Discovery of BUB1 kinase inhibitors for the treatment of cancer

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Development of a cellular BUB1 target engagement assay

Introduction

Target engagement (also termed target occupancy) is a concept in drug discovery that describes the physical interaction of a small molecule with its intended protein target in a specific biological system (e.g. in living cells, animals or humans).[1] Insufficient target engagement leads to a lack of drug efficacy in preclinical and clinical studies.[1] Therefore, investigating which compound concentration is required to obtain complete target occupancy is important.[1] Several methods have recently been developed to study target engagement[2], such as thermal protein shift[3], bioluminescence resonance energy transfer (BRET)[4] and activity-based protein profiling (ABPP) assays.[5]

ABPP relies on a chemical probe that forms a covalent bond with a protein of interest. Such probes consist of a recognition element, ligation tag and an electrophilic moiety, termed 'the warhead'.[6] The recognition element provides affinity for the target (or family of targets), whereas the electrophilic moiety reacts with a nucleophilic amino acid in close proximity. The ligation handle, such as an alkyne or an azide, enables the introduction of a fluorescent or biotin reporter group using copper-catalyzed azide-alkyne cycloaddition (CuAAC, 'click'-reaction).[7,8] This allows for visualization or identification of proteins using gel-based or mass spectrometry-based proteomics methods, respectively. Broad-spectrum probes are used to investigate cellular selectivity of small molecule inhibitors across an entire protein family. For example, Zhao *et al.*[9] published XO44 as a cell permeable broad-spectrum kinase probe, which provided the cellular selectivity profile of the approved kinase drug dasatinib on 133 kinases in a cell line using competitive chemical proteomics.[5] Alternatively, tailor-made probes can be used to determine the target engagement of a single target in a competitive, gel-based ABPP assay. Van der Wel *et al.* determined, for instance, cellular target engagement of FES kinase using a highly selective probe in a chemical genetic study.[10]

Chapters 2 – 4 described the discovery of substituted quinazolines and benzimidazoles as novel and potent inhibitors of budding uninhibited by benzimidazole 1 (BUB1) kinase for the treatment of cancer. To further evaluate these compounds in living cells, a BUB1 target engagement assay is highly desirable to correlate their target occupancy with cellular effects. To this end, a tailor-made probe targeting BUB1 is required. Recently, Shindo *et al.*[11] reported NS-062 as part of a series of close analogs of afatinib (**Figure 1A**), which is a covalent inhibitor of the epidermal growth factor receptor (EGFR) approved for the treatment of non-small cell lung cancer.[12] Of interest, the alkynylated-derivative of NS-062, compound **1** (**Figure 1A**), was found to bind BUB1[12] and resembles the quinazoline inhibitors described in **Chapter 3**. In this chapter, it was investigated whether compounds from this chemical series are suitable as chemical probes for a BUB1 engagement assay.

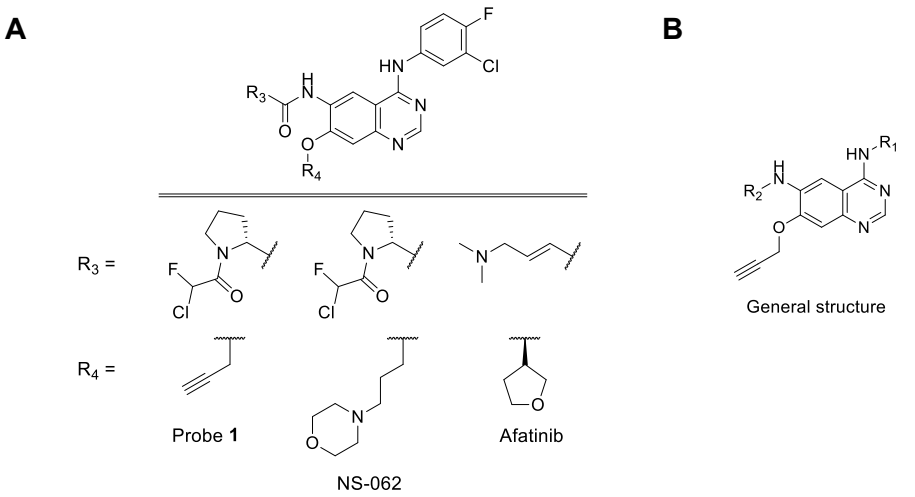


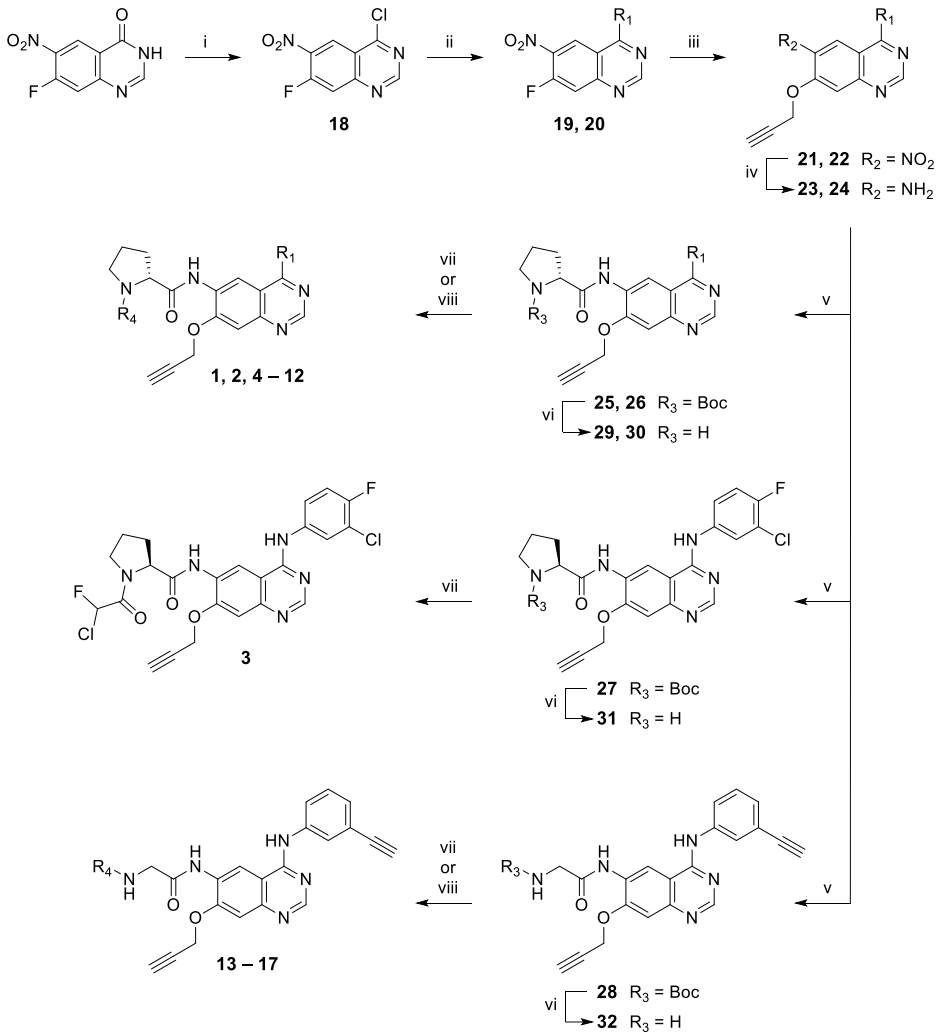
Figure 1 | (A) Chemical structures of probe **1**, NS-062 (both published by Shindo *et al.*[12]) and afatinib. **(B)** General structure of probe **1** and regions R_1 and R_2 of the quinazoline scaffold used to investigate the structure-activity relationship of **1**.

Results & Discussion

Design and synthesis of compounds 1 – 17

To study the potential of NS-062 analogues as BUB1 probes, compounds **1** – **17** were synthesized. Of note, a subset of these molecules (**1**, **3**, **4**, **8**, **10** and **12**) was previously reported.[12] Based on the biochemical activities of the quinazoline inhibitors reported in **Chapter 3**, the chloro-fluorophenyl of **1** at R_1 (**Figure 1B**) was substituted for a phenylacetylene in analogues **2**, **5**, **7**, **9**, **11** and **13** – **17**. The chloro-fluoroacetamide moiety was replaced by other warheads employed in approved kinase inhibitors, such as 2-butynamide[13] (in **5**, **6** and **14**), 4-dimethylaminocrotonamide[14] (in **7**, **8** and **15**) and an acrylamide[15] (in **9**, **10** and **16**). The chirality of the amino acid linker at R_2 (**Figure 1B**) was also investigated by substituting the D-proline for its L-enantiomer in compound **3**.[12] For analogues (**13** – **17**) glycine was used as a more flexible linker instead of a proline. Finally, to obtain negative control compounds, the warhead was replaced by an acetyl group in inhibitors **11**, **12** and **17**.[12]

Compounds **1** – **17** were synthesized as depicted in **Scheme 5.1**. Briefly, commercially available 7-fluoro-6-nitroquinazolin-4(3*H*)-one was chlorinated to obtain **18**.[16] A nucleophilic aromatic substitution with either 3-chloro-4-fluoroaniline or 3-ethynylaniline resulted in the formation of **19** and **20**, respectively.[16] Subsequent nucleophilic aromatic substitution was performed with propargyl alcohol to introduce the alkyne ligation handle.[17] Reduction of the nitro group yielded amines **23** and **24**,[17] which served as building blocks for the synthesis of **25** – **28**. The linkers were introduced by performing peptide coupling reactions using *N*-Boc-protected amino acids and pivaloyl chloride.[18] Boc deprotection yielded intermediates **29** – **32** and using acyl chlorides of the warhead or applying a peptide coupling method as mentioned above afforded the desired compounds.



Scheme 5.1 | Synthesis of 1 – 17. Reagents and conditions: **i)** $SOCl_2$, cat. DMF, 75°C, 99%. **ii)** 3-chloro-4-fluoroaniline (for **19**) or 3-ethynylaniline (for **20**), DIPEA, 2-propanol, 37 – 81%. **iii)** propargyl alcohol, KO*t*Bu, THF, 0°C – RT, 91% – quant. **iv)** Fe, NH_4Cl, EtOH/H_2O (30:1), 80°C, 66% – quant. **v)** Boc-D-Pro-OH (for **25** and **26**), Boc-L-Pro-OH (for **27**) or Boc-Gly-OH (for **28**), PivCl, DIPEA, cat. DMF, DCM, 0°C – RT, 74 – 97%. **vi)** TFA, DCM, 56% – quant. **vii)** 2-chloro-2-fluoroacetic acid (for **1** – **3** and **13**), 2-butynoic acid (for **5**, **6** and **14**) or (*E*)-4-(dimethylamino)but-2-enoic acid·HCl (for **7**, **8** and **15**), PivCl, DIPEA, cat. DMF, DCM, 0°C – RT, 5 – 73%. **viii)** 2-chloroacetyl chloride (for **4**), acryloyl chloride (for **9**, **10** or **16**) or acetyl chloride (for **11**, **12** and **17**), DIPEA, DCM, 0°C – RT, 13 – 72%.

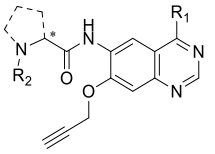
Biochemical evaluation of compound 1 – 17

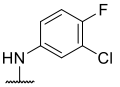
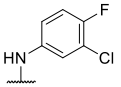
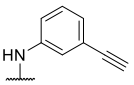
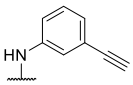
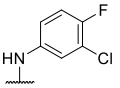
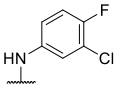
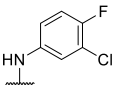
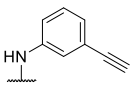
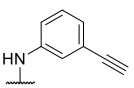
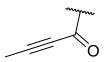
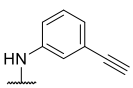
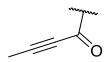
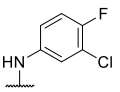
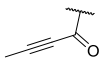
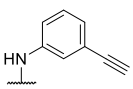
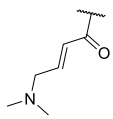
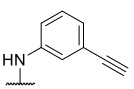
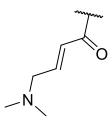
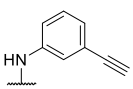
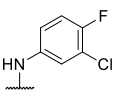
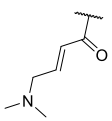
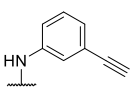
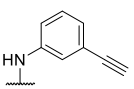
Compounds **1** – **17** were evaluated in a biochemical fluorescence polarization BUB1 activity assay to determine the half maximal inhibitory concentrations (IC_{50}) as described in **Chapter 2**. The data reported in **Table 5.1** are expressed as pIC_{50} ± SEM (N=2, n=2). The activity of the compounds with an electrophilic warhead (**1** – **10**, **13** – **16**) were compared to corresponding control compounds (**11**, **12**, **17**). Compound **4** was the most active inhibitor with a pIC_{50} of 8.95. It was over 4000-fold more potent than negative control compound **12**, which suggested that it forms a covalent bond with BUB1. Compound **4** contained a chloroacetamide, which was the most reactive electrophilic warhead in this series of inhibitors. In line, compounds with other, less reactive warheads, such as a chloro-fluoroacetamide (**1**), 2-butynamide (**5**, **6**), dimethylaminocrotonamide (**7**, **8**) or acrylamide (**9**,**10**) were significantly less potent. Furthermore, phenylacetylene **2** showed a 5-fold reduction in potency compared to chloro-fluorophenyl **1**, which indicated that the SAR of this series was different from the quinazolines reported in **Chapter 3**. Inverting the stereochemistry of the linker from D-proline (**1**) to L-proline (**3**) dramatically reduced potency, revealing the importance of this stereocenter for BUB1 activity. D-proline was the most optimal linker, since the glycine-containing compounds (**13** – **16**) did not show any increased activity compared to their negative control (**17**).

