Characterization of Covalent-Reversible EGFR Inhibitors

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Supporting Information

ABSTRACT: Within the spectrum of kinase inhibitors, covalent-reversible inhibitors (CRIs) provide a valuable alternative approach to classical covalent inhibitors. This special class of inhibitors can be optimized for an extended drug-target residence time. For CRIs, it was shown that the fast addition of thiols to electron-deficient olefins leads to a covalent bond that can break reversibly under proteolytic conditions. Research groups are just beginning to include CRIs in their arsenal of compound classes, and, with that, the understanding of this interesting set of chemical warheads is growing. However, systems to assess both characteristics of the covalent-reversible bond in a simple experimental setting are sparse. Here, we have developed an efficient methodology to characterize the covalent and reversible properties of CRIs and to investigate their potential in targeting clinically relevant variants of the receptor tyrosine kinase EGFR.

INTRODUCTION

Pinpointing the molecular mechanisms of cancer has led to the identification of protein kinases that can be targeted selectively for a more effective treatment.1,2 Covalent targeting of noncatalytic cysteines in dysregulated kinases represents a successful strategy in a broad variety of indications,3 including chronic lymphocytic leukemia,4,5 as well as afatinib6 and AZD9291 (osimertinib)7 in EGFR-positive non-small cell lung cancer (NSCLC) to name just a few. In the case of EGFR-dependent NSCLC, the sequential occurrence of somatic activating mutations in the kinase domain exemplified the need for an ever-evolving generation of new inhibitors.8−14 The 4-aminoquinazoline-based first-generation reversible EGFR inhibitors, such as erlotinib and gefitinib,10 showed significant clinical response rates between 50 and 80%. However, owing to a secondary point mutation located at the gatekeeper position (T790M) in the kinase domain, patients acquired resistance and suffered from a dramatic relapse within 18 months of treatment.13,15,16 Inhibitors of the second generation (e.g., afatinib17) contain electrophilic Michael-acceptor systems to address a unique cysteine (Cys797) located at the lip of the ATP-binding side on top of αHelix-D. The covalent modification resulting in an increased target residence time was thought to overcome T790M drug resistance.3,17,18 Afatinib proved to be equally potent against EGFRL858R/T790M and EGFRWT resulting in various side effects at clinical doses.19−24 The benefits of afatinib compared to the first-generation reversible inhibitors was considered slight.25 The third-generation inhibitors (e.g., osimertinib), characterized by its pyrimidine scaffold and therefore different orientation in the active site, were demonstrated to effectively inhibit the proliferation of drug-resistant EGFRL858R/T790M cell lines.26−29,33 Osimertinib showed excellent results in clinical studies as well as reduced on-target toxicity and was approved by the FDA as tagrisso in 2015.28−30

Despite the success of targeted covalent inhibitors, the alternative concept of covalent-reversible modification31−35 was recently introduced to the field of kinase inhibitors by Taunton et al.36,37 Numerous studies have shown that compounds equipped with the α-cyano-α,β-unsaturated carbonyl moiety can undergo rapid, reversible Michael addition with thiols.17−40 The reversible nature of this modification is hoped to reduce the risk of unspecific covalent modification and, therefore, potentially lowers the risk of severe side effects while maintaining the desirable maximum drug-target residence time on the target of interest.17,31,41,42

The electron-withdrawing substituents in these compounds render the β-keto position more susceptible to nucleophilic attack, thus accelerating the addition reaction to form a covalent bond with the target protein. In addition, these substituents increase the acidity of the Cα-H in the covalently bound inhibitor, which, in turn, facilitates a rapid elimination of the proton upon changes in the protein-inhibitor environment.37,43 Thus, the presence of electron-withdrawing groups could eliminate the disadvantages of covalent inhibitors, namely, the generation of non-endogenous protein fragments after proteasomal degradation.40 In various mass spectrometry (MS) experiments, the covalent nature of these compounds was confirmed, whereas the reversible features could be
observed via UV−vis spectroscopy by treating the cyanoacrylamides with β-mercaptoethanol, which led to a disappearance of the characteristic absorption peak.\(^{37}\) Dilution of the sample resulted in a reappearance or increase of this peak.\(^{37}\) This two-step characterization of CRIs still raises many questions, for example, whether the reversible properties of these cyanoacrylamides can also be observed when bound to a protein? Given the limited availability of methodologies to rapidly characterize the binding properties of CRIs,\(^ {44,45}\) we set out to develop a straightforward MS-based approach, which is based on competition experiments with covalent-irreversible inhibitors and allows to dissect the covalent and reversible binding characteristics of CRIs.

