesium bromide solution (3 M in diethyl ether 2.30 mL, 6.90 mmol) dropwise at 0 °C. After stirred for 30 min at 0 °C, 2,4-dichloro-5-trifluoromethylpyrimidine (**S27**) (985 mg, 4.54 mmol) in dry CH_2Cl_2 (1.0 mL) was added dropwise and the mixture was further stirred for 1.5 h at ambient temperature. The reaction mixture was diluted with water and the aqueous phase was extracted twice with AcOEt. The combined organic layers were washed with water and brine, dried over Na_2SO_4, filetered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (hexane/AcOEt = 4:1) to give **S28** (799 mg, 60% yield) as a yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.22 (bs, 1H), 9.01 (s, 1H), 8.26 (d, J = 3.2 Hz, 1H), 8.08 (s, 1H), 7.55 (d, J = 3.2 Hz, 1H), 7.25–7.29 (m, 2H).
LRMS (ESI) m/z: $[M+Na]^+$ calcd for $C_{13}H_7ClF_3N_3Na$ 320.02; Found 320.02.

***N*^{4}-[4-(1*H*-Indol-3-yl)-5-(trifluoromethyl)pyrimidin-2-yl]-*N*^{1}-[2-(dimethylamino)ethyl]-5-methoxy-*N*^{1}-methylbenzene-1,2,4-triamine (S30)**

S28 (799 mg, 2.68 mmol), **S3** (500 mg, 2.69 mmol), and *p*-toluenesulfonic acid monohydrate (521 mg, 2.74 mmol) were dissolved in 2-pentanol (20 mL) and stirred for 3 h at 120 °C. After cooling to ambient temperature, the precipitate was collected by suction filtration, washed with 2-pentanol and diethyl ether, and dried under vacuum at 50 °C to give the intermediate **S29** (1.20 g as a 1:1 complex with *p*-TsOH, 72% yield) as a light-yellow solid, which was used in the next step without further purification.

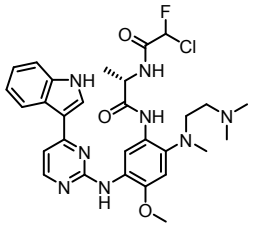
^{1}H NMR (500 MHz, DMSO-d_6) δ 11.88 (s, 1H), 9.31 (s, 1H), 8.75 (s, 1H), 8.57 (d, J = 8.5 Hz, 1H), 8.06 (brs, 1H), 7.89 (d, J = 2.5 Hz, 1H), 7.50–7.41 (m, 4H), 7.20 (t, J = 8.0 Hz, 1H), 7.13 (d, J = 8.5 Hz, 2H), 7.00 (t, J = 7.0 Hz, 1H), 3.97 (s, 3H), 2.30 (s, 3H).
LRMS (ESI) m/z: $[M+Na]^+$ calcd for $C_{20}H_{13}F_4N_5O_3Na$ 470.08; Found 470.09.

To a stirred solution of **S29**•*p*-TsOH (821 mg, 1.32 mmol) in dry DMA (3.0 mL) was added *N*,*N*,*N'*-trimethylethylenediamine (680 µL, 5.23 mmol) at ambient temperature. After stirred for 0.5 h at 120 °C, the reaction mixture was cooled to ambient temperature and diluted with sat. $NaHCO_3$. The aqueous phase was extracted twice with AcOEt. The combined organic layers were washed sequentially with sat. $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give an orange solid. The solid was redissolved in EtOH (30 mL) and water (10 mL). To the solution was added iron powder (371 mg, 6.65 mmol) and NH_4Cl (71.4 mg, 1.33 mmol) and refluxed for 3.5 h. After cooling to ambient temperature, the solid material was removed by filtration and the filtrate was concentrated in vacuo. The residue was diluted with sat. $NaHCO_3$ and extracted thrice with 4:1 $CHCl_3$/2-propanol. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH = 4:1 to 3:1) to give **S30** (486 mg, 74% yield over 2 steps) as a light-brown foam.

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.78 (s, 1H), 8.82 (s, 1H), 8.60 (s, 1H), 8.14 (brs, 1H), 7.86 (s, 1H), 7.44 (d, *J* = 8.0 Hz, 1H), 7.15 (t, *J* = 8.0 Hz, 1H), 7.02 (t, *J* = 6.5 Hz, 1H), 6.93 (s, 1H), 6.77 (s, 1H), 4.67 (brs, 2H), 3.68 (s, 3H), 2.92 (d, *J* = 6.5 Hz, 2H), 2.64 (s, 3H), 2.42 (brs, 2H), 2.21 (s, 6H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{25}H_{29}F_3N_7O$ 500.24; Found 500.24.