Evaluation of compounds 1 – 17 in living cells

To assess the ability of compounds **1** – **17** to covalently label BUB1, a U2OS cell line stably overexpressing N-terminal GFP-FLAG-tagged BUB1 (U2OS-BUB1^{GFP_FLAG} cells) was generated. Briefly, U2OS-BUB1^{GFP_FLAG} cells were incubated with probes **1** – **17** at 0.1 or 1 µM for 60 min after which the cells were harvested and lysed. Probe labeled proteins were ligated to a Cy5-fluorophore using click chemistry. The proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by in-gel fluorescence scanning (**Figure 5.2**). A fluorescent band at an apparent molecular weight of 175 kDa that corresponded to the BUB1^{GFP_FLAG} protein was detected for probes **1**, **2** and **4**, but not for **3** and **5** – **17**. Compounds **1** and **2** showed a similar overall labeling profile, however, the fluorescent intensity for the band at the apparent molecular weight of 175 kDa was less intense for probe **2**. This is in agreement with its reduced potency in the biochemical assay. Compound **4** was tested at 100 nM in view of its high potency. Indeed, the fluorescence intensity of the band at 175 kDa was the highest among all compounds, but significant labeling of other proteins was also observed. Of note, probe **5**, and to lesser extent compound **6**, showed strong fluorescent labeling of a protein with a lower apparent molecular weight in both U2OS-BUB1^{GFP_FLAG} (**Figure 5.2**) and non-transfected U2OS cells (**Supplementary Figure 5.1**, p.178). Taken all data together, compound **1** was selected for further evaluation as a chemical probe to study BUB1 target engagement.

Table 5.1 | Half maximal inhibitory concentrations (expressed as pIC_{50} ± SEM) of **1** – **17** determined by a fluorescence polarization assay on BUB1 kinase activity (N=2, n=2).



ID	R_1	Linker (*)	R_2	pIC_{50} ± SEM	ID	R_1	Linker (*)	R_2	pIC_{50} ± SEM
1		D-Pro		6.96 ± 0.04	**10**		D-Pro		< 5
2		D-Pro		6.26 ± 0.06	**11**		D-Pro		5.63 ± 0.04
3		L-Pro		< 5	**12**		D-Pro		5.30 ± 0.04
4		D-Pro		8.95 ± 0.05	**13**		Gly		5.97 ± 0.07
5		D-Pro		5.84 ± 0.03	**14**		Gly		6.24 ± 0.07
6		D-Pro		5.68 ± 0.04	**15**		Gly		6.25 ± 0.03
7		D-Pro		5.08 ± 0.04	**16**		Gly		6.34 ± 0.04
8		D-Pro		5.01 ± 0.04	**17**		Gly		6.47 ± 0.03
9		D-Pro		5.93 ± 0.02					

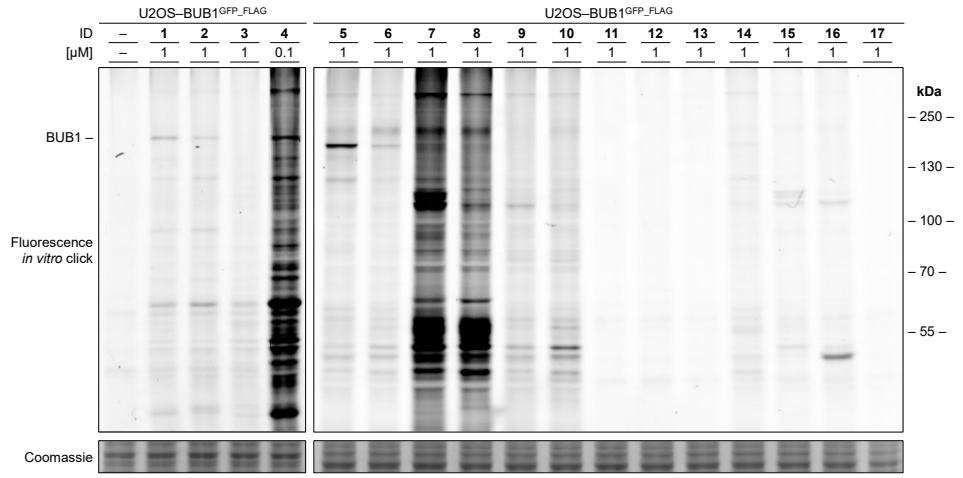


Figure 5.2 | Labeling by probes 1 – 10 and 13 – 16 and acetamide (11, 12, 17) controls. U2OS-BUB1^{GFP_FLAG} cells were incubated with probe (indicated concentration, 1 h, 37°C) after which the cells were lysed. Proteins labeled by probe were visualized by conjugation to a Cy5 fluorophore using click chemistry, SDS-PAGE and in-gel fluorescence scanning. Coomassie staining served as protein loading control.

Development of a cellular BUB1 engagement assay with probe 1.

To validate compound **1** as a BUB1 sensitive chemical probe, it was investigated which amino acid of BUB1 is responsible for the formation of a covalent bond with **1**. NS-062 covalently binds to Cys797 of EGFR (**Figure 5.3A**).[12] Based on a structural overlay of BUB1[19] and EFGR, it was hypothesized that Cys1080 of BUB1 could also react with the warhead of NS-062 and probe **1** (**Figure 5.3A**). Therefore, a C1080A point mutant was generated by site-directed mutagenesis of human BUB1 fused to an N-terminal FLAG-tag. U2OS cells were transiently transfected with wild-type (BUB1^{WT}) or mutant BUB1 (BUB1^{C1080A}) and incubated with **1**. Whereas BUB^{WT} was labeled by probe **1**, BUB1^{C1080A} was not (**Figure 5.3B**). Of note, expression of both BUB1 constructs was comparable as determined by immunoblotting against the FLAG-tag (**Figure 5.3B**). This confirmed that probe **1** specifically reacted with Cys1080. BUB1 labeling was concentration- and time-dependent (**Figure 5.3C–F**) and was optimal at 1 µM and 60 min. Finally, to study whether this probe could be used to study BUB1 target engagement, U2OS-BUB1^{GFP_FLAG} cells were pre-incubated with BAY1816032[20] at different concentrations. BAY1816032 was able to dose-dependently reduce the fluorescent labeling (**Figure 5.3G**) with an apparent target occupancy (expressed as pTE_{50}) of 6.45±0.10 (**Figure 5.3H**). Of note, the obtained pTE_{50} value is dependent on the kinetic conditions of the experiment since probe labeling occurs in a irreversible fashion, whereas BAY1816032 binds reversibly. Taken together, these results provide proof-of-principle that chemical probe **1** can be used to study BUB1 target engagement in living cells.

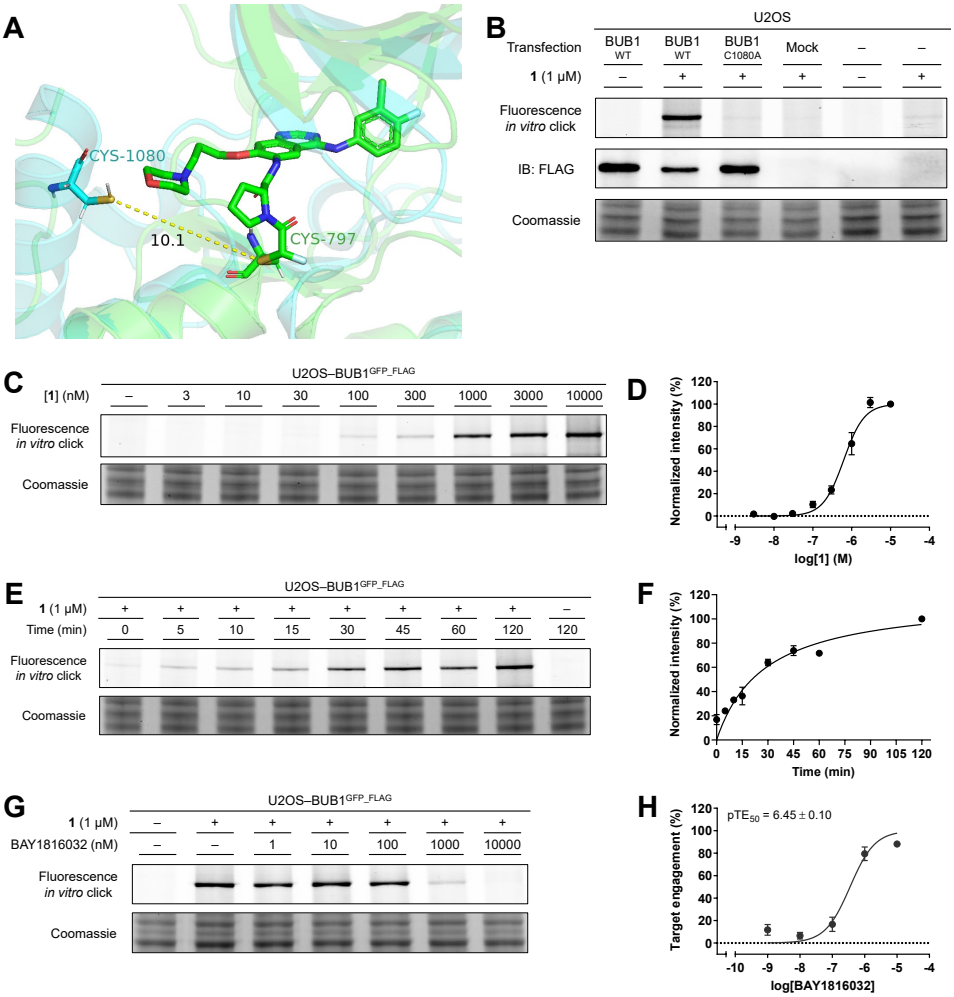


Figure 5.3 | Validation of 1 as BUB1 probe. (A) Crystal structure of NS-062 covalently bound to Cys797 of EGFR (PDB code: 5Y25, green) aligned with the crystal structure of BUB1 (PDB code: 4QPM, blue). The proposed nucleophilic amino acid of BUB1, Cys1080, is represented as sticks and its distance to Cys797 of EGFR is indicated (in angstrom, dashed line). **(B)** Labeling of $BUB1^{WT}$ but not $BUB1^{C1080A}$ in U2OS cells by **1**. U2OS cells were transfected with $BUB1^{WT}$, $BUB1^{C1080A}$ or mock control. 48 h post-transfection cells were treated with **1** (1 µM, 1 h, 37°C) after which the cells were lysed. Proteins labeled by **1** were visualized by conjugation to a Cy5 fluorophore using click chemistry, SDS-PAGE and in-gel fluorescence scanning. The top part of the gel was used for verification of protein expression by immunoblot (IB) against the N-terminal FLAG-tag, the bottom part of the gel was stained by Coomassie and served as loading control. **(C)** Representative visualization of dose-dependent labeling of GFP-FLAG-tagged BUB1 in U2OS-$BUB1^{GFP\_FLAG}$ cells by **1**. U2OS-$BUB1^{GFP\_FLAG}$ cells were incubated with vehicle (–) or **1** (indicated concentration, 1 h, 37°C) and labeling was visualized as described in (B). **(D)** Dose-response curve of **1** corresponding to experiments as performed in (C), normalized between vehicle control (–) and highest concentration of **1** (10 µM) and corrected for protein loading. Data represents mean ± SEM (N=3). **(E)** Representative visualization of time-dependent labeling of GFP-FLAG-tagged BUB1 in U2OS-$BUB1^{GFP\_FLAG}$ cells by **1**. U2OS-$BUB1^{GFP\_FLAG}$ cells were incubated with **1** (1 µM, indicated time, 37°C) and proteins labeled by **1** were visualized as described in (B). **(F)** Time-response curve of **1** corresponding to experiments as performed in (E), normalized between vehicle control (–) and longest incubation time with **1** (120 min) and corrected for protein loading. Data represents mean ± SEM (N=3). **(G)** Representative visualization of GFP-FLAG-tagged BUB1 target engagement in U2OS-$BUB1^{GFP\_FLAG}$ cells by BAY1816032. U2OS-$BUB1^{GFP\_FLAG}$ cells were pre-incubated with BAY1816032 (indicated concentration, 1 h, 37°C) followed by incubation with **1** (1 µM, 1 h, 37°C). Proteins labeled by **1** were visualized as described in (B). **(H)** Target engagement curve of BAY1816032 corresponding to experiments as performed in (G) (pTE_{50} = 6.45 ± 0.10). Data represents mean ± SEM (N=3).

Conclusion

Cellular target engagement studies are required to determine which compound concentration is sufficient to fully inhibit BUB1 activity in a living cell. Here, a series of quinazoline derivatives were designed, synthesized and tested as chemical probes of BUB1 suitable for gel-based ABPP studies. Compound **1**, which reacted with Cys1080 of BUB1, was validated as a chemical probe suitable for BUB1 engagement studies in U2OS cells. Probe **1** allows to correlate target engagement of BUB1 inhibitors with their phenotypic effects in cancer cells (**Chapter 6**). This will expand the understanding of the biological mode of action of BUB1 inhibitors and may help to further investigate BUB1 inhibitors as a potential treatment of cancer.

Acknowledgements

Tom van der Wel is kindly acknowledged for cloning and preparation of the U2OS-BUB1^{GFP_FLAG} stable cell line, Joel Rüegger for his contribution with regard to compound synthesis and biological evaluation of the probes, Bas de Man for his contribution regarding compound synthesis and biochemical evaluation and Hans van den Elst for HPLC purifications and HRMS measurements.

Experimental – Biochemistry

Biochemical evaluation of BUB1 inhibitors

Assays were performed in 384-well plates (Greiner, black, flat bottom, 781076) by sequential addition (final concentrations are indicated) of inhibitor (5 µL, 0.003 nM – 10 nM or 3 nM – 10 µM), BUB1/BUB3 (5 µL, 3.26 nM, Carna Biosciences (05-187), lot: 15CBS-0644 D), ATP (5 µL, 15 µM) and BUB1/BUB3 substrate (5 µL, 75 nM, Carna Biosciences (05-187MSSU)), all as 4x working solutions. The final concentration of DMSO was 1%. Assay reactions were stopped by addition of IMAP progressive binding reagent (20 µL, 1200x diluted (see below), Molecular Devices (R8155), lot: 3117896). Each assay included the following controls: (i) a background control (treated with vehicle instead of inhibitor and BUB1/BUB3 substrate), (ii) MIN controls (treated with 5 µM BAY1816032 (MedChem Express) as inhibitor, defined as 0% BUB1 activity) and (iii) MAX controls (treated with vehicle instead of inhibitor, defined as 100% BUB1 activity). All inhibitors were tested in two separate assays and all inhibitor concentrations were tested in duplicate per assay (N=2, n=2).