In the search for a suitable set of probe molecules to investigate the covalent and reversible properties, we combined the established synthesis schemes from previous publications of our group. We used 4-amino pyrazolopyrimidines that feature an acrylamide warhead as a Michael acceptor and bulky aromatic ring systems in the 3-position as a starting point. We could show that these inhibitors showed excellent inhibitory effects against drug-resistant EGFR\(^ {L858R/T790M}\) cell lines, as well as demonstrating a highly promising selectivity toward the drug-resistant variants and a favorable kinetic profile.\(^ {46}\) We combined these findings with lessons learned from Basu et al., wherein we developed CRIs based upon the third-generation EGFR\(^ {L858R/T790M}\) inhibitor, WZ 4002.\(^ {48}\) Herein, we were able to show that a small library of CRIs inhibited the drug-resistant variants of EGFR better than their reversible analogs. MS experiments with cSrc-mutants showed that these inhibitors alkylated the protein of interest. Furthermore, we verified the predominant E-configuration of the double bond of the Michael acceptor through nuclear Overhauser effect (NOE) experiments.\(^ {38}\) In the present study, we investigated the inhibitory impact of our newly developed probe molecules toward EGFR and its mutant variants (L858R, L858R/T790M). Subsequently, we used this focused library to investigate the covalent and reversible properties of CRIs with a single MS experiment.

### RESULTS AND DISCUSSION

#### Structure-Based Design of Covalent-Reversible Inhibitors (CRIs)

We designed CRIs based on the crystal structures of covalent pyrazolo[3,2-d]pyrimidines (PDB: 5j9y), which showed excellent inhibition of drug-resistant EGFR\(^ {T790M}\) both in biochemical and cellular assays (Figure 1).\(^ {46}\) We chose to focus on bulky aromatic ring systems as substituents at the 3-position to extend the inhibitor into the lipophilic pocket adjacent to the gatekeeper residue (Met790) and to achieve mutant selectivity by favorable interactions. To render the inhibitor covalent-reversible, we modified the attached Michael acceptor by inserting an electron-withdrawing group. We chose the cyano moiety, which was superior with respect to potency when compared to that of alternative moieties such as trifluoromethyl.\(^ {58}\) To avoid possible unfavorable interactions...
Table 1. Half-Maximal Inhibitory Concentrations (IC_{50}) Determined for Reference Compounds and CRIs 1a–e and Their Covalent Analogues 7a–f