Preparation of CFA-pyrimidines **11**–**19**



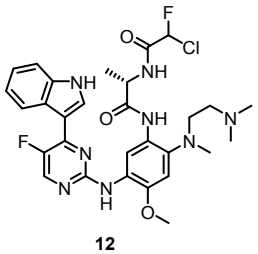
11

(2*S*)-*N*-(5-((4-(1*H*-Indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)-2-(2-chloro-2-fluoroacetamido)propanamide (11)

11 was prepared in the similar manner to **3** starting from **S8**.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.73 (s, 1H), 9.84 (d, J = 1.8 Hz, 1H), 9.01 (dd, J = 8.8 Hz, 1.3 Hz, 1H), 8.77 (s, 1H), 8.38 (d, J = 1.5 Hz, 1H), 8.30 (d, J = 4.0 Hz, 1H), 8.27 (dd, J = 2.6 Hz, 0.5 Hz, 1H), 7.98 (s, 1H), 7.44 (d, J = 4.3 Hz, 1H), 7.24 (dd, J = 2.8 Hz, 0.3 Hz, 1H), 7.15 (dt, J = 7.0 Hz, 1.0 Hz, 1H), 7.07 (dt, J = 7.5 Hz, 0.5 Hz, 1H), 7.02 (s, 1H), 6.81 and 6.79 (d, $J_{(H–F)}$ = 49.5 Hz, 1H), 4.55—4.51 (m, 1H), 3.82 (s, 3H), 2.96–2.94 (m, 2H), 2.20 (s, 6H), 2.68 (s, 3H), 2.31 (m, 2H),1.38 (t, J = 6.8 Hz, 3H).

LRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{29}H_{35}ClFN_8O_3$ 597.25; Found 597.25.



12

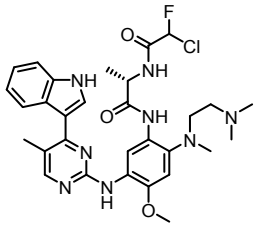
(2*S*)-2-(2-Chloro-2-fluoroacetamido)-*N*-(2-((2-(dimethylamino)ethyl)(methyl)amino)-5-((5-fluoro-4-(1*H*-indol-3-yl)pyrimidin-2-yl)amino)-4-methoxyphenyl)propanamide (12)

12 was prepared in the similar manner to **3** starting from **S12**.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.88 (s, 1H), 9.83 (s, 1H), 8.99 (t, J = 7.8 Hz, 1H), 8.46 (s, 1H), 8.36 (d, J = 3.8 Hz, 1H), 8.32 (d, J = 1.8 Hz, 1H), 8.27 (s, 1H), 8.15 (s, 1H), 7.46 (d, J = 4.0 Hz, 1H), 7.17 (t, J = 7.0 Hz, 1H), 7.04—7.01 (m, 2H), 6.79 and 6.78 (d, $J_{(H–F)}$ = 49.5 Hz, 1H), 4.52–4.49

(m, 1H), 3.77 (s, 3H), 2.97–2.96 (m, 2H), 2.70 (s, 3H), 2.33—2.26 (m, 2H), 2.20 (s, 6H), 1.34 (t, *J* = 6.5 Hz, 3H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{29}H_{34}ClF_2N_8O_3$ 615.24; Found 615.24.



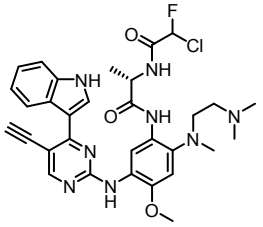
13

(2*S*)-*N*-(5-((4-(1*H*-Indol-3-yl)-5-methylpyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)-(methyl)amino)-4-methoxyphenyl)-2-(2-chloro-2-fluoroacetamido)propanamide (13)

13 was prepared in the similar manner to **3** starting from **S16**.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.67 (s, 1H) 9.79 (s, 1H), 8.98 (t, *J* = 7.5 Hz, 1H), 8.62 (s, 1H), 8.59 (s, 1H), 8.34 (d, *J* = 4.0 Hz, 1H), 8.20 (s, 1H), 7.98 (d, *J* = 1.5 Hz, 1H), 7.87 (s, 1H), 7.43 (d, *J* = 4.0 Hz, 1H), 7.03–7.00 (m, 2H), 6.79 (d x 2, $J_{(H-F)}$ = 49.5 Hz, 1H), 6.55 (s, 1H), 4.51–4.48 (m, 1H), 3.81 (s, 3H), 2.95 (brs, 2H), 2.67 (s, 3H), 2.39–2.27 (m, 5H), 2.22 (s, 6H), 1.32 (t, *J* = 5.4 Hz, 3H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{30}H_{37}ClFN_8O_3$ 611.27; Found 611.26.



14

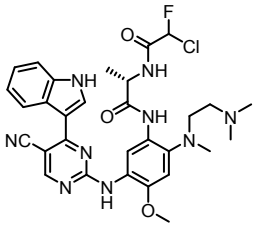
(2*S*)-2-(2-Chloro-2-fluoroacetamido)-*N*-(2-((2-(dimethylamino)ethyl)(methyl)amino)-5-((5-ethynyl-4-(1H-indol-3-yl)pyrimidin-2-yl)amino)-4-methoxyphenyl)propanamide (14)

14 was prepared in the similar manner to **3** starting from **S21**.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.73 (s, 1H), 9.81 (s, 1H), 8.99 (t, *J* = 7.8 Hz, 1H), 8.79 (d, *J* = 1.5 Hz, 1H), 8.70 (s, 1H), 8.40 (d, *J* = 1.8 Hz, 1H), 8.36—8.21 (m, 2H), 7.42 (d, *J* = 4.0 Hz, 1H), 7.11 (brs, 1H), 7.04 (s, 1H), 6.96 (brs, 1H), 6.77 (d x 2, $J_{(H-F)}$ = 49.5 Hz, 1H), 4.62 (s, 1H),

4.51–4.49 (m, 1H), 3.74 (s, 3H), 2.98 (brs, 2H), 2.71 (s, 3H), 2.37 (brs, 2H), 2.20 (s, 6H), 1.33 (t, *J* = 6.3 Hz, 3H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{31}H_{35}ClFN_8O_3$ 621.25; Found 621.25.