For each assay, assay buffer (AB) was freshly prepared and consisted of 20 mM HEPES (prepared by diluting 1 M HEPES, pH 7.2), 5 mM $MgCl_2$, 0.01% (v/v) Tween-20 and 1 mM DTT. Stocks of inhibitors (in DMSO) were diluted in AB to obtain 4x working solutions (4% DMSO) and 5 µL was added to the assay plate. BUB1/BUB3 (3.26 µM (486 µg/mL) in storage buffer) was diluted in AB to obtain 13.0 nM of which 5 µL was added to all wells of the assay plate. The assay plate was centrifuged (1 min, 200 *g*) and incubated at RT for 30 min. ATP (4 mM in MilliQ) was diluted in AB to obtain 60 µM of which 5 µL was added to each well. BUB1/BUB3 substrate (1 mM) was diluted in 20 mM HEPES (prepared by diluting 1 M HEPES (pH 7.2) in MilliQ) to obtain 80 µM (this solution was freshly prepared every assay) and further diluted in AB to obtain 300 nM after which 5 µL was added to each well of the assay plate except for background control wells. The assay plate was centrifuged (1 min, 200 *g*) and incubated at RT in the dark for 180 min. IMAP progressive binding buffer A (5x) and IMAP progressive binding buffer B (5x) were mixed in a ratio to obtain 30% buffer A and 70% buffer B, which was subsequently diluted 5x in MilliQ. IMAP progressive binding reagent was diluted 600x in aforementioned mixture of buffer A and B (to obtain a 2x working solution) of which 20 µL was added to each well of the assay plate. The assay plate was centrifuged (1 min, 200 *g*) and incubated at RT in the dark for 90 min. Fluorescence polarization was measured on a CLARIOstar plate reader using the following settings: (i) optic settings → excitation = F: 482-16, dichroic = F: LP 504, emission = F: 530-40, (ii) optic = top optic, (iii) speed/precision = maximum precision, (iv) focus adjustment was performed for every assay and (v) gain adjustment was done by setting the target mP value to 35 mP for one of the MIN control wells. Data was normalized between MIN and MAX controls and data was plotted using GraphPad Prism 8.0 using "Nonlinear regression (curve fit)" and "log(inhibitor) vs. normalized response – Variable slope" to determine pIC_{50} values.

General biology

DNA oligos were purchased at Integrated DNA Technologies and sequences can be found in **Supplementary Table 5.1** below. Cloning reagents were from Thermo Fisher. All cell culture disposables were from Sarstedt.

Cloning

The pDONR223-construct with full-length human cDNA of BUB1 was a gift from William Hahn & David Root (Addgene Human Kinase ORF Collection). Eukaryotic expression constructs of BUB1 were generated by PCR amplification and restriction/ligation cloning into a pcDNA3.1 vector, in frame with an N-terminal FLAG-tag or N-terminal GFP-FLAG-tag. Point mutations were introduced by site-directed mutagenesis. All plasmids were isolated from transformed XL10-Gold competent cells (prepared using *E. coli* transformation buffer set; Zymo Research) using plasmid isolation kits following the supplier's protocol (Qiagen). All sequences were verified by Sanger sequencing (Macrogen).

Supplementary Table 5.1 | List of oligonucleotide sequences.

ID	Name	Sequence
P1	FLAG-BUB1_forw	TGGTACCGCCGCCACCATGGACTACAAGGATGACGATGACAAGATGGACACCCCGGAAAA
P2	BUB1_stop_rev	TAGATCACTCGAGACCTCATTTTCGTGAACGCTTACATT
P3	BUB1_C1080A_stop_rev	TAGATCACTCGAGACCTCATTTTCGTGAACGCTTCGCTTCTAAGAGCAGTACAA
P4	XhoI-BUB1_forw	GCCCTCGAGATGGACACCCCGGAAAATGT

Cell culture

U2OS (human osteosarcoma) cells were purchased at ATCC and were tested on regular basis for mycoplasma contamination. Cultures were discarded after 2–3 months of use. Cells were cultured at 37°C under 7% CO_2 in DMEM (Sigma Aldrich, D6546) supplemented with GlutaMAX (2 mM, Thermo Fisher), 10% (v/v) heat-inactivated newborn calf serum (Seradigm), penicillin and streptomycin (200 µg/mL each, Duchefa) (complete medium). Growth medium was supplemented with G418 (600 µg/mL) (selection medium) for stable BUB1-overexpressing (U2OS-BUB1^{GFP_FLAG}) cells. Medium was refreshed every 2–3 days and cells were passaged by trypsinization twice a week at 80–90% confluence. Cell viability was assessed by Trypan Blue exclusion and cell quantification using a TC20™ Automated Cell Counter (Bio-Rad).

Generation of stable BUB1-overexpressing U2OS (U2OS-BUB1^{GFP_FLAG}) cells

One day prior to transfection, U2OS cells were transferred from confluent 10 cm dishes to 6-well plates (1:40 dilution). Before transfection, medium was refreshed (1 mL). A 3:1 (m/m) mixture of polyethyleneimine (PEI; 6 µg/well) and plasmid DNA (2 µg/well) was prepared in serum-free medium (200 µL) and incubated for 15 min at RT, after which the mixture was added dropwise to the cells. After 48 h, cells were passaged and grown in selection medium containing G418 (600 µg/mL) until the majority of cells was GFP-positive as determined by fluorescence microscopy. Cells were then single-cell diluted in 96-well plates and expanded to generate monoclonal cell lines stably overexpressing GFP-FLAG-BUB1. Expression was verified by immunoblot analysis using anti-FLAG antibody.

Overexpression of BUB1^{WT} and BUB1^{C1080A} and subsequent probe labeling

U2OS cells were seeded into 6-well plates (400,000 cells/well for transfections, 250,000 cells/well for non-transfected U2OS control) and incubated overnight to allow for cell adherence. Cell medium was aspirated and refreshed with complete medium (2 mL). A 3:1 (m/m) mixture of polyethyleneimine (PEI; 6 µg/well) and plasmid DNA (2 µg/well) was prepared in serum-free medium (200 µL) and incubated for 15 min at RT, after which the mixture was added dropwise to the cells. After 48 h, cells were treated with probe as described in "Probe labeling in living cells" (final probe concentration: 1 µM, incubation time: 1 h, lysis buffer: 60 µL for transfections, 120 µL for non-transfected cells, lysate concentration was adjusted to 1.15 mg/mL, 10 µg/lane for SDS-PAGE). After scanning fluorescence, the top part of the gel was immunoblotted as described in "Immunoblot", the bottom part of the gel was used for protein loading control as determined by Coomassie Brilliant Blue R-250 staining.

Probe labeling in living cells

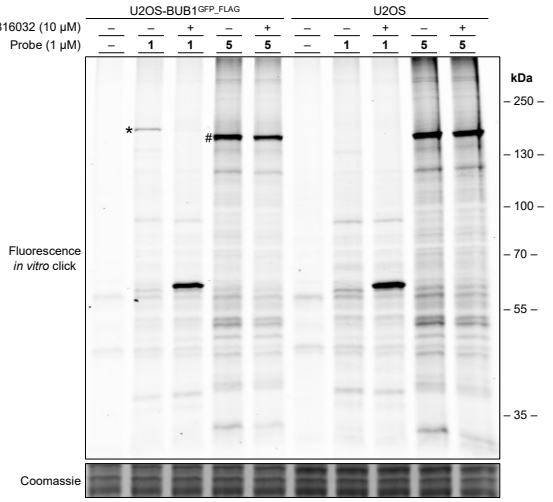
U2OS-BUB1^{GFP_FLAG} or U2OS cells from 10 cm dishes with low cell density (<50% confluence) were seeded into 6-well plates (500,000 cells/well) and incubated overnight to allow for cell adherence. Probe or competitor (stock solutions in DMSO) were diluted 100x in complete medium to obtain 10x working solutions (1% DMSO). For dose-response experiments, compounds were further diluted in complete medium containing 1% DMSO. Cell medium was aspirated and complete medium (900 µL for probe labeling only, 800 µL for competition experiments) was added. Either competitor (100 µL, 10x working solution) or probe (100 µL, 10x working solution) was added and cells were incubated at 37°C for 1 h (or for indicated time in case of time-response experiments). For competition experiments, probe (100 µL, 10x working solution) was subsequently added and cells were incubated at 37°C for 1 h. Medium was aspirated and cells were washed with PBS (1 mL). Cells were harvested by trypsinization and centrifuged (500 *g*, 3 min). Pellets were washed with PBS (1 mL), centrifuged (500 *g*, 3 min) and supernatant was removed. Pellets were snap-frozen in liquid nitrogen and subsequently thawed on ice (cell pellet can optionally be stored at –80°C). Cells were lysed by suspending the pellet in 60 µL M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher), supplemented with 1x Halt™ protease inhibitor cocktail

(EDTA-free) (Thermo Fisher) and 1x Halt™ phosphatase inhibitor cocktail (Thermo Fisher), after which the samples were incubated on ice for 15 min. Samples were vortexed at medium speed and centrifuged (14,000 *g*, 10 min, 4°C). The supernatant was collected and protein concentration determined by a Quick Start™ Bradford Protein Assay (Bio-Rad). Lysates were diluted to 1.15 mg/mL in M-PER™ Mammalian Protein Extraction Reagent (lysates can optionally be snap-frozen and stored at –80°C). "Click-mix" was prepared freshly by mixing $CuSO_4$ (42 µL of 15 mM in H_2O) and sodium ascorbate (21 µL of 150 mM in H_2O) until yellow, followed by the addition of THPTA (7 µL of 15 mM in H_2O) and Cy5-N_3 (7 µL of 82.5 µM in DMSO). To 26 µL lysate was added 4 µL click-mix and samples were incubated at 37°C for 30 min. Samples were denatured by the addition of 4x Laemmli buffer (10 µL of 240 mM Tris-HCl pH 6.8, 8% w/v SDS, 40% v/v glycerol, 5% v/v β-mercaptoethanol, 0.04% v/v/ bromophenol blue) and incubated at 95°C for 3 min. Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% polyacrylamide gel (180 V, 70 min, 10 or 20 µL/lane). Gels were scanned using Cy2, Cy3 and Cy5 multichannel settings (532/28, 602/50 and 700/50 filters, respectively) on a ChemiDoc™ MP imager (Bio-Rad). Fluorescence intensity was quantified using Image Lab 6.0.1 (Bio-Rad) and corrected for protein loading as determined by Coomassie Brilliant Blue R-250 staining. Data was plotted using GraphPad Prism 8.0.

Immunoblot

Samples were resolved by SDS-PAGE as described above and transferred to 0.2 µm polyvinylidene difluoride membranes by a Trans-Blot Turbo™ Transfer system (Bio-Rad) directly after fluorescence scanning. Membranes were washed with TBS (50 mM Tris pH 7.5, 150 mM NaCl) and blocked with 5% milk in TBS-T (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h at RT. Membranes were then incubated with primary antibody (monoclonal mouse anti-FLAG M2 (1:5000, Sigma Aldrich, F3156)) in 5% milk in TBS-T (overnight at 4 °C). Membranes were washed three times with TBS-T, incubated with secondary antibody (goat anti-mouse-HRP (1:5000, Santa Cruz, sc-2005)) in 5% milk in TBS-T (1 h at RT) and then washed three times with TBS-T and twice with TBS. Luminol development solution (10 mL of 1.4 mM luminol in 100 mM Tris pH 8.8 + 100 µL of 6.7 mM p-coumaric acid in DMSO + 3 µL of 30% (v/v) H_2O_2) was added after which chemiluminescence and Cy3 were detected on a ChemiDoc™ MP imager. For $BUB1^{WT}$ and $BUB1^{C1080A}$ transfections, development was performed using Clarity Max Western ECL Substrate (Bio-Rad).

Supplementary figures



Supplementary Figure 5.1 | Competition with BAY1816032 abolishes labeling of GFP-FLAG-tagged BUB1 by **1** (*), but not the labeling of a protein with a lower apparent molecular weight by **5** (#). U2OS-$BUB1^{GFP\_FLAG}$ or U2OS cells were pre-incubated with vehicle (–) or BAY1816032 (10 µM, 1 h, 37°C) followed by incubation with vehicle (–) or indicated probe (1 µM, 1 h, 37°C). Proteins labeled by probe were visualized by conjugation to a Cy5 fluorophore using click chemistry, SDS-PAGE and in-gel fluorescence scanning. Coomassie staining served as protein loading control.

Experimental – Chemistry

General synthesis

All reagents and solvents were purchased from chemical suppliers (Fluorochem, Sigma-Aldrich, Merck, Fisher Scientific, Honeywelel, VWR, Biosolve) and used without further purification. All reactions were performed at room temperature (RT) under a nitrogen atmosphere, unless stated otherwise. Reactions were monitored by thin layer chromatography (TLC, silica gel 60, UV_{254}, Macherey-Nagel, ref: 818333) and compounds were visualized by UV absorption (254 nm and/or 366 nm) or spray reagent (permanganate (5 g/L $KMnO_4$, 25 g/L K_2CO_3)) followed by heating. Alternatively, reactions were monitored by liquid chromatography-mass spectrometry (LCMS), either on a Thermo Finnigan (Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer (ESI+) coupled to a Surveyor HPLC system (Thermo Finnigan) equipped with a Nucleodur C18 Gravity column (50x4.6 mm, 3 µm particle size, Macherey-Nagel)) or a Thermo Fleet (Thermo LCQ Fleet ion-trap mass spectrometer (ESI+) coupled to a Vanquish UHPLC system). LCMS eluent consisted of MeCN in 0.1% TFA (aq.) and LCMS methods were as follows: 0.5 min cleaning with starting gradient, 8 min using specified gradient (linear), 2 min cleaning with 90% MeCN in 0.1% TFA (aq.). LCMS data is reported as follows: instrument (Finnigan or Fleet), gradient (% MeCN in 0.1% TFA (aq.)), retention time (t_r) and mass (as m/z: $[M+H]^+$). Purity of final compounds was determined to be ≥ 95% by integrating UV intensity of spectra generated by either of the LCMS instruments. ^1H and ^{13}C NMR spectra were recorded on a Bruker AV400 (400 and 101 MHz, respectively) or Bruker AV500 (500 and 126 MHz, respectively) NMR spectrometer. NMR samples were prepared in deuterated DMSO. Chemical shifts are given in ppm (δ) relative to residual protonated solvent signals (DMSO → δ 2.500 (^1H), δ 39.520 (^{13}C)). Data was processed by using MestReNova (v. 14) and is reported as follows: chemical shift (δ), multiplicity, coupling constant (*J* in Hz) and integration. Multiplicities are abbreviated as follows: s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, t = triplet, dt = doublet of triplets, p = pentet, m = multiplet. For some molecules rotamer peaks were observed, resulting in extra splitting of peaks. For these compounds, chemical shifts were reported as ranges and multiplicity was denoted by "2x", followed by the multiplicities specified above (i.e. 2x d = twice a doublet). The reported coupling constant corresponds to either of the multiplet peaks (of note, coupling constants were the same for both multiplet peaks). Purification was done either by manual silica gel column chromatography (using 40-63 µm, 60 Å silica gel, Macherey-Nagel) or automated flash column chromatography on a Biotage Isolera machine (using pre-packed cartridges with 40-63 µm, 60 Å silica gel (4, 12, 25 or 40 g), Screening Devices). High-performance liquid chromatography (HPLC) purifications were performed on either an Agilent 1200 preparative HPLC system (equipped with a Gemini C18 column (250x10 mm, 5 µm particle size, Phenomenex) coupled to a 6130 quadrupole mass spectrometer) or a Waters Acquity UPLC system (equipped with a Gemini C18 column (150x21 mm, 5 µm particle size, Phenomenex) coupled to a SQ mass spectrometer). Specified gradients for HPLC purifications (MeCN in 0.2% TFA (aq.)) were linear (5 mL/min for 12 min (Agilent) or 25 mL/min for 10 min (Waters)). High resolution mass spectrometry (HRMS) spectra were recorded through direct injection of a 1 µM sample either on a Thermo Scientific Q Exactive Orbitrap equipped with an electrospray ion source in positive mode coupled to an Ultimate 3000 system (source voltage = 3.5 kV, capillary temperature = 275 °C, resolution R = 240,000 at m/z 400, external lock, mass range m/z = 150-2000) or on a Synapt G2-Si high definition mass spectrometer (Waters) equipped with an electrospray ion source in positive mode (ESI-TOF) coupled to a NanoEquity system with Leu-enkephalin (m/z = 556.2771) as internal lock mass. The eluent for HRMS measurements consisted of a 1:1 (v/v) mixture of MeCN in 0.1% formic acid (aq.) using a flow of 25 mL/min. Compound names were generated by ChemDraw (v. 19.1.21).