| compound | EGFR | IC_{50} (nM) | K_i (nM) | k_{on,act} (min^{-1}) | k_{on,act}/K_i (µM^{-1} s^{-1}) |
|----------|-------|-------------|----------|----------------------|---------------------------------|
| 1a       | WT    | 5385 ± 3889 |          |                      |                                 |
|          | L858R | 229 ± 62    |          |                      |                                 |
|          | L858R/T790M | 4541 ± 543 |          |                      |                                 |
| 1b       | WT    | >10 µM      |          |                      |                                 |
|          | L858R | 385 ± 207   |          |                      |                                 |
|          | L858R/T790M | 2543 ± 420 |          |                      |                                 |
| 1c       | WT    | 102 ± 75    | 64 ± 1   | 0.03 ± 0.01          | 0.01 ± 0.01                     |
|          | L858R | 21 ± 20     | 14 ± 2   | 0.04 ± 0.03          | 0.68 ± 0.25                     |
|          | L858R/T790M | 80 ± 51    | 15 ± 3   | 0.11 ± 0.01          | 0.73 ± 0.16                     |
| 1d       | WT    | 96 ± 26     | 265 ± 1  | 0.04 ± 0.01          | 0.01 ± 0.01                     |
|          | L858R | 10 ± 8      | 37 ± 6   | 0.18 ± 0.02          | 0.20 ± 0.01                     |
|          | L858R/T790M | 20 ± 13    | 51 ± 2   | 0.17 ± 0.08          | 0.05 ± 0.02                     |
| 1e       | WT    | 338 ± 69    | 1674 ± 450 | 0.17 ± 0.06        | 0.01 ± 0.01                     |
|          | L858R | 85 ± 34     | 79 ± 9   | 0.12 ± 0.01          | 0.26 ± 0.07                     |
|          | L858R/T790M | 213 ± 49   | 83 ± 10  | 0.13 ± 0.04          | 0.36 ± 0.02                     |
| 7a       | WT    | 136 ± 58    |          |                      |                                 |
|          | L858R | 11 ± 3.0    |          |                      |                                 |
|          | L858R/T790M | 243 ± 42   |          |                      |                                 |
| 7b       | WT    | 366 ± 91    |          |                      |                                 |
|          | L858R | 27 ± 1.9    |          |                      |                                 |
|          | L858R/T790M | 223 ± 75   |          |                      |                                 |
| 7c       | WT    | 58 ± 13     | 47 ± 5.8 | 0.13 ± 0.05          | 0.05 ± 0.04                     |
|          | L858R | 1.0 ± 0.3   | 44 ± 6.5 | 0.25 ± 0.06          | 0.10 ± 0.08                     |
|          | L858R/T790M | 1.9 ± 0.8  | 58 ± 3.3 | 0.31 ± 0.04          | 0.09 ± 0.02                     |
| 7d       | WT    | 13 ± 3.0    |          |                      |                                 |
|          | L858R | 2.0 ± 0.8   |          |                      |                                 |
|          | L858R/T790M | 2.7 ± 0.3  |          |                      |                                 |
| 7e       | WT    | 35 ± 14     | 25 ± 7.2 | 0.11 ± 0.03          | 0.08 ± 0.02                     |
|          | L858R | 3.8 ± 2.8   | 19 ± 3.1 | 0.17 ± 0.08          | 0.14 ± 0.07                     |
|          | L858R/T790M | 2.5 ± 1.4  | 16 ± 5.2 | 0.29 ± 0.02          | 0.36 ± 0.09                     |
| 7f       | WT    | 16 ± 5.6    | 15 ± 3.2 | 0.19 ± 0.12          | 0.21 ± 0.05                     |
|          | L858R | 1.1 ± 0.6   | 1.6 ± 0.4 | 0.14 ± 0.01        | 1.60 ± 0.53                     |
|          | L858R/T790M | <1         | 1.5 ± 0.8 | 0.17 ± 0.02          | 3.42 ± 1.44                     |
| osimertinib | WT   | 1.6 ± 0.2   | 13.5 ± 3.0 | 0.43 ± 0.11        | 0.52 ± 0.05                     |
|          | L858R | 1.3 ± 0.4   | 1.58 ± 0.19 | 0.30 ± 0.01       | 3.24 ± 0.46                     |
|          | L858R/T790M | 0.3 ± 0.02 | 1.46 ± 0.07 | 0.33 ± 0.06      | 3.75 ± 0.39                     |
| WZ 4002  | WT    | 14 ± 7     |          |                      |                                 |
|          | L858R | 0.06 ± 0.04 |          |                      |                                 |
|          | L858R/T790M | 0.9 ± 1.2  |          |                      |                                 |
| gefitinib | WT    | 274 ± 151   |          |                      |                                 |
|          | L858R | 0.02 ± 0.004 |          |                      |                                 |
|          | L858R/T790M | 55.27 ± 48.03 |          |                      |                                 |

With Arg841 near Cys797 (Figure S2), while hoping to maintain the activity against the different EGFR mutant variants, as shown by Basu et al., we chose cyclopropyl over different aromatic substituents or small aliphatic groups as a second electron-withdrawing group.38

**Synthesis of a Focused Library.** We synthesized a focused library of EGFR inhibitors (Table 1). The synthesis began with 4-amino-1H-pyrazolo-[3,4-d]-pyrimidine (2), which was treated with N-iodosuccinimide to accomplish a regioselective iodination at the 3-position affording 3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine (3, Scheme 1). To bring about the essential R-configuration of the linker segment that introduced the covalent-reversible warhead, tert-butyl-(S)-3-hydroxypiperidine-1-carboxylate was coupled to N1 by the Mitsunobu reaction. In line with the mechanism, the reaction proceeded with a clean inversion of the stereogenic center, and, therefore, the right orientation (R) was ensured. Highly pure tert-butyl-(R)-3-(4-amino-3-ido-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidine-1-carboxylate (4) was obtained by conducting multiple cycles of column chromatography using different solvent systems to eliminate the inevitably formed triphenylphosphine oxide. Subsequent Suzuki coupling was performed under standard coupling conditions using Pd(PPh3)$_4$ and sodium carbonate in dimethyl glycol and ethanol (3:1 v/v) to obtain 5a–e. After acidic Boc-deprotection of the secondary piperidine-amine, the attachment of the covalent-reversible
warhead was achieved in a two-step sequence. First, the electron-withdrawing cyano-group was introduced with cyanoacetic acid via a standard acid−amine coupling protocol (EDC, HOBt, DIPEA). To finally obtain our desired CRIs, we performed an aldol condensation with cyclopropanecarbaldehyde and compound 6 using piperidine as a base in refluxing ethanol to afford the desired products predominantly as trans-olfs.38 The exclusive (E) stereochemistry was determined in preceding experiments by monitoring NOE cross peaks in NMR studies to discriminate cis- and trans-geometry (1, Scheme 1).38