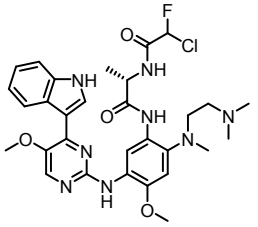
15

(2*S*)-2-(2-Chloro-2-fluoroacetamido)-*N*-(5-((5-cyano-4-(1H-indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)propanamide (15)

15 was prepared in the similar manner to **3** starting from **S22**.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.97 (s, 1H), 9.80 (d, *J* = 3.0 Hz, 1H), 9.41 (s, 1H), 9,00 (t, *J* = 8.5 Hz, 1H), 8.66 (s, 1H), 8.51 (d, *J* = 1.5 Hz, 1H), 8.17 (s, 1H), 7.46 (d, *J* = 3.8 Hz, 1H), 7.16 (brs, 1H), 7.06 (s, 1H), 6.98 (brs, 1H), 6.77 (d x 2, $J_{(H–F)}$ = 49.5 Hz, 1H), 4.52–4.49 (m, 1H), 3.73 (s, 3H), 3.00 (brs, 2H), 2.72 (s, 3H), 2.38–2.36 (m, 2H), 2.22 (s, 6H), 1.34 (t, *J* = 6.5 Hz, 3H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{30}H_{34}ClFN_9O_3$ 622.25; Found 622.24.



16

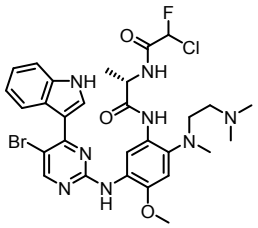
(2*S*)-*N*-(5-((4-(1*H*-Indol-3-yl)-5-methoxypyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)-(methyl)amino)-4-methoxyphenyl)-2-(2-chloro-2-fluoroacetamido)propanamide (16)

16 was prepared in the similar manner to **3** starting from **S26**.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.69 (s, 1H), 9.83 (s, 1H), 8.99 (t, *J* = 7.3 Hz, 1H), 8.68 (s, 1H), 8.54 (d, *J* = 4.0 Hz, 1H), 8.34 (d, *J* = 1.5 Hz, 1H), 8.17 (d, *J* = 0.75 Hz, 1H), 7.78 (s, 1H), 7.44 (d, *J* = 4.0 Hz, 1H), 7.04 (t, *J* = 7.3 Hz, 1H), 7.01 (s, 1H), 6.79 and 6.78 (d, $J_{(H–F)}$ = 49.5 Hz, 1H),

4.52–4.48 (m, 1H), 3.96 (s, 3H), 3.82 (s, 3H), 2.96—2.94 (m, 2H), 2.68 (s, 3H), 2.34—2.27 (m, 2H), 2.20 (s, 6H), 1.34 (t, *J* = 6.8 Hz, 3H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{30}H_{37}ClFN_8O_4$ 627.26; Found 627.26.



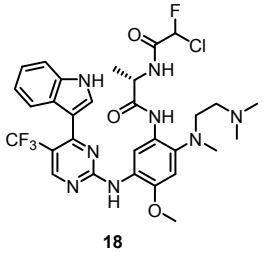
17

(2*S*)-*N*-(5-((5-Bromo-4-(1*H*-indol-3-yl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)-(methyl)amino)-4-methoxyphenyl)-2-(2-chloro-2-fluoroacetamido)propanamide (17)

17 was prepared in the similar manner to **3** starting from **S20**.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.78 (s, 1H), 9.78 (s, 1H), 8.99 (t, *J* = 8.5 Hz, 1H), 8.54 (1.5 Hz, 1H), 8.51 (s, 1H), 8.43 (s, 1H), 8.31 (s, 1H), 8.17 (d, *J* = 3.8 Hz, 1H), 7.43 (d, *J* = 4.3 Hz, 1H), 7.13 (t, *J* = 7.5 Hz, 1H), 7.03 (s, 1H), 6.97 (t, *J* = 7.5 Hz, 1H), 6.78 (d x 2, $J_{(H-F)}$ = 49.5 Hz, 1H), 4.52–4.48 (m, 1H), 3.76 (s, 3H), 2.97—2.95 (m, 2H), 2.69 (s, 2H), 2.37—2.31 (m, 2H), 2.20 (s, 6H), 1.33 (t, *J* = 6.8 Hz).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{29}H_{34}{}^{81}BrClFN_8O_3$ 677.16; Found 677.16.