General procedure A – Peptide coupling

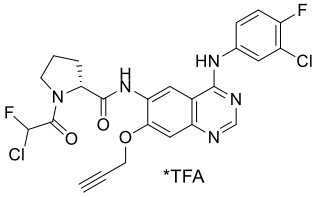
Acid derivative (3.3 eq.) was dissolved in DCM (0.15 M based on amine analogue) after which DIPEA (4 eq.) and DMF (0.1 eq.) were added. The mixture was cooled down to 0°C, pivaloyl chloride (3 eq.) was added and the mixture was stirred at 0°C for 1.5 h. Amine analogue (1 eq.) was added, the mixture was allowed to warm to RT and stirred for 16 h. The mixture was poured into 1 M $NaHCO_3$ (aq.) (50 or 100 mL) and the product extracted with DCM (3x50 or 3x100 mL). The combined organic layers were washed

with brine (100 mL), dried over Na_2SO_4, filtered and concentrated. Purification was performed as indicated.

General procedure B – Peptide coupling

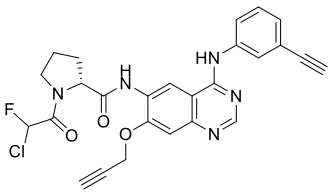
Amine analogue (1 eq.) was dissolved in DCM (0.15 M) and cooled down to 0°C. DIPEA (1 eq.) and acyl chloride derivative (1 eq.) were added after which the mixture was allowed to warm to RT and stirred for 16 h. The mixture was poured into 1 M $NaHCO_3$ (aq.) (20 mL) and the product extracted with DCM (3x20 mL). The combined organic layers were washed with brine (20 mL), dried over Na_2SO_4, filtered and concentrated. Purification was performed as indicated.

(2*R*)-1-(2-Chloro-2-fluoroacetyl)-*N*-(4-((3-chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (1)



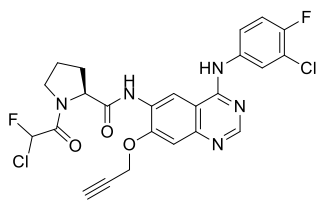
The title compound was synthesized from **29** (100 mg, 227 µmol) and 2-chloro-2-fluoroacetic acid according to general procedure A. The crude was purified by HPLC (Agilent, 35 – 38% MeCN in 0.2% TFA (aq.)) to afford the product as TFA salt (25.6 mg, 39.4 µmol, 17%). ^{1}H NMR (500 MHz, DMSO) (as a mixture of two diastereomers) δ 10.77 (br s, 1H), 9.90 – 9.82 (2x s, 1H), 9.05 – 8.93 (2x s, 1H), 8.74 (s, 1H), 8.02 – 7.98 (2x dd, *J* = 6.8, 2.6 Hz, 1H), 7.72 – 7.67 (2x ddd, *J* = 9.0, 4.6, 2.6 Hz, 1H), 7.51 – 7.45 (m, 2H), 7.27 – 7.09 (2x d, *J* = 48.5 Hz, 1H), 5.17 – 5.11 (2x d, *J* = 2.4 Hz, 2H), 4.88 – 4.79 (2x dd, *J* = 8.6, 3.3 Hz, 1H), 3.84 – 3.73 (m, 2H), 3.58 – 3.49 (m, 1H), 2.31 – 2.20 (m, 1H), 2.11 – 1.89 (m, 3H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (126 MHz, DMSO) δ 170.29, 170.11, 161.75 (d, $J_{(C–F)}$ = 24.8 Hz), 161.71 (d, $J_{(C–F)}$ = 24.5 Hz), 158.61, 158.36, 158.11, 158.06, 157.85, 155.40, 155.34, 154.35, 153.45, 153.40, 152.75 (d, $J_{(C–F)}$ = 244.5 Hz), 152.01, 135.33, 128.51, 128.31, 125.67, 125.51, 124.43 (d, $J_{(C–F)}$ = 7.2 Hz), 124.26 (d, $J_{(C–F)}$ = 7.2 Hz), 119.00 (d, $J_{(C–F)}$ = 18.6 Hz), 118.20, 116.85, 116.72 (d, $J_{(C–F)}$ = 21.8 Hz), 115.83, 115.55, 108.36, 104.52, 104.30, 92.23 (d, $J_{(C–F)}$ = 245.7 Hz), 92.01 (d, $J_{(C–F)}$ = 245.9 Hz), 79.95, 79.87, 77.80, 77.77, 60.61, 60.43, 56.99, 46.75, 46.67, 29.38, 29.24, 24.57, 24.26 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 5.31 min, m/z: 534.2. HRMS $[C_{24}H_{19}Cl_2F_2N_5O_3 + H]^+$: 534.09058 calculated, 534.09075 found.

(2*R*)-1-(2-Chloro-2-fluoroacetyl)-*N*-(4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (2)



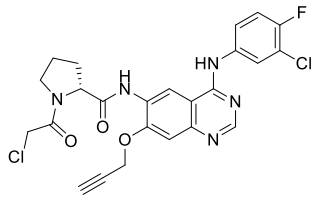
The title compound was synthesized from **30** (65.0 mg, 158 µmol) and 2-chloro-2-fluoroacetic acid according to general procedure A. The crude was purified by automated column chromatography (0 – 20% MeOH/DCM) to afford the product (3.8 mg, 7.5 µmol, 5%). ^{1}H NMR (500 MHz, DMSO) (as a mixture of two diastereomers) δ 9.84 (s, 1H), 9.73 – 9.64 (2x s, 1H), 8.89 – 8.79 (2x s, 1H), 8.55 – 8.53 (2x s, 1H), 7.99 – 7.96 (2x t, *J* = 1.9 Hz, 1H), 7.87 – 7.83 (m, 1H), 7.41 – 7.36 (m, 2H), 7.21 – 7.19 (m, 1H), 7.25 – 7.08 (2x d, *J* = 48.5 Hz, 1H), 5.11 – 5.07 (2x d, *J* = 2.4 Hz, 2H), 4.86 – 4.75 (2x dd, *J* = 8.7, 3.2 Hz, 1H), 4.17 – 4.17 (2x s, 1H), 3.84 – 3.73 (m, 1H), 3.72 – 3.71 (2x t, *J* = 2.3 Hz, 1H), 3.61 – 3.48 (m, 1H), 2.28 – 2.18 (m, 1H), 2.11 – 1.90 (m, 3H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (126 MHz, DMSO) δ 170.14, 169.90, 161.79 (d, $J_{(C–F)}$ = 24.3 Hz), 161.73 (d, $J_{(C–F)}$ = 24.5 Hz), 157.06, 154.21, 154.08, 153.57, 152.82, 149.03, 148.69, 139.80, 139.77, 128.82, 127.25, 127.09, 126.53, 125.13, 125.08, 122.98, 122.91, 121.68, 117.07, 115.62, 109.55, 109.48, 108.32, 108.28, 92.17 (d, $J_{(C–F)}$ = 245.8 Hz), 92.09 (d, $J_{(C–F)}$ = 245.8 Hz), 83.59, 80.48, 79.38, 79.31, 78.32, 60.64, 60.47, 56.59, 46.72, 46.64, 29.41, 29.23, 24.54, 24.21 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 5.13 min, m/z: 506.2. HRMS $[C_{26}H_{21}ClFN_5O_3 + H]^+$: 506.13897 calculated, 506.13866 found.

(2*S*)-1-(2-Chloro-2-fluoroacetyl)-*N*-(4-((3-chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (3)



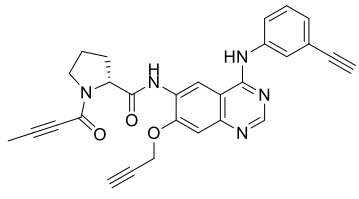
The title compound was synthesized from **31** (83.0 mg, 189 µmol) and 2-chloro-2-fluoroacetic acid according to general procedure A. The crude was purified by automated column chromatography (0 – 20% MeOH/DCM) to afford the product (42.7 mg, 79.9 µmol, 42%). ^{1}H NMR (400 MHz, DMSO) (as a mixture of two diastereomers) δ 9.93 (s, 1H), 9.71 (d, *J* = 29.3 Hz, 1H), 8.83 (d, *J* = 38.0 Hz, 1H), 8.56 – 8.51 (2x s, 1H), 8.12 – 8.05 (2x dd, *J* = 6.9, 2.6 Hz, 1H), 7.80 – 7.74 (m, 1H), 7.45 – 7.38 (m, 2H), 7.28 – 7.06 (2x d, *J* = 48.5 Hz, 1H), 5.13 – 5.05 (2x d, *J* = 2.5 Hz, 2H), 4.86 – 4.75 (2x dd, *J* = 8.5, 3.1 Hz, 1H), 3.84 – 3.70 (m, 2H), 3.61 – 3.47 (m, 1H), 2.30 – 2.16 (m, 1H), 2.11 – 1.92 (m, 3H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (101 MHz, DMSO) δ 170.18, 169.96, 161.80 (d, $J_{(C–F)}$ = 24.6 Hz), 161.74 (d, $J_{(C–F)}$ = 24.6 Hz), 156.98, 154.08, 153.94, 153.57, 153.31 (d, $J_{(C–F)}$ = 242.9 Hz), 152.82, 148.79, 136.74, 127.39, 127.23, 123.90, 123.82, 122.79 (d, $J_{(C–F)}$ = 7.0 Hz), 122.70 (d, $J_{(C–F)}$ = 6.8 Hz), 118.73 (d, $J_{(C–F)}$ = 18.3 Hz), 116.83, 116.48 (d, $J_{(C–F)}$ = 21.3 Hz), 115.37, 109.39, 109.32, 108.25, 92.18 (d, $J_{(C–F)}$ = 246.0 Hz), 92.08 (d, $J_{(C–F)}$ = 245.9 Hz), 79.46, 79.39, 78.31, 60.64, 60.47, 56.63, 46.74, 46.66, 29.43, 29.26, 24.56, 24.24 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 5.32 min, m/z: 534.2. HRMS $[C_{24}H_{19}Cl_2F_2N_5O_3 + H]^+$: 534.09058 calculated, 534.09040 found.

(*R*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)-1-(2-chloroacetyl)pyrrolidine-2-carboxamide (4)



The title compound was synthesized from **29** (108 mg, 246 µmol) and 2-chloroacetyl chloride (1.5 eq.) according to general procedure B (using 1.6 eq. DIPEA). The crude was purified by automated column chromatography (0 – 20% MeOH/DCM) to afford the product (36.6 mg, 70.9 µmol, 29%). ^{1}H NMR (400 MHz, DMSO) δ 9.91 (s, 1H), 9.61 (s, 1H), 8.83 (s, 1H), 8.54 (s, 1H), 8.10 (dd, *J* = 6.9, 2.7 Hz, 1H), 7.78 (ddd, *J* = 9.0, 4.3, 2.6 Hz, 1H), 7.47 – 7.36 (m, 2H), 5.09 (d, *J* = 2.5 Hz, 2H), 4.87 – 4.72 (2x dd, *J* = 8.4, 2.6 Hz, 1H), 4.44 – 4.07 (m, 2H), 3.72 (t, *J* = 2.2 Hz, 1H), 3.71 – 3.64 (m, 1H), 3.62 – 3.55 (m, 1H), 2.23 – 2.11 (m, 1H), 2.09 – 1.87 (m, 3H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (101 MHz, DMSO) δ 170.62, 164.81, 156.94, 154.01, 153.27 (d, *J* = 242.9 Hz), 153.19, 148.72, 136.79 (d, $J_{(C–F)}$ = 3.1 Hz), 127.37, 123.76, 122.64 (d, $J_{(C–F)}$ = 6.8 Hz), 118.72 (d, $J_{(C–F)}$ = 18.3 Hz), 116.45 (d, $J_{(C–F)}$ = 21.6 Hz), 116.04, 109.39, 108.26, 79.38, 78.31, 60.46, 56.63, 46.75, 42.89, 29.48, 24.40 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 5.10 min, m/z: 516.1. HRMS $[C_{24}H_{20}Cl_2FN_5O_3 + H]^+$: 516.10000 calculated, 516.10012 found.