Biochemical Characterization of CRIs toward EGFR. The binding properties of the focused CRI library toward the different EGFR mutants were evaluated using an activity-based assay quantifying the relative phosphorylation of an artificial peptidic substrate in a time-resolved FRET manner. Positive controls were defined by WZ 4002, gefitinib, and osimertinib. The determined IC50 values (Table 1) were consistent with data reported in the literature.38

We found that all CRIs inhibited different EGFR mutants. To our dismay, we did not achieve selectivity toward EGFRL858R/T790M. Compared to our previously reported inhibitors (7a–f), we observed a more than 20-fold decrease in activity. We speculate that the proximity between Cys797 and Arg841, observed in previously published crystal structures (PDB: 5j9z and 5j9y),36 could force the covalent-reversible warhead, with its bulky cyclopropyl moiety, into a slightly different conformation, which, in turn, leads to a repositioning near the gatekeeper residue. Further experiments were conducted to determine the kinetic parameters, K_i and k_{inact} wherein K_i reflects the reversible binding affinity and k_{inact} describes the rate of covalent bond formation. To analyze binding characteristics of our best compounds, we monitored IC50 values of 1c−e in different EGFR variants in a time-dependent manner, from which K_i and k_{inact} can be deduced as described previously.47 As the kinetic parameters of 1c−e suggest a rather slow covalent bond formation, with k_{inact} values of 0.13 min⁻¹ against the double mutant of EGFR (1e), one could speculate that the competition with ATP in the active site plays a role in the rather modest activity values of this compound.48 Comparison between 1a and 1c provided clear evidence that bulkier substituents were crucial for potent inhibition of the different EGFR mutants. Inhibitor 1d illustrated the most potent inhibitory effect on each EGFR mutant. A feasible explanation for this observation might be based on the straight orientation of the azaindole moiety pointing into the back pocket and stacking favorably between Met790 and Lys745 (Figure S2). In addition, the secondary amine is spatially oriented in proximity to Glu762, thereby forming an additional hydrogen bond and ultimately stabilizing the conformation of the protein−ligand complex. For the investigation of the selectivity profile of our CRI scaffold, we implemented SelectScreen profiling (Life Technologies) of compound 1d at a concentration of 1 μM against a panel of 100 kinases (including selected mutants; Figure S1). Compound 1d inhibited EGFRL858R/T790M and EGFRL858R/T790M.

Scheme 1. Synthetic Route To Generate CRIs 1a−e

"Reagents and conditions: (i) N-iodosuccinimide, DMF, 85 °C, 20 h; (ii) tert-butyl-(S)-3-hydroxypiperidine-1-carboxylate, triphenylphosphine, disopropyl azodicarboxylate (DIAD), 0 °C to room temperature (rt), 18 h; (iii) boronic acid/pinacol ester, Pd(PPh3)4, sat. Na2CO3, DME/EtOH (3:1, v/v), 90 °C, 12 h; (iv) 20% TFA in DCM, 12 h; (v) cyanoacetic acid, EDC·HCl, HOBt, DIPEA, DCM, rt, 12 h; (vi) cyclopropanecarbaldehyde, piperidine, EtOH, 80 °C, 16 h."
Almost all kinases that harbor a cysteine comparable to C797 in the hinge region (see Table S1) were inhibited more than 50% and rank in the top 25% of the tested kinase set. The biochemically observed trend that 1d shows a good selectivity toward the gatekeeper mutant variant of EGFR was confirmed in this screen. Thus, the selectivity profile of 1d underlines the biochemically observed selectivity toward EGFR<sub>T790M</sub>.