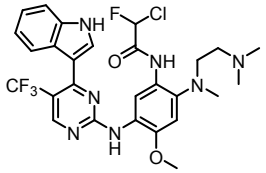


18

(2*S*)-*N*-(5-((4-(1*H*-Indol-3-yl)-5-(trifluoromethyl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)-ethyl)(methyl)amino)-4-methoxyphenyl)-2-(2-chloro-2-fluoroacetamido)propanamide (18) (NSP-037)

18 was prepared in the similar manner to **3** starting from **S30**.

^{1}H NMR (500 MHz, DMSO-d_6, as a mixture of two diastereomers) δ 11.78 (s, 1H), 9.80 and 9.79 (s, 1H), 9.11 (s, 1H), 9.01 (t, J = 8.0 Hz, 1H), 8.62 (s, 1H), 8.23 (s, 1H), 8.06 (brs, 1H), 7.86 (s, 1H), 7.43 (d, J = 8.0 Hz, 1H), 7.13 (t, J = 7.5 Hz, 1H), 7.04 (s, 1H), 6.97 (brs, 1H), 6.79 and 6.78 (d, $J_{(H–F)}$ = 49.5 Hz, 1H), 4.56–4.46 (m, 1H), 3.76 (s, 3H), 3.05–2.91 (m, 2H), 2.70 (s, 3H), 2.40–2.29 (m, 2H), 2.20 (s, 6H), 1.37–1.32 (m, 3H).
LRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{30}H_{34}ClF_4N_8O_3$ 665.24; Found 665.24.



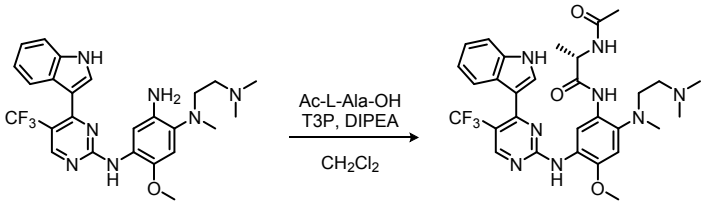
19

***N*-(5-((4-(1*H*-Indol-3-yl)-5-(trifluoromethyl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)-(methyl)amino)-4-methoxyphenyl)-2-chloro-2-fluoroacetamide (19)**

19 was prepared in the similar manner to **2** starting from **S30**.

^{1}H NMR (400 MHz, DMSO-d_6): δ 9.38 (s, 1H), 8.79 (s, 1H), 8.55 (s, 1H), 8.26 (d, J = 8.4 Hz, 2H), 7.84 (d, J = 6.0 Hz, 2H), 7.44 (d, J = 7.6 Hz, 1H), 7.18–7.30 (m, 2H), 6.78 (s, 1H), 6.38 (d, $J_{(H–F)}$ = 51.0 Hz, 1H), 3.90 (s, 3H), 3.02 (s, 2H), 2.71 (s, 3H), 2.69 (t, 2H), 2.47 (s, 3H).
HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{27}H_{29}ClF_4N_7O_2$ 594.2002; Found 594.2018.

Preparation of **20**



S30 **20**

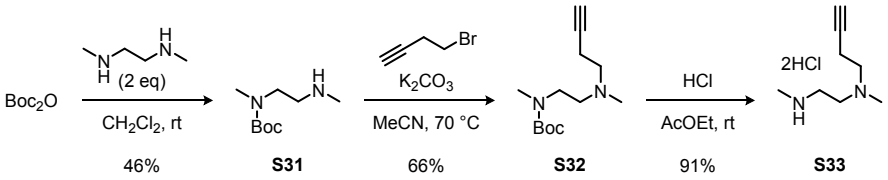
(*S*)-*N*-(5-{[4-(1*H*-Indol-3-yl)-5-(trifluoromethyl)pyrimidin-2-yl]amino}-2-{[2-(dimethylamino)-ethyl](methyl)amino}-4-methoxyphenyl)-2-acetamidopropanamide (20)

To a stirred solution of **S29** (24.2 mg, 0.0484 mmol) and Ac-L-Ala-OH (22.2 mg, 0.169 mmol) in dry CH_2Cl_2 (0.5 mL) was added DIPEA (51.0 µL, 0.293 mmol) and propylphosphonic anhydride (T3P) (50 wt. % in AcOEt, 87.0 µL, 0.146 mmol) at ambient temperature. After stirred for 3 h, the

reaction mixture was diluted with sat. $NaHCO_3$ and the aqueous phase was extracted thrice with AcOEt. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH/aq. NH_3 = 150:10:1 to 100:10:1) to give **20** (19.5 mg, 66% yield) as a light-brown foam.

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.80 (s, 1H), 9.64 (s, 1H), 9.16 (s, 1H), 8.62 (s, 1H), 8.32 (d, *J* = 7.5 Hz, 1H), 8.26 (s, 1H), 8.04 (brs, 1H), 7.85 (d, *J* = 2.0 Hz, 1H), 7.43 (d, *J* = 8.0 Hz, 1H), 7.13 (t, *J* = 7.5 Hz, 1H), 7.05 (s, 1H), 6.96 (brs, 1H), 4.38 (p, *J* = 7.5 Hz, 1H), 3.75 (s, 3H), 3.03–2.92 (m, 2H), 2.67 (s, 3H), 2.39–2.30 (m, 2H), 2.20 (s, 6H), 1.90 (s, 3H), 1.27 (d, *J* = 7.5 Hz, 3H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{30}H_{36}F_3N_8O_3$ 613.29; Found 613.29.