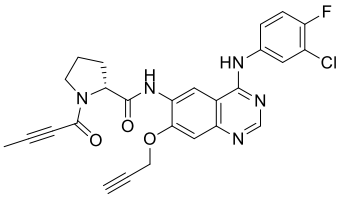
(*R*)-1-(But-2-ynoyl)-*N*-(4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (5)



The title compound was synthesized from **30** (80.0 mg, 194 µmol) and but-2-ynoic acid according to general procedure A. The crude was purified by automated column chromatography (0 – 12% MeOH/DCM) to afford the product (58.0 mg, 121 µmol, 63%). ^{1}H NMR (400 MHz, DMSO) δ 9.86 – 9.83 (2x s, 1H), 9.82 – 9.67 (2x s, 1H), 8.89 – 8.83 (2x s, 1H), 8.56 – 8.54 (2x s, 1H), 8.00 – 7.97 (2x t, *J* = 1.9 Hz, 1H), 7.88 – 7.83 (2x ddd, *J* = 8.3, 2.2, 1.1 Hz, 1H), 7.42 – 7.35 (m, 2H), 7.23 – 7.19 (m, 1H), 5.13 – 5.08 (2x d, *J* = 2.4 Hz, 2H), 4.98 – 4.72 (2x dd, *J* = 8.7, 3.4 Hz, 1H), 4.19 – 4.18 (2x s, 1H), 3.76 – 3.67 (m, 2H), 3.49 (dd, *J* = 7.7, 6.0 Hz, 1H), 2.41 – 2.19 (m, 1H), 2.13 – 1.85 (m, 6H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (101 MHz, DMSO) δ 171.15, 170.30, 157.04, 154.17, 154.08, 153.14, 153.00, 152.11, 152.05, 148.82, 148.74, 139.81, 139.71, 128.82, 127.30, 127.07, 126.63, 126.51, 125.30, 125.09, 123.14, 122.93, 121.68, 116.47, 115.87, 109.54, 109.50, 108.35, 108.26, 88.37, 87.94, 83.61, 83.56, 80.55, 80.50, 79.38, 79.35, 78.35, 78.32, 74.41, 74.31, 61.43, 59.29, 56.59, 56.55, 48.60, 46.08, 31.16, 29.98, 23.77, 22.87, 3.33, 3.31 (the spectrum was

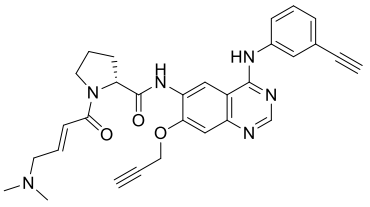
accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 4.92 min, m/z: 478.2. HRMS [$C_{28}H_{23}N_5O_3$ + H]$^+$: 478.18737 calculated, 478.18743 found.

(*R*)-1-(But-2-ynoyl)-*N*-(4-((3-chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (6)



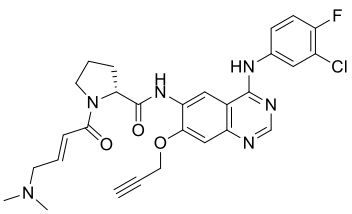
The title compound was synthesized from **29** (52.0 mg, 118 µmol) and but-2-ynoic acid according to general procedure A. The crude was purified by HPLC (Agilent, 35 – 38% MeCN in 0.2% TFA (aq.)) after which the fractions were concentrated and traces of TFA were removed by coevaporation with 1:1 MeCN/H_2O (20 mL). The residue was dissolved in DCM (15 mL) and washed with 1 M $NaHCO_3$ (aq.) (3x5 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated to afford the product (6.0 mg, 12 µmol, 10%). ^{1}H NMR (500 MHz, DMSO) δ 9.89 – 9.87 (2x s, 1H), 9.81 – 9.66 (2x s, 1H), 8.85 – 8.81 (2x s, 1H), 8.54 – 8.53 (2x s, 1H), 8.11 – 8.07 (2x dd, *J* = 6.9, 2.6 Hz, 1H), 7.80 – 7.75 (2x ddd, *J* = 9.1, 4.4, 2.6 Hz, 1H), 7.45 – 7.37 (m, 2H), 5.13 – 5.07 (2x d, *J* = 2.4 Hz, 2H), 4.97 – 4.72 (2x dd, *J* = 8.7, 3.5 Hz, 1H), 3.74 – 3.68 (m, 2H), 3.48 (dd, *J* = 7.8, 6.0 Hz, 1H), 2.37 – 2.19 (m, 1H), 2.10 – 1.86 (m, 6H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (126 MHz, DMSO) δ 171.13, 170.28, 156.90, 154.04, 153.05, 152.98 (d, $J_{(C–F)}$ = 245.8 Hz), 152.96, 152.05, 148.70, 148.64, 136.77 (d, $J_{(C–F)}$ = 3.4 Hz), 127.37, 127.15, 123.99, 123.76, 122.88 (d, $J_{(C–F)}$ = 6.8 Hz), 122.64 (d, $J_{(C–F)}$ = 6.7 Hz), 118.66 (d, $J_{(C–F)}$ = 19.5 Hz), 116.43 (d, $J_{(C–F)}$ = 21.6 Hz), 116.09, 115.58, 109.35, 109.33, 108.37, 108.27, 88.33, 87.87, 79.37, 78.30, 74.28, 61.38, 59.24, 56.58, 48.56, 46.03, 31.12, 29.94, 23.73, 22.82, 3.28. LCMS (Fleet, 10 → 90%): t_r = 5.10 min, m/z: 506.2. HRMS [$C_{26}H_{21}ClFN_5O_3$ + H]$^+$: 506.13897 calculated, 506.13910 found.

(*R*,*E*)-1-(4-(Dimethylamino)but-2-enoyl)-*N*-(4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (7)



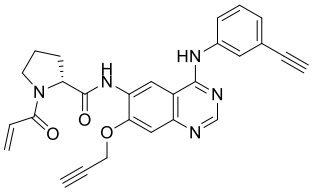
The title compound was synthesized from **30** (80.0 mg, 194 µmol) and (*E*)-4-(dimethylamino)but-2-enoic acid hydrochloride according to general procedure A (using 5 eq. DIPEA). The crude was purified by automated column chromatography (10 – 20% MeOH/DCM) to afford the product (74.0 mg, 142 µmol, 73%). ^{1}H NMR (400 MHz, DMSO) δ 9.85 (s, 1H), 9.89 – 9.69 (2x s, 1H), 8.92 – 8.84 (2x s, 1H), 8.55 – 8.53 (2x s, 1H), 7.99 (t, *J* = 1.9 Hz, 1H), 7.90 – 7.84 (2x ddd, *J* = 8.3, 2.3, 1.1 Hz, 1H), 7.41 – 7.35 (m, 2H), 7.22 – 7.18 (2x dt, *J* = 7.6, 1.3 Hz, 1H), 6.74 – 6.57 (2x dt, *J* = 15.2, 6.2 Hz, 1H), 6.52 – 6.28 (2x dt, *J* = 15.0, 1.5 Hz, 1H), 5.12 – 5.07 (2x d, *J* = 2.4 Hz, 2H), 4.98 – 4.79 (2x dd, *J* = 8.3, 3.1 Hz, 1H), 4.20 – 4.19 (2x s, 1H), 3.78 – 3.46 (m, 3H), 3.18 – 3.07 (m, 2H), 2.28 – 1.85 (m, 10H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (101 MHz, DMSO) δ 171.42, 170.83, 163.75, 157.01, 154.25, 153.98, 153.48, 152.82, 149.02, 148.61, 141.05, 140.53, 139.84, 139.71, 128.79, 127.46, 126.88, 126.57, 126.45, 125.20, 125.04, 124.21, 124.18, 123.03, 122.88, 121.66, 117.10, 115.44, 109.57, 109.44, 108.36, 108.19, 83.61, 83.56, 80.54, 80.50, 79.47, 79.33, 78.35, 78.30, 60.07, 59.79, 59.52, 59.45, 56.61, 47.05, 46.74, 44.66, 44.58, 31.99, 29.05, 24.41, 22.34 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 3.89 min, m/z: 523.4. HRMS [$C_{30}H_{30}N_6O_3$ + H]$^+$: 523.24522 calculated, 523.24527 found.

(*R*,*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)-1-(4-(dimethylamino)but-2-enoyl)pyrrolidine-2-carboxamide (8)



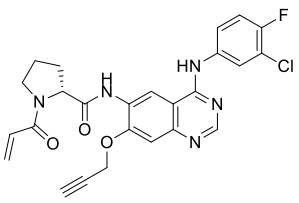
The title compound was synthesized from **29** (80.0 mg, 182 µmol) and (*E*)-4-(dimethylamino)but-2-enoic acid hydrochloride according to general procedure A (using 5 eq. DIPEA). The crude was purified by automated column chromatography (10 – 20% MeOH/DCM) to afford the product (41 mg, 74 µmol, 41%). ^{1}H NMR (400 MHz, DMSO) δ 9.91 (br s, 1H), 9.85 – 9.69 (2x s, 1H), 8.90 – 8.80 (2x s, 1H), 8.54 – 8.51 (2x s, 1H), 8.10 (dd, *J* = 6.9, 2.5 Hz, 1H), 7.77 (ddd, *J* = 9.2, 4.2, 2.5 Hz, 1H), 7.44 – 7.37 (m, 2H), 6.73 – 6.58 (2x dt, *J* = 15.1, 6.1 Hz, 1H), 6.48 – 6.22 (2x dt, *J* = 15.1, 1.4 Hz, 1H), 5.12 – 5.07 (2x d, *J* = 2.4 Hz, 2H), 4.98 – 4.78 (2x dd, *J* = 8.4, 3.1 Hz, 1H), 3.79 – 3.48 (m, 3H), 3.09 – 2.94 (m, 2H), 2.09 (d, *J* = 41.8 Hz, 10H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (101 MHz, DMSO) δ 171.46, 170.87, 163.90, 163.87, 156.89, 154.11, 153.87, 153.26, 153.20 (d, $J_{(C–F)}$ = 242.8 Hz), 152.74, 148.50, 142.44, 141.87, 136.87, 127.55, 127.02, 123.89, 123.72, 123.30, 122.77 (d, *J* = 6.5 Hz), 122.62 (d, $J_{(C–F)}$ = 6.8 Hz), 118.66 (d, $J_{(C–F)}$ = 18.3 Hz), 116.42 (d, $J_{(C–F)}$ = 21.5 Hz), 115.10, 109.45, 108.37, 108.20, 79.49, 79.34, 78.33, 78.29, 62.49, 60.05, 59.94, 59.91, 59.76, 56.61, 47.02, 46.71, 45.15, 45.03, 31.97, 29.02, 24.41, 22.34 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 4.02 min, m/z: 551.2. HRMS $[C_{28}H_{28}ClFN_6O_3 + H]^+$: 551.19682 calculated, 551.19663 found.

(*R*)-1-Acryloyl-*N*-(4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (9)



The title compound was synthesized from **30** (80.0 mg, 194 µmol) and acryloyl chloride according to general procedure B. The crude was purified by automated column chromatography (0 – 10% MeOH/DCM) to afford the product (47.0 mg, 101 µmol, 52%). ^{1}H NMR (400 MHz, DMSO) δ 9.87 – 9.82 (2x s, 1H), 9.82 – 9.67 (2x s, 1H), 8.91 – 8.77 (2x s, 1H), 8.56 – 8.53 (2x s, 1H), 7.99 (t, *J* = 1.9 Hz, 1H), 7.85 (ddd, *J* = 8.4, 2.3, 1.1 Hz, 1H), 7.41 – 7.35 (m, 2H), 7.23 – 7.18 (2x dt, *J* = 7.6, 1.3 Hz, 1H), 6.70 – 6.43 (2x dd, *J* = 16.7, 10.3 Hz, 1H), 6.24 – 6.16 (2x dd, *J* = 16.7, 2.4 Hz, 1H), 5.75 – 5.70 (2x dd, *J* = 10.3, 2.4 Hz, 1H), 5.09 (d, *J* = 2.5 Hz, 2H), 4.97 – 4.80 (2x dd, *J* = 8.5, 3.0 Hz, 1H), 4.22 – 4.17 (2x s, 1H), 3.78 – 3.48 (m, 3H), 2.42 – 1.85 (m, 4H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (101 MHz, DMSO) δ 171.47, 170.79, 163.87, 163.81, 157.02, 154.34, 154.00, 153.76, 152.82, 149.20, 148.62, 139.83, 139.67, 129.27, 129.08, 128.83, 128.79, 127.65, 127.48, 127.45, 126.84, 126.61, 126.47, 125.18, 125.06, 123.01, 122.91, 121.69, 121.66, 117.46, 115.41, 109.56, 109.42, 108.44, 108.20, 83.61, 83.55, 80.56, 80.50, 79.35, 78.34, 78.32, 60.07, 59.75, 56.64, 56.61, 47.03, 46.75, 32.00, 29.09, 24.40, 22.29 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 4.78 min, m/z: 466.4. HRMS $[C_{27}H_{23}N_5O_3 + H]^+$: 466.18737 calculated, 466.18736 found.

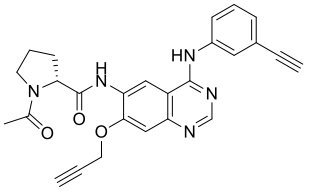
(*R*)-1-Acryloyl-*N*-(4-((3-chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (10)



The title compound was synthesized from **29** (80.0 mg, 182 µmol) and acryloyl chloride according to general procedure B. The crude was purified by automated column chromatography (0 – 10% MeOH/DCM) to afford the product (19 mg, 38 µmol, 21%). ^{1}H NMR (400 MHz, DMSO) δ 9.90 – 9.87 (2x s, 1H), 9.83 – 9.68 (2x s, 1H), 8.90 – 8.75 (2x s, 1H), 8.55 – 8.52 (2x s, 1H), 8.09 (dd, *J* = 6.9, 2.6 Hz, 1H), 7.78 (ddd, *J* = 9.1, 4.4, 2.6 Hz, 1H), 7.45 – 7.37 (m, 2H), 6.71 – 6.43 (2x dd, *J* = 16.7, 10.3 Hz, 1H), 6.23 – 6.15 (2x dd, *J* = 16.6, 2.4 Hz, 1H), 5.75 – 5.69 (2x dd, *J* = 10.3, 2.3 Hz, 1H), 5.11 – 5.08 (2x d, *J* = 2.4 Hz, 2H), 4.97 – 4.80 (2x dd, *J* = 8.5, 2.9 Hz, 1H), 3.77 – 3.48 (m, 3H), 2.41 – 1.84 (m, 4H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (101 MHz, DMSO) δ 171.48, 170.81, 163.86, 163.78, 156.91, 153.90, 153.67, 153.22 (d, $J_{(C–F)}$ = 242.9 Hz), 152.79, 152.10, 149.10, 148.54, 136.81 (d, $J_{(C–F)}$ = 2.9 Hz), 129.30, 129.07, 127.66, 127.54, 127.49, 126.94, 123.89, 123.74, 122.77 (d, $J_{(C–F)}$ = 6.6 Hz), 122.63 (d, $J_{(C–F)}$ = 6.9 Hz), 118.69 (d, $J_{(C–F)}$ = 17.9 Hz), 118.66 (d, $J_{(C–F)}$ = 18.3 Hz), 117.11, 116.45 (d,

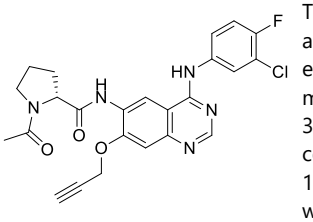
$J_{(C–F)}$ = 21.5 Hz), 116.42 (d, $J_{(C–F)}$ = 21.4 Hz), 115.14, 109.41, 109.28, 108.45, 108.22, 79.38, 78.33, 78.30, 60.06, 59.73, 56.61, 47.03, 46.74, 31.99, 29.09, 24.39, 22.27 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 4.95 min, m/z: 494.2. HRMS [$C_{25}H_{21}ClFN_5O_3$ + H]$^+$: 494.13897 calculated, 494.13893 found.