**MS Experiments.** To verify the covalent-reversible nature of the synthesized inhibitors, 1a–e, we developed a new MS experiment (Figure 2). We claim that the reversible properties of these inhibitors make it possible to displace the CRIs with classical covalent inhibitors. By measuring the protein mass before and after the addition of the classical covalent inhibitors, it should be possible to show the covalent and reversible properties of this novel class of inhibitors.

In a newly developed experimental setting, we were able to verify our hypothesis. The entire set of CRIs (1a–e) showed complete single-labeled EGFR<sub>L858R/T790M</sub>, as compared to control EGFR<sub>L858R/T790M</sub> treated with DMSO, indicating a covalent adduct with our protein of interest. The subsequent treatment with covalent inhibitors (7a–f, Figure 3) displaced the CRI and led to a shift in protein mass.

This shift was consistent with the expected mass values of single-labeled EGFR<sub>L858R/T790M</sub> with only the classical covalent inhibitors (Figure 4). For comparison, we incubated EGFR<sub>L858R/T790M</sub> with a moderately active covalent inhibitor (7b) and tried to displace it with a covalent inhibitor that was biochemically more active (7f). Here, no shift in protein mass could be observed, which leads to the conclusion that the shift in protein mass for the CRIs can only be attributed to the reversible nature of this class of compounds (Figure 4).

To further validate our methodology, we incubated the protein of interest with reversible ATP-competitive inhibitors (a pyrazolopyrimidine and gefitinib) and added to each experiment either a CRI (1a, 1c) or the covalent inhibitor (8c) and analyzed the samples via MS-spectrometry.
displaced it with a covalent inhibitor of a different core motif (amino pyrimidine, osimertinib) (Figures S9−S12).

**CONCLUSIONS**

The occurrence of drug-resistant mutations in EGFR during the progression of NSCLS still poses a major challenge in the development of effective inhibitors. Here, we present a subset of EGFR inhibitors that contain chemically tuned electrophilic moieties that combine the advantageous properties of both covalent and reversible inhibition strategies. To determine the biochemical potential, our focused library of synthesized CRIs was evaluated in an activity-based assay system and confirmed moderate to good inhibitory potential. SAR studies clearly illustrated that bulkier substituents at the 3-position of the pyrazolo[3,4-d]pyrimidine scaffold considerably increased the inhibitory potential. Starting with the phenyl moiety, we extended the molecule with a trifluoromethyl moiety, further into the pocket next to the gatekeeper residue. The rotatable

**Figure 4.** Deconvoluted mass spectra of EGFR<sup>L858R/T790M</sup> incubated with DMSO (top), CRI (middle), and a covalent counterpart (bottom). Mass differences in relation to the DMSO-treated control are displayed as Δ values and demonstrate covalent-reversible modification of EGFR<sup>L858R/T790M</sup> with compounds 1a−e and the subsequent displacement with 7a−e. (A−E, CRIs 1a−e; F, control experiment). For full spectra, see Figures S3−S8.
residue did not improve the selectivity between the EGFR variants. Therefore, we chose the rigid bicyclic aromatic moieties to facilitate the positioning between the gatekeeper and Lys845. With these compounds, we were able to achieve a minimum 20-fold improvement of the activity compared to that of the monocyclic aromatic residues. Although the mutant selectivity of the compounds revealed a distinct trend, further optimization is needed to improve the activity and selectivity for the different EGFR variants. A promising starting point for further developments could be 1d. The optimization of 1d would lead to a better understanding of the narrow back pocket close to the gatekeeper. This would help to elucidate the binding modes in different oncogenic mutants. Furthermore, the area surrounding Arg848 is speculated to be crucial for generating additional contacts to stabilize a prearrangement of the CRI before covalently modifying its target. Therefore, a reassessment of the covalent-reversible warhead could lead to more selective inhibitors owing to the possible repositioning of the molecule in its binding pocket. Finally, we were able to verify the covalent and reversible nature of this novel class of inhibitors within the setting of an MS experiment. We were able to develop a method that can show the covalent modification of drug-resistant EGFR variants with CRIs and their subsequent displacement with classical covalent inhibitors, which proves their reversible nature. This new methodology will help characterize the binding characteristics of CRIs in a simple and straightforward manner.

Further investigations of the covalent-reversible warhead are needed to achieve a better inhibitory effect when targeting EGFR and its mutants. However, our newly developed methodology offers an elegant and easy way to characterize the complete set of binding characteristics of CRIs.