Preparation of the alkynylated amine **S33** for probes **21** and **22**



***tert*-Butyl methyl[2-(methylamino)ethyl]carbamate (S31)**
To a stirred solution of *N*,*N*'-dimethylethylenediamine (4.22 g, 47.9 mmol) in dry CH_2Cl_2 (80 mL) was added Boc_2O (4.97 g, 22.7 mmol) in CH_2Cl_2 (100 mL) dropwise over 3.5 h at ambient temperature. After stirred for 1 h, the reaction mixture was washed sequentially with sat. $NaHCO_3$ x2, water, and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH/aq. NH_3 = 200:10:1 to 50:10:1) to give **S31** (1.97 g, 46% yield) as colorless oil.

***tert*-Butyl {2-[but-3-yn-1-yl(methyl)amino]ethyl}(methyl)carbamate (S32)**
To a stirred solution of 4-bromo-1-butyne (900 µL, 9.59 mmol) in dry MeCN (24 mL) was added **S31** (1.50 g, 7.99 mmol) in dry MeCN (8.0 mL) and solid K_2CO_3 (2.48 g, 18.0 mmol) at ambient temperature. After stirred for 10 h at 70 °C, the reaction mixture was diluted with AcOEt, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (hexane/AcOEt = 1:1) to give **S32** (1.26 g, 66% yield) as pale-yellow oil.

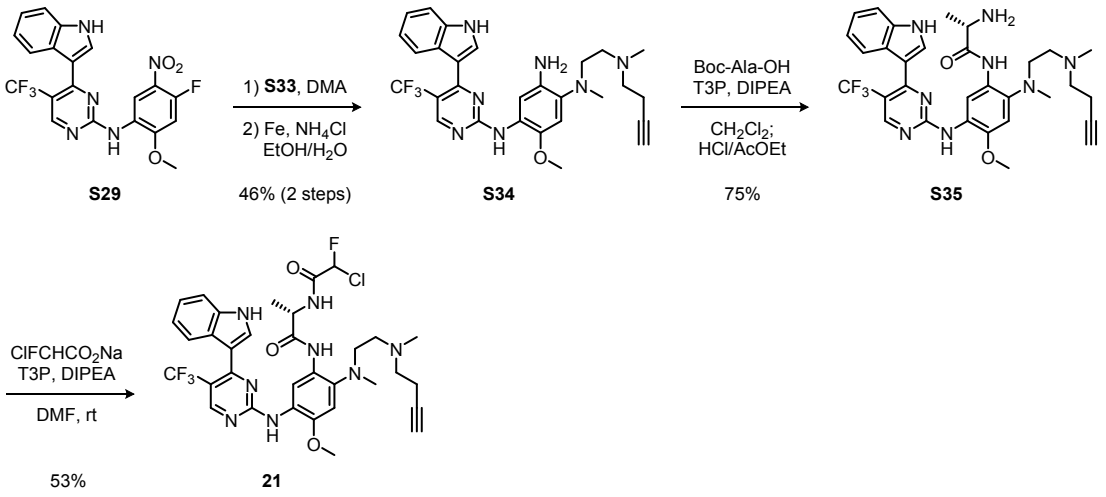
^{1}H NMR (500 MHz, $CDCl_3$) δ 3.36–3.26 (m, 2H), 2.87 (s, 3H), 2.64 (t, *J* = 7.5 Hz, 2H), 2.53 (d, *J* = 5.0 Hz, 2H), 2.34 (td, *J* = 7.5, 2.5 Hz, 2H), 2.31 (s, 3H), 1.98 (brs, 1H), 1.46 (s, 9H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{13}H_{25}N_2O_2$ 241.19; Found 241.19.

N^1-(But-3-yn-1-yl)-N^1,N^2-dimethylethane-1,2-diamine dihydrochloride (S33)

To a round-bottom flask charged with **S32** (1.24 g, 5.16 mmol) was added 4*N* HCl/AcOEt (20.0 mL) dropwise at ambient temperature. After stirred for 45 min, the volatiles were removed in vacuo and the residue was dried at 80 °C under vacuum to give the title compound (**S33**) (997 mg, 91% yield) as a white solid.

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.20 (brs, 1H), 9.34 (brs, 2H), 3.38 (brs, 4H), 3.29 (brs, 2H), 3.12 (s, 1H), 2.84 (brs, 3H), 2.76 (d, *J* = 5.0 Hz, 2H), 2.59 (s, 3H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_8H_{17}N_2$ 141.14; Found 141.14.