(*R*)-1-Acetyl-*N*-(4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (11)



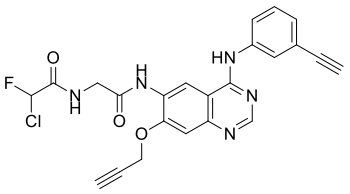
The title compound was synthesized from **30** (80.0 mg, 194 µmol) and acetyl chloride according to general procedure B. The crude was purified by HPLC (Agilent, 29 – 35% MeCN in 0.2% TFA (aq.)) after which the fractions were concentrated and traces of TFA were removed by coevaporation with 1:1 MeCN/H_2O (20 mL). The residue was dissolved in DCM (15 mL) and washed with 1 M $NaHCO_3$ (aq.) (3x5 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated to afford the product (63.0 mg, 139 µmol, 72%). ^{1}H NMR (400 MHz, DMSO) δ 11.46 (br s, 1H), 10.11 – 9.90 (2x s, 1H), 9.18 – 9.01 (2x s, 1H), 8.92 – 8.88 (2x s, 1H), 7.80 – 7.78 (2x t, *J* = 1.9 Hz, 1H), 7.69 – 7.65 (m, 1H), 7.56 – 7.53 (2x s, 1H), 7.52 – 7.47 (2x t, *J* = 7.9 Hz, 1H), 7.44 – 7.40 (2x dt, *J* = 7.7, 1.4 Hz, 1H), 5.17 (d, *J* = 2.4 Hz, 2H), 4.83 – 4.72 (2x dd, *J* = 8.5, 3.0 Hz, 1H), 4.28 – 4.26 (2x s, 1H), 3.85 – 3.83 (2x t, *J* = 2.4 Hz, 1H), 3.66 – 3.39 (m, 2H), 2.21 – 1.84 (m, 7H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (101 MHz, DMSO) δ 171.89, 171.29, 169.23, 168.68, 159.14, 159.09, 159.04, 158.78, 158.42, 158.06, 155.85, 154.54, 150.53, 150.06, 137.78, 137.28, 137.14, 136.94, 129.81, 129.69, 129.58, 129.34, 129.28, 128.92, 128.13, 128.05, 125.70, 125.65, 122.22, 122.16, 120.10, 118.60, 117.21, 115.73, 114.32, 111.42, 107.64, 107.54, 101.37, 101.06, 82.90, 82.86, 81.49, 81.43, 80.33, 80.30, 77.42, 60.68, 59.75, 57.39, 47.79, 46.48, 32.11, 29.31, 24.48, 22.61, 22.36, 22.28 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 4.52 min, m/z: 454.2. HRMS [$C_{26}H_{23}N_5O_3$ + H]$^+$: 454.18737 calculated, 454.18728 found.

(*R*)-1-Acetyl-*N*-(4-((3-chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (12)



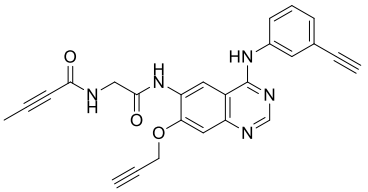
The title compound was synthesized from **29** (43 mg, 98 µmol) and acetyl chloride according to general procedure B (after 16 h of stirring, extra DIPEA (0.56 eq.) and acetyl chloride (0.56 eq.) were added and the mixture stirred for 4 h). The crude was purified by HPLC (Agilent, 30 – 32% MeCN in 0.2% TFA (aq.)) after which the fractions were concentrated and traces of TFA were removed by coevaporation with 1:1 MeCN/H_2O (20 mL). The residue was dissolved in DCM (15 mL) and washed with 1 M $NaHCO_3$ (aq.) (3x5 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated to afford the product (23 mg, 48 µmol, 49%). ^{1}H NMR (400 MHz, DMSO) δ 11.42 – 11.37 (2x s, 1H), 10.09 – 9.89 (2x s, 1H), 9.16 – 8.98 (2x s, 1H), 8.91 – 8.88 (2x s, 1H), 7.96 – 7.92 (2x dd, *J* = 6.8, 2.6 Hz, 1H), 7.67 – 7.62 (2x ddd, *J* = 9.1, 4.4, 2.5 Hz, 1H), 7.58 – 7.50 (m, 2H), 5.18 – 5.16 (2x d, *J* = 2.5 Hz, 2H), 4.82 – 4.71 (2x dd, *J* = 8.6, 3.0 Hz, 1H), 3.87 – 3.84 (2x t, *J* = 2.2 Hz, 1H), 3.65 – 3.39 (m, 2H), 2.42 – 1.82 (m, 7H) (the spectrum was accompanied by rotamer peaks). ^{13}C NMR (101 MHz, DMSO) δ 171.86, 171.28, 169.15, 168.59, 158.96, 158.54, 158.19, 157.83, 154.40, 154.03, 150.24, 138.35, 137.43, 134.24 (d, $J_{(C–F)}$ = 3.4 Hz), 129.51, 128.80, 127.03, 126.97, 125.61 (d, $J_{(C–F)}$ = 7.2 Hz), 119.23 (d, $J_{(C–F)}$ = 18.8 Hz), 118.27, 116.94 (d, $J_{(C–F)}$ = 22.1 Hz), 115.45, 107.62, 101.39, 80.32, 80.29, 77.46, 77.44, 59.68, 57.33, 47.74, 46.43, 29.28, 24.44, 22.33, 22.27 (the spectrum was accompanied by rotamer peaks). LCMS (Fleet, 10 → 90%): t_r = 4.71 min, m/z: 482.2. HRMS [$C_{24}H_{21}ClFN_5O_3$ + H]$^+$: 482.13897 calculated, 482.13884 found.

2-Chloro-*N*-(2-((4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)amino)-2-oxoethyl)-2-fluoroacetamide (13)



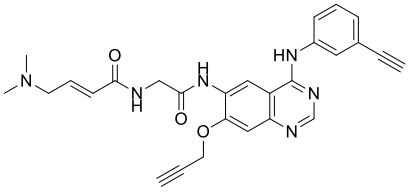
The title compound was synthesized from **32** (60.0 mg, 162 µmol) and 2-chloro-2-fluoroacetic acid according to general procedure A. The crude was purified by HPLC (Waters, 30 – 40% MeCN in 0.2% TFA (aq.)) after which the fractions were concentrated and traces of TFA were removed by coevaporation with 1:1 MeCN/H_2O (20 mL). The residue was dissolved in DCM (15 mL) and washed with 1 M $NaHCO_3$ (aq.) (3x5 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated to afford the product (16 mg, 34 µmol, 21%). ^{1}H NMR (400 MHz, DMSO) δ 9.84 (s, 1H), 9.73 (s, 1H), 9.12 – 9.07 (m, 1H), 8.88 (s, 1H), 8.54 (s, 1H), 7.98 (t, *J* = 1.9 Hz, 1H), 7.84 (ddd, *J* = 8.3, 2.3, 1.1 Hz, 1H), 7.40 (s, 1H), 7.38 (t, *J* = 8.0 Hz, 1H), 7.20 (dt, *J* = 7.6, 1.3 Hz, 1H), 6.88 (d, *J* = 49.3 Hz, 1H), 5.11 (d, *J* = 2.4 Hz, 2H), 4.19 (s, 1H), 4.15 (d, *J* = 5.4 Hz, 2H), 3.76 – 3.70 (m, 1H). ^{13}C NMR (101 MHz, DMSO) δ 167.14, 164.13 (d, $J_{(C-F)}$ = 23.3 Hz), 157.00, 154.14, 152.81, 148.68, 139.76, 128.84, 126.87, 126.52, 125.05, 122.90, 121.68, 116.06, 109.50, 108.37, 93.86 (d, $J_{(C-F)}$ = 251.0 Hz), 83.59, 80.56, 79.49, 78.34, 56.47, 42.46. LCMS (Fleet, 10 → 90%): t_r = 4.76 min, m/z: 466.2. HRMS [$C_{23}H_{17}ClFN_5O_3$ + H]$^+$: 466.10767 calculated, 466.10730 found.

***N*-(2-((4-((3-Ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)amino)-2-oxoethyl)but-2-ynamide (14)**



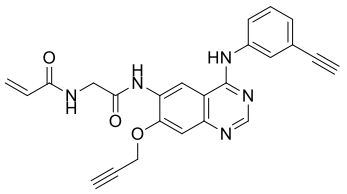
The title compound was synthesized from **32** (60.0 mg, 162 µmol) and but-2-ynoic acid according to general procedure A. The crude was loaded onto silica gel and purified by automated column chromatography (3 – 10% MeOH/DCM) to afford the product (9.0 mg, 21 µmol, 13%). ^{1}H NMR (500 MHz, DMSO) δ 9.83 (s, 1H), 9.61 (s, 1H), 8.89 – 8.85 (m, 2H), 8.53 (s, 1H), 7.98 (t, *J* = 1.9 Hz, 1H), 7.84 (ddd, *J* = 8.3, 2.3, 1.1 Hz, 1H), 7.40 – 7.36 (m, 2H), 7.20 (dt, *J* = 7.6, 1.3 Hz, 1H), 5.10 (d, *J* = 2.4 Hz, 2H), 4.20 (s, 1H), 4.03 (d, *J* = 6.1 Hz, 2H), 3.73 (t, *J* = 2.3 Hz, 1H), 1.99 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 167.60, 156.97, 154.08, 153.08, 152.74, 148.60, 139.77, 128.83, 126.95, 126.50, 125.02, 122.87, 121.67, 115.83, 109.50, 108.31, 83.59, 83.41, 80.55, 79.47, 78.33, 75.31, 56.49, 42.63, 3.09. LCMS (Fleet, 10 → 90%): t_r = 4.50 min, m/z: 438.2. HRMS [$C_{25}H_{19}N_5O_3$ + H]$^+$: 438.15607 calculated, 438.15611 found.

(*E*)-4-(Dimethylamino)-*N*-(2-((4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)amino)-2-oxoethyl)but-2-enamide (15)



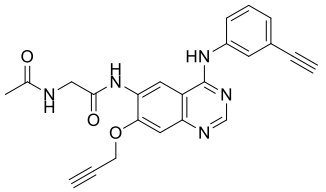
The title compound was synthesized from **32** (40.0 mg, 108 µmol) and (*E*)-4-(dimethylamino)but-2-enoic acid hydrochloride according to general procedure A (using 5 eq. DIPEA). The crude was purified by HPLC (Waters, 20–30% MeCN in 0.2% TFA (aq.)) after which the fractions were concentrated and traces of TFA were removed by coevaporation with 1:1 MeCN/H_2O (20 mL). The residue was dissolved in DCM (15 mL) and washed with 1 M $NaHCO_3$ (aq.) (3x5 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated to afford the product (19 mg, 39 µmol, 37%). ^{1}H NMR (400 MHz, DMSO) δ 9.82 (s, 1H), 9.60 (s, 1H), 8.89 (s, 1H), 8.53 (s, 1H), 8.51 (t, *J* = 6.1 Hz, 1H), 7.98 (t, *J* = 1.9 Hz, 1H), 7.84 (ddd, *J* = 8.2, 2.2, 1.1 Hz, 1H), 7.42 – 7.33 (m, 2H), 7.20 (dt, *J* = 7.6, 1.3 Hz, 1H), 6.63 (dt, *J* = 15.5, 6.1 Hz, 1H), 6.17 (dt, *J* = 15.5, 1.6 Hz, 1H), 5.10 (d, *J* = 2.4 Hz, 2H), 4.19 (s, 1H), 4.10 (d, *J* = 6.0 Hz, 2H), 3.74 – 3.70 (m, 1H), 3.00 (dd, *J* = 6.2, 1.6 Hz, 2H), 2.14 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 168.33, 165.27, 156.98, 154.06, 152.72, 148.59, 140.29, 139.79, 128.82, 127.02, 126.49, 125.49, 125.00, 122.85, 121.69, 115.66, 109.54, 108.30, 83.60, 80.52, 79.43, 78.30, 59.79, 56.52, 45.12, 42.77. LCMS (Fleet, 10 → 90%): t_r = 3.59 min, m/z: 483.2. HRMS [$C_{27}H_{26}N_6O_3$ + H]$^+$: 483.21392 calculated, 483.21389 found.

N-(2-((4-((3-Ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)amino)-2-oxoethyl)acrylamide (16)



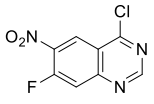
The title compound was synthesized from **32** (60.0 mg, 162 µmol) and acryloyl chloride according to general procedure B. The crude was loaded onto silica gel and purified by automated column chromatography (3 – 10% MeOH/DCM) to afford the product (9.0 mg, 21 µmol, 13%). ^{1}H NMR (500 MHz, DMSO) δ 9.83 (s, 1H), 9.64 (s, 1H), 8.89 (s, 1H), 8.60 (t, *J* = 6.0 Hz, 1H), 8.54 (s, 1H), 7.98 (t, *J* = 1.9 Hz, 1H), 7.84 (ddd, *J* = 8.3, 2.3, 1.1 Hz, 1H), 7.40 (s, 1H), 7.38 (t, *J* = 8.0 Hz, 1H), 7.20 (dt, *J* = 7.6, 1.3 Hz, 1H), 6.37 (dd, *J* = 17.1, 10.2 Hz, 1H), 6.16 (dd, *J* = 17.1, 2.1 Hz, 1H), 5.66 (dd, *J* = 10.2, 2.1 Hz, 1H), 5.10 (d, *J* = 2.4 Hz, 2H), 4.19 (s, 1H), 4.13 (d, *J* = 6.0 Hz, 2H), 3.73 (t, *J* = 2.3 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 168.21, 165.21, 156.98, 154.08, 152.76, 148.62, 139.78, 131.36, 128.83, 126.98, 126.50, 125.89, 125.01, 122.86, 121.68, 115.76, 109.52, 108.30, 83.60, 80.54, 79.47, 78.32, 56.52, 42.73. LCMS (Fleet, 10 → 90%): t_r = 4.30 min, m/z: 426.2. HRMS $[C_{24}H_{19}N_5O_3 + H]^+$: 426.15607 calculated, 426.15604 found.