**Experimental Section**

**Activity-Based Assay for IC_{50} Determination and Kinetic Characterization.** IC_{50} determinations for EGFR and its mutants (Carna Biosciences, lot13CBS-0005K for EGFR\(^{WT}\), Invitrogen, lot279551C for EGFR\(^{L858R}\) and Invitrogen, lot350247C for EGFR\(^{L858R/T790M}\)) were performed with the HTRF KinEASE-TK assay from Cisbio according to the manufacturer’s instructions. Briefly, the amount of EGFR in each reaction well was set to 0.60 ng of EGFR\(^{WT}\) (0.67 nM), 0.10 ng of EGFR\(^{L858R}\) (0.11 nM), or 0.07 ng of EGFR\(^{L858R/T790M}\) (0.08 nM). An artificial substrate peptide (TK-substrate from Cisbio) was phosphorylated by EGFR. After completion of the reaction (reaction times: 25 min for WT, 15 min for L858R, and 20 min for T790M/L858R), the reaction was stopped by the addition of a buffer containing EDTA, as well as an antiphosphotyrosine antibody labeled with europium cryptate and streptavidin labeled with the fluorophore XL665. FRET between europium cryptate and XL665 was measured after an additional hour of incubation to quantify the phosphorylation of the substrate peptide. ATP concentrations were set at their respective \(K_m\) values (9.5 \(\mu\)M for EGFR\(^{WT}\), 25 \(\mu\)M for EGFR\(^{L858R}\), and 20 \(\mu\)M for EGFR\(^{L858R/T790M}\)) while a substrate concentration of 1 \(\mu\)M, 225 nM, and 275 nM, respectively, was used. Kinase and the inhibitor were preincubated for 30 min before the reaction was started by the addition of ATP and a substrate peptide. An EnVision multimode plate reader (Perkin Elmer) was used to measure the fluorescence of the samples at 620 nm (Eu-labeled antibody) and 665 nm (XL665 labeled streptavidin) 50 \(\mu\)s after excitation at 320 nm. The quotient of both intensities for reactions made with eight different inhibitor concentrations was then analyzed using the Quattro Software Suite for IC_{50} determination. Each reaction was performed in duplicate, and at least three independent determinations of each IC_{50} were made.

For kinetic characterization (\(K_v, k_{\text{cat}}\)) of covalent bond formation, the corresponding inhibitors were incubated with EGFR\(^{WT}\), EGFR\(^{L858R}\), and EGFR\(^{L858R/T790M}\) over different periods of time (0.5–90 min), whereas the duration of the enzymatic and stop reaction were kept constant. IC_{50} values were determined for the 12 different incubation times as described above and afterward plotted accordingly. Kinetic parameters were calculated with XLfit (version 5.4.0.8; IDBS, Munich, Germany) as reported by Krippendorff et al. \(^{47}\)

**MS Experiments.** We used the drug-resistant mutant variant, EGFR\(^{L858R/T790M}\), for MS experiments. We incubated 26 \(\mu\)M of the protein with 132 \(\mu\)M of the covalent-reversible inhibitor in a buffer (25 mM Tris, 250 mM NaCl, 10% glycerol, 1 mM TCEP, pH 8) on ice for 3 h. Then, an aliquot of 15 \(\mu\)L was treated with 66 \(\mu\)M of the covalent inhibitor and again incubated in ice for 1 h. We analyzed the aliquots by MS using a Thermo Fisher Scientific Ultimate 3000 HPLC system connected to a Thermo Fisher Scientific Velos Pro (2d ion trap). A 5 \(\mu\)L sample was injected and separated using a Vydac 214TP C4 5 \(\mu\)m column (150 mm × 2.1 mm) starting at 20% solvent B for 5 min, followed by a gradient up to 90% solvent B over 14 min with a flow rate of 210 \(\mu\)L/min, with 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. A mass range of 700–2000 m/z was scanned, and raw data were deconvoluted and analyzed with MagTran software (version 1.02). \(^{49}\) The deconvoluted spectra were smoothed and fitted to a mass range of 37750–38550 m/z with mMass software (version 5.50).