Preparation of probe **21**



N^4-[4-(1*H*-Indol-3-yl)-5-(trifluoromethyl)pyrimidin-2-yl]-N^1-{2-[but-3-yn-1-yl(methyl)amino]-ethyl}-5-methoxy-N^1-methylbenzene-1,2,4-triamine (S34)

To a stirred solution of **S29** (154 mg, 0.344 mmol) and **S33** (111 mg, 0.520 mmol) in dry DMA (1.5 mL) was added DIPEA (270 µL, 1.55 mmol) at ambient temperature. After stirred for 3 h at 120 °C, the reaction mixture was diluted with sat. $NaHCO_3$ and the aqueous phase was extracted

thrice with AcOEt. The combined organic layers were washed with sat. $NaHCO_3$ x2 and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was re-dissolved in EtOH (12 mL) and water (4.0 mL). To the solution was added iron powder (394 mg, 7.06 mmol) and NH_4Cl (43.6 mg, 0.815 mmol) and refluxed for 8 h. After cooling to ambient temperature, the solid material was removed by filtration and the filtrate was concentrated in vacuo. The residue was diluted with sat. $NaHCO_3$ and extracted thrice with 4:1 $CHCl_3$/2-propanol. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH/aq. NH_3 = 500:10:2) to give **S34** (86.0 mg, 46% yield over 2 steps) as a yellow foam.

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.80 (s, 1H), 8.87 (s, 1H), 8.61 (s, 1H), 8.14 (brs, 1H), 7.87 (d, *J* = 2.5 Hz, 1H), 7.45 (d, *J* = 8.0 Hz, 1H), 7.16 (t, *J* = 7.5 Hz, 1H), 7.02 (brs, 1H), 6.90 (s, 1H), 6.76 (s, 1H), 4.58 (brs, 2H), 3.68 (s, 3H), 3.39–3.30 (m, 2H), 2.89 (t, *J* = 6.5 Hz, 2H), 2.79 (t, *J* = 2.5 Hz, 1H), 2.66 (s, 3H), 2.55 (t, *J* = 7.5 Hz, 2H), 2.30 (td, *J* = 7.5, 2.5 Hz, 2H), 2.22 (s, 3H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{28}H_{31}F_3N_7O$ 538.25; Found 538.25.

(*S*)-*N*-(5-{[4-(1*H*-Indol-3-yl)-5-(trifluoromethyl)pyrimidin-2-yl]amino}-2-({2-[but-3-yn-1-yl-(methyl)amino]ethyl)(methyl)amino}-4-methoxyphenyl)-2-aminopropanamide (S35)
To a stirred solution of **S34** (63.0 mg, 0.117 mmol) and Boc-L-Ala-OH (68.8 mg, 0.364 mmol) in dry CH_2Cl_2 (2.0 mL) was added propylphosphonic anhydride (T3P) (50 wt. % in AcOEt, 209 μL, 0.351 mmol) and DIPEA (122 μL, 0.700 mmol) at ambient temperature. After stirred for 3 h, 4*N* HCl/AcOEt (2.0 mL) was added to the mixture and stirred for 45 min. The reaction mixture was basified with sat. $NaHCO_3$ and the aqueous phase was extracted thrice with 4:1 $CHCl_3$/2-propanol. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH/aq. NH_3 = 300:15:2) to give **S35** (53.5 mg, 75% yield) as a light-brown foam.

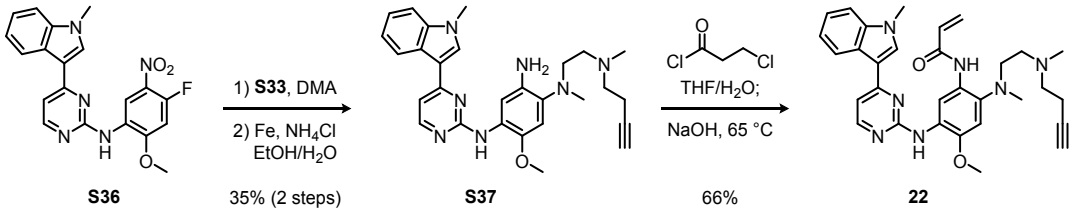
^{1}H NMR (500 MHz, DMSO-d_6) δ 11.80 (s, 1H), 10.21 (brs, 1H), 9.16 (s, 1H), 8.61 (s, 1H), 8.41 (brs, 1H), 8.05 (brs, 1H), 7.86 (s, 1H), 7.43 (d, *J* = 8.0 Hz, 1H), 7.14 (t, *J* = 7.5 Hz, 1H), 7.03 (brs, 1H), 6.95 (brs, 1H), 3.75 (s, 3H), 3.41 (q, *J* = 7.0 Hz, 1H), 3.35–3.31 (m, 2H), 2.99 (t, *J* = 7.0 Hz, 2H), 2.79 (t, *J* = 2.5 Hz, 1H), 2.71 (s, 3H), 2.57–2.52 (m, 2H), 2.27 (td, *J* = 7.5, 2.5 Hz, 2H), 2.19 (s, 3H), 1.22 (d, *J* = 7.0 Hz, 3H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{31}H_{36}F_3N_8O_2$ 609.29; Found 609.29.