2-Acetamido-*N*-(4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)acetamide (17)



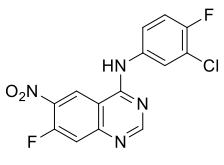
The title compound was synthesized from **32** (60.0 mg, 162 µmol) and acetyl chloride according to general procedure B. The crude was purified by HPLC (Agilent, 24 – 30% MeCN in 0.2% TFA (aq.)) after which the fractions were concentrated and traces of TFA were removed by coevaporation with 1:1 MeCN/H_2O (20 mL). The residue was dissolved in DCM (15 mL) and washed with 1 M $NaHCO_3$ (aq.) (3x5 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated to afford the product (14 mg, 34 µmol, 21%). ^{1}H NMR (400 MHz, DMSO) δ 9.83 (s, 1H), 9.51 (s, 1H), 8.89 (s, 1H), 8.53 (s, 1H), 8.38 (t, *J* = 5.9 Hz, 1H), 7.98 (t, *J* = 1.9 Hz, 1H), 7.85 (ddd, *J* = 8.2, 2.2, 1.1 Hz, 1H), 7.39 (s, 1H), 7.38 (t, *J* = 8.0 Hz, 1H), 7.20 (dt, *J* = 7.6, 1.3 Hz, 1H), 5.10 (d, *J* = 2.4 Hz, 2H), 4.20 (s, 1H), 4.00 (d, *J* = 6.0 Hz, 2H), 3.74 (t, *J* = 2.3 Hz, 1H), 1.93 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.96, 168.39, 156.98, 154.05, 152.67, 148.58, 139.79, 128.83, 126.97, 126.49, 125.00, 122.85, 121.68, 115.48, 109.53, 108.27, 83.59, 80.55, 79.47, 78.33, 56.58, 42.82, 22.46. LCMS (Fleet, 10 → 90%): t_r = 4.04 min, m/z: 414.2. HRMS $[C_{23}H_{19}N_5O_3 + H]^+$: 414.15607 calculated, 414.15607 found.

4-Chloro-7-fluoro-6-nitroquinazoline (18)



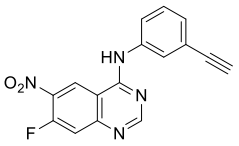
7-fluoro-6-nitroquinazolin-4(3*H*)-one (2.50 g, 12.0 mmol) was suspended in $SOCl_2$ (21.8 mL) and DMF (105 µL, 1.35 mmol). The mixture was heated to 75°C, stirred for 5 h and subsequently concentrated to afford the product (2.70 g, 11.9 mmol, 99%). ^{1}H NMR (500 MHz, DMSO) δ 8.72 (d, *J* = 8.2 Hz, 1H), 8.35 (s, 1H), 7.79 (d, *J* = 12.2 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 159.35, 157.68 (d, $J_{(C–F)}$ = 265.9 Hz), 153.69 (d, $J_{(C–F)}$ = 13.6 Hz), 150.18, 135.55 (d, $J_{(C–F)}$ = 9.5 Hz), 125.69, 119.32, 115.45 (d, $J_{(C–F)}$ = 21.3 Hz).

N-(3-Chloro-4-fluorophenyl)-7-fluoro-6-nitroquinazolin-4-amine (19)



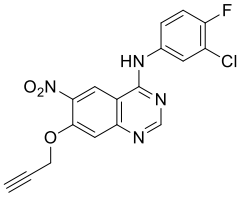
18 (1.40 g, 6.15 mmol) was mixed in 2-propanol (13.7 mL) after which DIPEA (2.15 mL, 12.3 mmol) and 3-chloro-4-fluoroaniline (895 mg, 6.15 mmol) were added. The mixture was stirred for 7 h, subsequently diluted in EtOAc (50 mL) and poured into H_2O (50 mL). The organic layer was isolated and the water layer extracted with EtOAc (2x50 mL). The combined organic layers were washed with brine (100 mL), dried over Na_2SO_4, filtered and concentrated. The crude was loaded onto silica gel and purified by automated column chromatography (25 – 75% EtOAc/pentane) to afford the product (760 mg, 2.26 mmol, 37%). ^{1}H NMR (500 MHz, DMSO) δ 10.49 (s, 1H), 9.56 (d, *J* = 7.9 Hz, 1H), 8.72 (s, 1H), 8.12 (dd, *J* = 7.0, 2.6 Hz, 1H), 7.84 (d, *J* = 12.4 Hz, 1H), 7.79 (ddd, *J* = 9.2, 4.5, 2.8 Hz, 1H), 7.48 (t, *J* = 9.1 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 158.38, 158.18, 156.46 (d, $J_{(C–F)}$ = 265.1 Hz), 154.04 (d, $J_{(C–F)}$ = 13.2 Hz), 153.96 (d, $J_{(C–F)}$ = 244.3 Hz), 135.53, 135.45, 124.41, 124.37, 123.13 (d, $J_{(C–F)}$ = 7.1 Hz), 119.00 (d, $J_{(C–F)}$ = 18.6 Hz), 116.77 (d, $J_{(C–F)}$ = 22.0 Hz), 115.07 (d, $J_{(C–F)}$ = 20.2 Hz), 111.27. LCMS (Fleet, 10 → 90%): t_r = 5.88 min, m/z: 337.2.

N-(3-Ethynylphenyl)-7-fluoro-6-nitroquinazolin-4-amine (20)



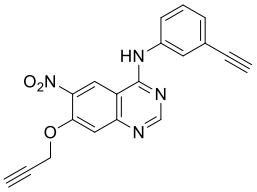
18 (2.20 g, 9.67 mmol) was mixed in 2-propanol (22 mL) after which DIPEA (3.38 mL, 19.3 mmol) and 3-ethynylaniline (985 µL, 9.67 mmol) were added. The mixture was stirred for 16 h, subsequently diluted in DCM (150 mL) and poured into H_2O (50 mL). The organic layer was isolated and the water layer extracted with DCM (2x50 mL). The combined organic layers were washed with brine (100 mL), dried over Na_2SO_4, filtered and concentrated. The crude was loaded onto silica gel and purified by automated column chromatography (25 – 75% EtOAc/pentane) to afford the product (2.40 g, 7.79 mmol, 81%). ^{1}H NMR (400 MHz, DMSO) δ 10.45 (s, 1H), 9.60 (d, *J* = 8.0 Hz, 1H), 8.72 (s, 1H), 7.99 (t, *J* = 1.8 Hz, 1H), 7.87 (ddd, *J* = 8.5, 2.2, 1.2 Hz, 1H), 7.82 (d, *J* = 12.5 Hz, 1H), 7.44 (t, *J* = 8.0 Hz, 1H), 7.29 (dt, *J* = 7.7, 1.2 Hz, 1H), 4.24 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 158.47, 158.28, 156.44 (d, $J_{(C–F)}$ = 264.8 Hz), 154.13 (d, $J_{(C–F)}$ = 13.0 Hz), 138.61, 135.47 (d, $J_{(C–F)}$ = 10.0 Hz), 129.10, 127.73, 125.58, 124.52, 123.29, 121.93, 115.02 (d, $J_{(C–F)}$ = 19.9 Hz), 111.40, 83.25, 80.95. LCMS (Fleet, 10 → 90%): t_r = 5.38 min, m/z: 309.2.

N-(3-Chloro-4-fluorophenyl)-6-nitro-7-(prop-2-yn-1-yloxy)quinazolin-4-amine (21)



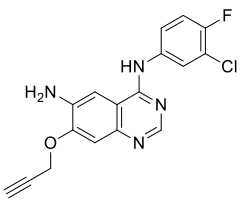
19 (730 mg, 2.17 mmol) was mixed in THF (10 mL) after which propargyl alcohol (0.5 mL, 8.67 mmol) was added. The mixture was cooled down to 0°C and potassium *tert*-butoxide (487 mg, 4.34 mmol) was added. After stirring for 5 min, the mixture was allowed to warm to RT and continued to stir for 16 h. The mixture was diluted in EtOAc (20 mL) and poured into H_2O (20 mL). The organic layer was isolated and the water layer extracted with EtOAc (2x20 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, filtered and concentrated. The crude loaded onto silica gel and purified by automated column chromatography (10 – 75% EtOAc/pentane) to afford the product (737 mg, 1.98 mmol, 91%). ^{1}H NMR (400 MHz, DMSO) δ 10.38 (br s, 1H), 9.24 (s, 1H), 8.64 (s, 1H), 8.13 (dd, *J* = 6.9, 2.6 Hz, 1H), 7.76 (ddd, *J* = 9.0, 4.3, 2.7 Hz, 1H), 7.52 (s, 1H), 7.43 (t, *J* = 9.1 Hz, 1H), 5.17 (d, *J* = 2.4 Hz, 2H), 3.77 (t, *J* = 2.3 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 157.86, 157.60, 153.54 (d, $J_{(C–F)}$ = 243.4 Hz), 153.15, 152.26, 138.69, 136.60, 123.88, 122.71 (d, $J_{(C–F)}$ = 6.9 Hz), 122.21, 118.87 (d, $J_{(C–F)}$ = 18.3 Hz), 116.63 (d, $J_{(C–F)}$ = 21.6 Hz), 110.98, 108.86, 79.93, 77.73, 57.43. LCMS (Fleet, 10 → 90%): t_r = 5.41 min, m/z: 373.3.

N-(3-Ethynylphenyl)-6-nitro-7-(prop-2-yn-1-yloxy)quinazolin-4-amine (22)



20 (2.27 g, 7.35 mmol) was mixed in THF (24 mL) after which propargyl alcohol (1.70 mL, 29.4 mmol) was added. The mixture was cooled down to 0°C and potassium *tert*-butoxide (1.65 g, 14.7 mmol) was added. After stirring for 5 min, the mixture was allowed to warm to RT and continued to stir for 22 h. The mixture was diluted in H_2O (25 mL) and filtered. The solids were collected and dried to afford the product (2.53 g, 7.35 mmol, quant.) which was used as such in subsequent reaction. ^{1}H NMR (400 MHz, DMSO) δ 10.13 (s, 1H), 9.28 (s, 1H), 8.68 (s, 1H), 8.03 (t, *J* = 1.8 Hz, 1H), 7.88 (ddd, *J* = 8.3, 2.3, 1.1 Hz, 1H), 7.56 (s, 1H), 7.42 (t, *J* = 7.9 Hz, 1H), 7.26 (dt, *J* = 7.6, 1.3 Hz, 1H), 5.19 (d, *J* = 2.4 Hz, 2H), 4.23 (s, 1H), 3.78 (t, *J* = 2.3 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 157.99, 157.62, 153.11, 152.24, 138.93, 138.89, 129.06, 127.29, 125.14, 122.86, 122.10, 121.88, 111.13, 108.59, 83.35, 80.83, 79.95, 77.70, 57.45. LCMS (Finnigan, 10 → 90%): t_r = 5.86 min, m/z: 345.1.

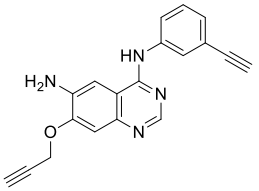
N^4-(3-Chloro-4-fluorophenyl)-7-(prop-2-yn-1-yloxy)quinazoline-4,6-diamine (23)



21 (717 mg, 1.92 mmol) was mixed in EtOH/H_2O (30:1, 43 mL) after which iron powder (537 mg, 9.62 mmol) and NH_4Cl (309 mg, 5.77 mmol) were added. The mixture was heated to 80°C and stirred for 2.5 h. The mixture was filtered over Celite and subsequently concentrated to afford the product (438 mg, 1.28 mmol, 66%). ^{1}H NMR (400 MHz, DMSO) δ 9.44 (s, 1H), 8.38 (s, 1H), 8.19 (dd, *J* = 6.9, 2.7 Hz, 1H), 7.81 (ddd, *J* = 9.1, 4.3, 2.7 Hz, 1H), 7.43 – 7.36 (m, 2H), 7.22 (s, 1H), 5.40 (s, 2H), 5.04 (d, *J* = 2.4 Hz, 2H), 3.73 – 3.67 (m, 1H). ^{13}C NMR (101 MHz, DMSO) δ 155.10, 152.71 (d, $J_{(C–F)}$ = 242.0 Hz), 150.39,

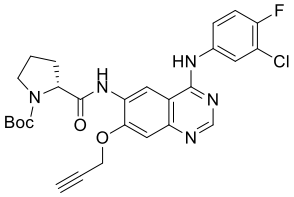
150.38, 144.42, 138.57, 137.49 (d, $J_{(C–F)}$ = 2.9 Hz), 122.54, 121.50 (d, $J_{(C–F)}$ = 6.7 Hz), 118.65 (d, $J_{(C–F)}$ = 18.2 Hz), 116.44 (d, $J_{(C–F)}$ = 21.6 Hz), 110.86, 107.55, 101.34, 79.04, 78.71, 56.06. LCMS (Fleet, 10 → 90%): t_r = 4.78 min, m/z: 343.3.