**Chemistry.** Unless otherwise noted, all reagents and solvents were purchased from Acros, Fluka, Sigma, Aldrich, or Merck and used without further purification. Dry solvents were purchased as anhydrous reagents from commercial suppliers. \(^1\)H and \(^13\)C NMR spectra were recorded on a Bruker Avance DRX 400 or Bruker Avance DRX 500 spectrometer at 400 or 500 MHz and 101 or 125 MHz, respectively. \(^1\)H chemical shifts are reported in \(\delta\) (ppm) as s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), m (multiplet), and bs (broad singlet) and are referenced to the residual solvent signal: CDCl\(_3\) (7.26), DMSO-\(d_6\) (2.50). Coupling constants (\(J\)) are expressed in hertz (Hz). \(^13\)C spectra are referenced to the residual solvent signal: CDCl\(_3\) (77.0) or DMSO-\(d_6\) (39.0). All final compounds were purified to >95% purity as determined by high-performance liquid chromatography (HPLC). The purity was measured using Agilent 1200 series HPLC systems with UV detection at 210 nm (system: Agilent Eclipse XDB-C18 4.6 mm × 150 mm, 5 \(\mu\)m, 10–100% CH\(_3\)CN in H\(_2\)O, with 0.1% TFA, for 15 min at 1.0 mL/min). Analytical TLC was carried out on Merck 62 F245 aluminum-backed silica gel plates. Compounds were purified by column chromatography using Baker silica gel (40–70 \(\mu\)m particle size) or Flash Chromatography on a Biotage Isolera One using Biotage SNAP, SNAP Ultra, ZIP Sphere, or ZIP KP-Sil columns (25, 10, 5, or 120 g, respectively) monitored by UV at \(\lambda = 254\) and 280 nm. Preparative HPLC was conducted on an Agilent HPLC system (1200 series) with a VP 250/21 nucleodur C18 column from Macherey-Nagel and monitored by UV at \(\lambda = 254\) nm.
General Procedure A. Suzuki Cross-Coupling of Iodinated Pyrazolopyrimidines with Various Boronic Acids. tert-Butyl-(R)-3-(4-amino-3-iodo-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidine-1-carboxylate (3). A solution of 3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine (3, 7.5 g, 28.7 mmol, 1 equiv) and tert-butyl-(S)-3-hydroxyperidine-1-carboxylate (8.3 g, 40.2 mmol, 1.4 equiv) in anhydrous THF was stirred at rt under argon atmosphere. To the pale yellow suspension, triphenylphosphine (11.3 g, 43.1 mmol, 1.5 equiv) was added, and the resulting solution was cooled to 0 °C in an ice bath. To this solution, DIAD (8.5 mL, 43.1 mmol, 1.5 equiv) was added. The reaction mixture was warmed to rt and stirred for 18 h under argon atmosphere. After completion of the reaction, the mixture was quenched using saturated sodium hydrogen carbonate and extracted three times with ethyl acetate and washed with water and saturated sodium chloride solution. The combined organic layers were dried over anhydrous sodium sulfate and evaporated to dryness.

General Procedure B. Deprotection of the Secondary Amine. The protected pyrazolopyrimidines (4a–e) were dissolved in DCM and cooled to 0 °C. To this stirred solution, TFA in DCM (20/80, v/v) was added dropwise. The reaction mixture was warmed to rt and stirred overnight. The volatiles were evaporated, and the residue was taken up in DCM. The product was separated between sodium hydrogen carbonate and a solution of 10% MeOH in DCM. The organic phase was separated, and the volatiles were removed in vacuo to obtain the designated product.

General Procedure C. Amide Coupling. A solution of (R)-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amines (5a–e) was charged with 2-cyanooacetic acid, HOBt, and DIPEA, and, after stirring under argon atmosphere for 10 min, EDC·HCl in DCM was added. The reaction mixture was stirred at rt for 12 h under argon atmosphere. The reaction mixture was quenched using saturated sodium hydrogen carbonate and extracted three times with ethyl acetate and washed with water and saturated sodium chloride solution. The combined organic layers were dried over anhydrous sodium sulfate and evaporated to dryness.

General Procedure D. Condensation with Cyclopropyl-carbaldehyde. The respective nitriles (6a–e) were dissolved in EtOH and treated with piperidine. After stirring under argon atmosphere for 5 min, cyclopropane-carbaldehyde was added at rt. The reaction mixture was then stirred at 80 °C for 16 h under argon atmosphere. The reaction mixture was quenched using saturated sodium hydrogen carbonate and extracted three times with ethyl acetate and washed with water and saturated sodium chloride solution. The combined organic layers were dried over anhydrous sodium sulfate and evaporated to dryness to obtain the crude product. The resulting crude product was purified via flash chromatography (normal phase) to obtain the title compound.