(2*S*)-*N*-(5-{[4-(1*H*-Indol-3-yl)-5-(trifluoromethyl)pyrimidin-2-yl]amino}-2-[{2-[but-3-yn-1-yl-(methyl)-amino]ethyl}(methyl)amino]-4-methoxyphenyl)-2-(2-chloro-2-fluoroacetamido)propanamide (21)

To a stirred solution of **S35** (44.3 mg, 0.0728 mmol) and sodium chlorofluoroacetate (35.5 mg, 0.264 mmol) in dry DMF (1.0 mL) was added DIPEA (76.0 µL, 0.436 mmol) and propylphosphonic anhydride (T3P) (50 wt. % in AcOEt, 130 µL, 0.218 mmol) at ambient temperature. After stirred for 2 h, the reaction mixture was diluted with sat. $NaHCO_3$ and the aqueous phase was extracted thrice with AcOEt. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (AcOEt/hexane/2 M ethanolic ammonia = 80:20:1) to give **21** (26.8 mg, 53% yield) as a pale-yellow foam.

^{1}H NMR (500 MHz, DMSO-d_6, as a mixture of two diastereomers) δ 11.80 (s, 1H), 9.44 (s, 1H), 9.14 (s, 1H), 9.11–9.05 (m, 1H), 8.62 (s, 1H), 8.16 (brs, 1H), 8.08 (brs, 1H), 7.86 (d, J = 2.0 Hz, 1H), 7.43 (d, J = 8.0 Hz, 1H), 7.14 (t, J = 7.5 Hz, 1H), 7.03 (s, 1H), 6.99 (brs, 1H), 6.80 (d, $J_{(H–F)}$ = 49.5 Hz, 1H), 4.54 (q, J = 7.0 Hz, 1H), 3.77 (s, 3H), 3.35–3.32 (m, 2H), 3.00 (brs, 2H), 2.81 (brs, 1H), 2.69 (s, 3H), 2.51 (brs, 2H), 2.31 (brs, 2H), 2.21 (s, 3H), 1.37–1.32 (m, 3H).
LRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{33}H_{36}ClF_4N_8O_3$ 703.25; Found 703.25.

Preparation of probe **22**



***N*^{1}-{2-[But-3-yn-1-yl(methyl)amino]ethyl}-5-methoxy-*N*^{1}-methyl-*N*^{4}-[4-(1-methyl-1*H*-indol-3-yl)-pyrimidin-2-yl]benzene-1,2,4-triamine (S37)**

To a stirred solution of **S36**[S5] (107 mg, 0.272 mmol) and **S33** (89.3 mg, 0.419 mmol) in dry DMA (1.0 mL) was added DIPEA (213 µL, 1.22 mmol) at ambient temperature. After stirred for 3 h at 120 °C, the reaction mixture was diluted with sat. $NaHCO_3$ and the aqueous phase was extracted thrice with AcOEt. The combined organic layers were washed with sat. $NaHCO_3$ x2 and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was re-dissolved in EtOH (9.0 mL) and water (3.0 mL). To the solution was added iron powder (233 mg, 4.17 mmol) and

NH_4Cl (25.0 mg, 0.468 mmol) and refluxed for 10 h. After cooling to ambient temperature, the solid material was removed by filtration and the filtrate was concentrated in vacuo. The residue was diluted with sat. $NaHCO_3$ and extracted thrice with AcOEt. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (AcOEt/MeOH/2 M ethanolic ammonia = 100:4:1) to give **S37** (45.8 mg, 35% yield over 2 steps) as brown viscous oil.

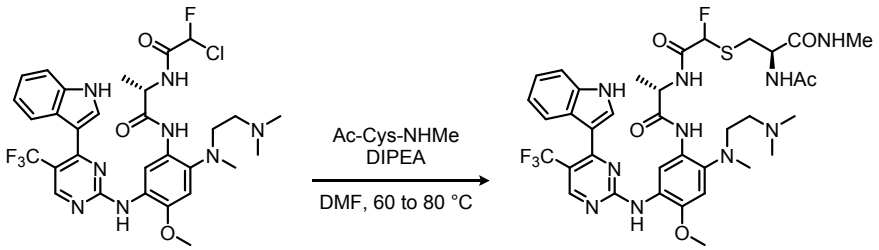
^{1}H NMR (500 MHz, DMSO-d_6) δ 8.44 (d, *J* = 8.0 Hz, 1H), 8.32 (s, 1H), 8.27 (d, *J* = 5.0 Hz, 1H), 7.80 (s, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.48 (s, 1H), 7.26 (td, *J* = 8.0, 1.0 Hz, 1H), 7.18 (td, *J* = 8.0, 1.0 Hz, 1H), 7.15 (d, *J* = 5.0 Hz, 1H), 6.76 (s, 1H), 4.60 (brs, 2H), 3.89 (s, 3H), 3.75 (s, 3H), 2.89 (t, *J* = 6.5 Hz, 2H), 2.79 (t, *J* = 2.5 Hz, 1H), 2.64 (s, 3H), 2.55 (t, *J* = 7.5 Hz, 2H), 2.50 (t, *J* = 6.5 Hz, 2H), 2.30 (td, *J* = 7.5, 2.5 Hz, 2H), 2.20 (s, 3H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{28}H_{34}N_7O$ 484.28; Found 484.28.