*N*⁴-(3-Ethynylphenyl)-7-(prop-2-yn-1-yloxy)quinazoline-4,6-diamine (24)



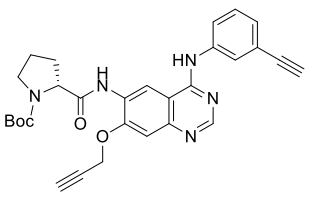
22 (140 mg, 407 µmol) was mixed in EtOH/H_2O (30:1, 9 mL) after which iron powder (114 mg, 2.03 mmol) and NH_4Cl (65.2 mg, 1.22 mmol) were added. The mixture was heated to 80°C and stirred for 2.5 h. The mixture was filtered over Celite and subsequently concentrated to afford the product (128 mg, 407 µmol, quant.). ^{1}H NMR (500 MHz, DMSO) δ 10.18 (br s, 1H), 8.56 (s, 1H), 7.95 (t, *J* = 1.9 Hz, 1H), 7.82 – 7.78 (m, 1H), 7.58 (s, 1H), 7.41 (t, *J* = 7.9 Hz, 1H), 7.29 (s, 1H), 7.26 (dt, *J* = 7.6, 1.3 Hz, 1H), 5.68 (br s, 2H), 5.07 (d, *J* = 2.4 Hz, 2H), 4.23 (s, 1H), 3.76 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 156.32, 151.15, 148.29, 139.67, 139.04, 129.00, 127.42, 125.73, 123.53, 121.83, 110.09, 103.63, 101.77, 83.38, 80.85, 79.52, 78.22, 56.39. LCMS (Fleet, 10 → 90%): t_r = 4.54 min, m/z: 315.2.

tert-Butyl (*R*)-2-((4-((3-chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)carbamoyl)pyrrolidine-1-carboxylate (25)



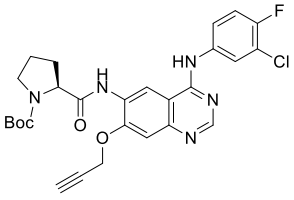
The title compound was synthesized from **23** (418 mg, 1.22 mmol) and Boc-D-Pro-OH according to general procedure A. The crude was purified by automated column chromatography (2 – 10% MeOH/DCM) to afford the product (488 mg, 0.904 mmol, 74%). ^{1}H NMR (400 MHz, DMSO) δ 9.89 (s, 1H), 9.56 – 9.45 (2x s, 1H), 8.93 – 8.88 (2x s, 1H), 8.53 (s, 1H), 8.08 (s, 1H), 7.81 – 7.73 (m, 1H), 7.42 (t, *J* = 9.2 Hz, 1H), 7.39 (s, 1H), 5.09 (d, *J* = 2.4 Hz, 2H), 4.58 – 4.44 (m, 1H), 3.75 – 3.69 (m, 1H), 3.50 – 3.38 (m, 1H), 2.37 – 2.08 (m, 1H), 2.07 – 1.81 (m, 1H), 1.43 – 1.28 (2x s, 9H) (three proline protons were not observed). LCMS (Finnigan, 10 → 90%): t_r = 6.65 min, m/z: 540.1.

tert-Butyl (*R*)-2-((4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)carbamoyl)pyrrolidine-1-carboxylate (26)



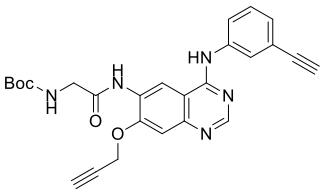
The title compound was synthesized from **24** (837 mg, 2.66 mmol) and Boc-D-Pro-OH according to general procedure A. The crude was purified by automated column chromatography (2 – 10% MeOH/DCM) to afford the product (1.32 g, 2.58 mmol, 97%). ^{1}H NMR (400 MHz, DMSO) δ 9.83 (s, 1H), 9.54 – 9.44 (2x s, 1H), 8.94 – 8.88 (2x s, 1H), 8.53 (s, 1H), 7.99 – 7.93 (m, 1H), 7.87 – 7.82 (m, 1H), 7.39 (s, 1H), 7.38 (t, *J* = 7.9 Hz, 1H), 7.20 (d, *J* = 7.6 Hz, 1H), 5.09 (d, *J* = 2.4 Hz, 2H), 4.56 – 4.45 (m, 1H), 4.19 (s, 1H), 3.75 – 3.69 (m, 1H), 3.46 – 3.38 (m, 2H), 2.31 – 2.11 (m, 1H), 2.09 – 1.80 (m, 3H), 1.44 – 1.29 (2x s, 9H). LCMS (Fleet, 10 → 90%): t_r = 5.70 min, m/z: 512.3.

tert-Butyl (*S*)-2-((4-((3-chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)carbamoyl)pyrrolidine-1-carboxylate (27)



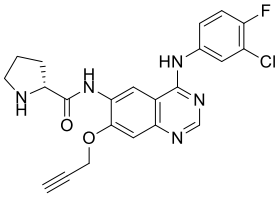
The title compound was synthesized from **23** (104 mg, 303 µmol) and Boc-L-Pro-OH according to general procedure A. The crude purified by automated column chromatography (2 – 10% MeOH/DCM) and used as such in subsequent reaction (yield: 131 mg). LCMS (Finnigan, 10 → 90%): t_r = 6.87 min, m/z: 540.2.

***tert*-Butyl (2-((4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)amino)-2-oxoethyl)carbamate (28)**



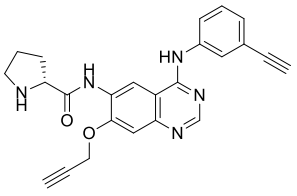
The title compound was synthesized from **24** (749 mg, 2.38 mmol) and Boc-Gly-OH according to general procedure A. The crude was loaded onto Celite and purified by automated column chromatography (2 – 10% MeOH/DCM) to afford the product (910 mg, 1.93 mmol, 81%). ^{1}H NMR (400 MHz, DMSO) δ 9.84 (s, 1H), 9.44 (s, 1H), 8.95 (s, 1H), 8.54 (s, 1H), 7.98 (t, *J* = 1.9 Hz, 1H), 7.85 (ddd, *J* = 8.3, 2.3, 1.1 Hz, 1H), 7.41 (s, 1H), 7.38 (t, *J* = 7.9 Hz, 1H), 7.30 (t, *J* = 6.1 Hz, 1H), 7.20 (dt, *J* = 7.6, 1.3 Hz, 1H), 5.11 (d, *J* = 2.4 Hz, 2H), 4.18 (s, 1H), 3.87 (d, *J* = 6.1 Hz, 2H), 3.73 (t, *J* = 2.3 Hz, 1H), 1.42 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 168.74, 157.01, 156.05, 154.01, 152.35, 148.46, 139.80, 128.82, 127.04, 126.51, 125.07, 122.92, 121.69, 114.93, 109.60, 108.27, 83.61, 80.51, 79.47, 78.40, 78.26, 56.58, 44.06, 28.22. LCMS (Fleet, 10 → 90%): t_r = 5.31 min, m/z: 472.2.

(*R*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (29)



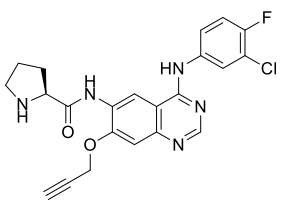
25 (468 mg, 867 µmol) was dissolved in DCM (8.6 mL) and cooled down to 0°C. TFA (2.6 mL) was added and the mixture was stirred at 0°C for 2.5 h. The mixture was quenched with 1 M $NaHCO_3$ (aq.) (50 mL) and the product extracted with DCM (3x50 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, filtered and concentrated. The crude was loaded onto silica gel and purified by automated column chromatography (0 – 10% MeOH/DCM) to afford the product (381 mg, 867 µmol, quant.). ^{1}H NMR (400 MHz, DMSO) δ 10.52 (s, 1H), 9.89 (s, 1H), 9.10 (s, 1H), 8.52 (s, 1H), 8.08 (dd, *J* = 6.9, 2.7 Hz, 1H), 7.77 (ddd, *J* = 9.1, 4.4, 2.7 Hz, 1H), 7.41 (t, *J* = 9.1 Hz, 1H), 7.40 (s, 1H), 5.14 (d, *J* = 2.4 Hz, 2H), 3.83 (dd, *J* = 9.1, 5.1 Hz, 1H), 3.75 (t, *J* = 2.3 Hz, 1H), 3.02 (dt, *J* = 10.2, 6.6 Hz, 1H), 2.84 (dt, *J* = 10.2, 6.4 Hz, 1H), 2.14 – 2.05 (m, 1H), 1.93 – 1.84 (m, 1H), 1.67 (p, *J* = 6.8 Hz, 2H) (the proline –NH was not observed). ^{13}C NMR (101 MHz, DMSO) δ 173.54, 156.89, 153.59, 153.18 (d, $J_{(C–F)}$ = 242.5 Hz), 151.50, 147.96, 136.87 (d, $J_{(C–F)}$ = 3.0 Hz), 127.28, 123.73, 122.63 (d, $J_{(C–F)}$ = 6.8 Hz), 118.65 (d, $J_{(C–F)}$ = 18.4 Hz), 116.41 (d, $J_{(C–F)}$ = 21.7 Hz), 111.36, 109.69, 108.20, 79.44, 78.19, 60.98, 56.90, 46.74, 30.37, 26.11. LCMS (Finnigan, 10 → 90%): t_r = 4.76 min, m/z: 440.2.

(*R*)-*N*-(4-((3-Ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (30)



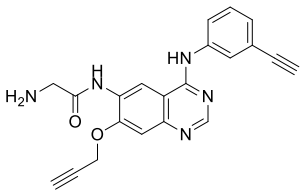
26 (1.25 g, 2.43 mmol) was dissolved in DCM (24 mL) and cooled down to 0°C. TFA (7.5 mL) was added and the mixture was stirred at 0°C for 3 h. The mixture was quenched with 1 M $NaHCO_3$ (aq.) (100 mL) and the product extracted with DCM (2x100 mL). The combined organic layers were washed with brine (100 mL), dried over Na_2SO_4, filtered and concentrated. The crude was loaded onto silica gel and purified by automated column chromatography (0 – 10% MeOH/DCM) to afford the product (561 mg, 1.36 mmol, 56%). ^{1}H NMR (400 MHz, DMSO) δ 10.51 (s, 1H), 9.83 (s, 1H), 9.11 (s, 1H), 8.52 (s, 1H), 7.96 (t, *J* = 1.9 Hz, 1H), 7.84 (ddd, *J* = 8.3, 2.3, 1.1 Hz, 1H), 7.42 – 7.32 (m, 2H), 7.20 (dt, *J* = 7.6, 1.3 Hz, 1H), 5.14 (d, *J* = 2.4 Hz, 2H), 4.19 (s, 1H), 3.84 (dd, *J* = 9.1, 5.1 Hz, 1H), 3.75 (t, *J* = 2.2 Hz, 1H), 3.02 (dt, *J* = 10.2, 6.6 Hz, 1H), 2.85 (dt, *J* = 10.2, 6.4 Hz, 1H), 2.16 – 2.04 (m, 1H), 1.96 – 1.83 (m, 1H), 1.67 (p, *J* = 6.8 Hz, 2H) (the proline –NH was not observed). ^{13}C NMR (101 MHz, DMSO) δ 173.52, 157.00, 153.69, 151.50, 148.02, 139.89, 128.80, 127.20, 126.41, 125.02, 122.88, 121.65, 111.60, 109.85, 108.17, 83.61, 80.50, 79.43, 78.21, 60.99, 56.89, 46.76, 30.38, 26.12. LCMS (Fleet, 10 → 90%): t_r = 3.63 min, m/z: 412.3.

(*S*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)pyrrolidine-2-carboxamide (31)



27 (120 mg, 222 µmol) was dissolved in DCM (0.7 mL) and cooled down to 0°C. TFA (0.7 mL) was added and the mixture was stirred at 0°C for 2 h. The mixture was quenched with 1 M $NaHCO_3$ (aq.) (30 mL) and the product extracted with EtOAc (3x30 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated. The crude purified by automated column chromatography (0 – 10% MeOH/DCM) to afford the product (83.0 mg, 189 µmol, 85%). ^{1}H NMR (400 MHz, DMSO) δ 10.52 (s, 1H), 9.88 (s, 1H), 9.08 (s, 1H), 8.51 (s, 1H), 8.07 (dd, *J* = 6.9, 2.7 Hz, 1H), 7.76 (ddd, *J* = 9.1, 4.4, 2.7 Hz, 1H), 7.39 (t, *J* = 9.1 Hz, 1H), 7.38 (s, 1H), 5.13 (d, *J* = 2.4 Hz, 2H), 3.84 (dd, *J* = 9.1, 5.1 Hz, 1H), 3.74 (t, *J* = 2.3 Hz, 1H), 3.02 (dt, *J* = 10.2, 6.6 Hz, 1H), 2.85 (dt, *J* = 10.2, 6.7 Hz, 1H), 2.15 – 2.05 (m, 1H), 1.93 – 1.84 (m, 1H), 1.67 (p, *J* = 6.8 Hz, 2H) (the proline –NH was not observed). ^{13}C NMR (101 MHz, DMSO) δ 173.63, 156.94, 153.65, 153.27 (d, $J_{(C–F)}$ = 242.8 Hz), 151.59, 148.02, 136.91 (d, $J_{(C–F)}$ = 3.1 Hz), 127.31, 123.76, 122.64 (d, $J_{(C–F)}$ = 6.8 Hz), 118.75 (d, $J_{(C–F)}$ = 18.4 Hz), 116.44 (d, $J_{(C–F)}$ = 21.5 Hz), 111.50, 109.75, 108.21, 79.47, 78.22, 61.04, 56.96, 46.82, 30.45, 26.16. LCMS (Finnigan, 10 → 90%): t_r = 4,92 min, m/z: 440.2.

2-Amino-*N*-(4-((3-ethynylphenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)acetamide (32)



28 (810 mg, 1.72 mmol) was dissolved in DCM (8.5 mL) and cooled down to 0°C. TFA (5.2 mL) was added after which the mixture was allowed to warm to RT and stirred for 3 h. The mixture was quenched with 1 M $NaHCO_3$ (aq.) (100 mL) and DCM (100 mL) was added. The mixture was filtered and the solids were collected. From the filtrate, the layers were separated and the water layer was extracted with DCM (2x50 mL). The combined organic layers were washed with brine (100 mL), dried over Na_2SO_4, filtered and concentrated. The residue was combined with the collected solids, loaded onto silica gel and purified by automated column chromatography (0 – 5% MeOH/DCM) to afford the product (396 mg, 1.07 mmol, 62%). ^{1}H NMR (400 MHz, DMSO) δ 9.83 (s, 1H), 9.13 (s, 1H), 8.52 (s, 1H), 7.96 (t, *J* = 1.9 Hz, 1H), 7.84 (ddd, *J* = 8.2, 2.2, 1.1 Hz, 1H), 7.41 (s, 1H), 7.38 (t, *J* = 7.9 Hz, 1H), 7.20 (dt, *J* = 7.6, 1.3 Hz, 1H), 5.15 (d, *J* = 2.4 Hz, 2H), 4.19 (s, 1H), 3.74 (t, *J* = 2.3 Hz, 1H), 3.36 (s, 2H) (three –NHs were not observed). ^{13}C NMR (101 MHz, DMSO) δ 171.82, 157.00, 153.70, 151.44, 148.00, 139.87, 128.80, 127.24, 126.43, 125.07, 122.94, 121.65, 111.82, 109.82, 108.14, 83.61, 80.52, 79.49, 78.23, 56.74, 45.20. LCMS (Fleet, 10 → 90%): t_r = 3.31 min, m/z: 372.2.

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