***N*-[2-({2-[But-3-yn-1-yl(methyl)amino]ethyl}(methyl)amino)-4-methoxy-5-{[4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl]amino}phenyl]acrylamide (22)** [S6]

To a stirred solution of **S37** (34.5 mg, 0.0713 mmol) in THF/H_2O (10:1) (1.1 mL) was added 3-chloropropionyl chloride (10.2 µL, 0.107 mmol) dropwise at 0 °C. After stirred for 30 min, solid NaOH (18.2 mg, 0.455 mmol) was added to the mixture and stirred for 9 h at 65 °C. The reaction mixture was diluted with sat. $NaHCO_3$ and the aqueous phase was extracted thrice with AcOEt. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (AcOEt/hexane/2 M ethanolic ammonia = 80:20:1) to give **22** (25.8 mg, 76% yield) as a pale-yellow foam.

^{1}H NMR (500 MHz, DMSO-d_6) δ 9.75 (s, 1H), 9.09 (brs, 1H), 8.67 (brs, 1H), 8.33 (d, *J* = 5.0 Hz, 1H), 8.26 (d, *J* = 8.0 Hz, 1H), 7.92 (s, 1H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.25 (td, *J* = 7.0, 1.0 Hz, 1H), 7.23 (d, *J* = 5.0 Hz, 1H), 7.16 (td, *J* = 8.0, 1.0 Hz, 1H), 7.03 (s, 1H), 6.54 (dd, *J* = 17.0, 10.0 Hz, 1H), 6.27 (dd, *J* = 17.0, 2.0 Hz, 1H), 5.78 (dd, *J* = 10.0, 2.0 Hz, 1H), 3.92 (s, 3H), 3.87 (s, 3H), 2.90 (t, *J* = 5.5 Hz, 2H), 2.81 (t, *J* = 2.5 Hz, 1H), 2.73 (s, 3H), 2.61 (t, *J* = 7.5 Hz, 2H), 2.45 (t, *J* = 5.5 Hz, 2H), 2.33 (td, *J* = 7.5, 2.5 Hz, 2H), 2.23 (s, 3H).
LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{31}H_{36}N_7O_2$ 538.29; Found 538.29.

Preparation of compound **23**



18 47% **23**

(2*R*)-3-((2-(((*S*)-1-((5-((4-(1*H*-Indol-3-yl)-5-(trifluoromethyl)pyrimidin-2-yl)amino)-2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxyphenyl)amino)-1-oxopropan-2-yl)amino)-1-fluoro-2-oxoethyl)thio)-2-acetamido-*N*-methylpropanamide (23)

To a stirred solution of Ac-Cys-NHMe[S7] (9 mg, 0.0551 mmol) and **18** (30 mg, 0.0394) in dry DMF (20 mL) was added DIPEA (48 μL, 0.276 mmol). After stirred for 2 h at 60 °C, to the reaction mixture was added Ac-Cys-NHMe (3.5 mg, 0.0199 mmol) and DIPEA (30 μL, 0.173 mmol) and further stirred for 22 h at 80 °C. The mixture was concentrated in vacuo and the residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH/aq. NH_3 = 1600:10:1 to 400:10:1) to give a white solid, which was further purified by flash column chromatography on silica gel ($CHCl_3$/MeOH/aq. NH_3 = 400:10:1 to 200:10:1) to give **23** (15 mg, 47% yield) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.4 (brs, 1H), 9.42 (s, 1H), 8.74 (s, 1H), 8.22 (dd, *J* = 20, 8.0 Hz, 1H), 7.90–7.86 (m, 2H), 7.45 (t, *J* = 2.5 Hz, 1H), 7.23–7.14 (m, 1H), 7.14 (brs, 1H), 6.80–6.79 (m, 2H), 6.04 (dd, *J* = 35, 51 Hz, 1H), 4.57–4.52 (m, 2H), 3.89 (s, 3H), 2.95–2.87 (m, 3H), 2.81 (d, *J* = 5.0 Hz, 2H), 2.76 (d, *J* = 5.0 Hz, 1H), 2.70 (d, *J* = 15 Hz, 3H), 2.32 (s, 6H), 2.05 (d, *J* = 35 Hz, 3H), 1.48(s, 3H).

LRMS (ESI) *m*/*z*: $[M+H]^+$ calcd for $C_{36}H_{45}F_4N_{10}O_5S$ 805.32; Found 805.31.

Reference

S5. Butterworth, S. *et al.* 2-(2,4,5-Substituted-anilinopyrimidine derivatives as EGFR modulators useful for treating cancer. International patent WO 2013/014448 A1 filed 25 July 2012, and published 31 January 2013.

S6. Niessen, S. *et al*. Proteome-wide Map of Targets of T790M-EGFR-Directed Covalent Inhibitors. *Cell Chem. Biol.* **2017**, *24*, 1388–1400.

S7. Abuzar, S. & Kohn, H. Studies on the reactivity of bicyclomycin with nucleophilic amino acid derivatives. *J. Org. Chem.* **54**, 4000–4003 (1989).