3-Iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine (3). A solution of 3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine (2, 5.0 g, 37.0 mmol, 1 equiv) and N-iodosuccinimide (12.5 g, 55.5 mmol, 1.5 equiv) were dissolved in 40 mL of DMF, heated to 85 °C, and stirred for 20 h. After completion of the reaction, the mixture was cooled to rt to obtain a solid precipitate. The precipitate was filtered and thoroughly washed with 2-propanol and ice-cold methanol. The light yellow solid was dried in vacuo to afford title compound 3 (8.8 g, 32.8 mmol, 88.7% yield). The product was used without further purification.

LC–MS: R₁ = 3.61 min, [M + H]⁺ = 262 g/mol.
1H NMR (500 MHz, DMSO-d$_6$) δ 8.24 (s, 1H), 7.86 (d, J = 7.9 Hz, 1H), 7.68 (s, 1H), 7.54–7.50 (m, 1H), 7.27 (t, J = 7.6 Hz, 1H), 7.15 (t, J = 7.4 Hz, 1H), 6.70 (s, 2H), 4.70 (dd, J = 14.2, 9.8, 4.2 Hz, 1H), 4.12–4.00 (m, 2H), 3.89 (s, 3H), 3.86 (d, J = 13.1 Hz, 1H), 3.05–2.98 (m, 1H), 2.32–2.22 (m, 1H), 2.14 (dt, J = 17.6, 6.5 Hz, 1H), 2.01–1.95 (m, 2H), 1.37 (s, 9H).

13C NMR (126 MHz, DMSO-d$_6$) δ 158.8, 155.9, 137.3, 133.6, 132.8, 132.5, 132.0 (d, J = 9.7 Hz), 130.1, 129.2 (d, J = 11.8 Hz), 126.6, 126.2, 126.1, 120.0, 110.5, 107.7, 84.2–84.1 (m), 60.2, 52.3, 33.2, 28.4, 25.3–24.1 (m), 21.2.

(R)-3-Phenyl-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine. Deprotected 5a was prepared according to General Procedure B using 5a (300.9 mg, 0.79 mmol, 1.00 equiv) and treated with 2 mL of TFA solution as described. The solid obtained was dried in vacuo to afford the title compound in a quantitative yield. The product was used without further purification.

LC–MS: $R_t = 2.06$ min, [M + H] = 295 g/mol.

(R)-1-(Piperidin-3-yl)-3-((3-trifluoromethyl)phenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine. Deprotected 5b was prepared according to General Procedure B using 5b (373.2 mg, 0.76 mmol, 1.00 equiv) and treated with 2 mL of TFA solution as described. The solid obtained was dried in vacuo to afford the title compound in a quantitative yield. The product was used without further purification.

LC–MS: $R_t = 2.83$ min, [M + H] = 363 g/mol.

(R)-3-(Naphthalen-1-yl)-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine. Deprotected 5c was prepared according to General Procedure B using 5c (388.8 mg, 0.87 mmol, 1.00 equiv) and treated with 6 mL of TFA solution as described. The solid obtained was dried in vacuo to afford the title compound in a quantitative yield. The product was used without further purification.

LC–MS: $R_t = 2.61$ min, [M + H] = 345 g/mol.

(R)-1-(Piperidin-3-yl)-3-(1H-pyrrolo[2,3-b]pyridin-5-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine. Deprotected 5d was prepared according to General Procedure B using 5d (1.0 mmol, 1.00 equiv) and treated with 2 mL of TFA solution as described. The solid obtained was dried in vacuo to afford the title compound in a quantitative yield. The product was used without further purification.

LC–MS: $R_t = 1.94$ min, [M + H] = 335 g/mol.

(R)-3-(1-Methyl-1H-indol-3-yl)-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine. Deprotected 5e was prepared according to General Procedure B using 5e (222.7 mg, 0.50 mmol, 1.00 equiv) and treated with 2 mL of TFA solution as described. The solid obtained was dried in vacuo to afford the title compound in a quantitative yield. The product was used without further purification.

LC–MS: $R_t = 2.48$ min, [M + H] = 348 g/mol.

(R)-3-(3-(4-Amino-3-phenyl-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-oxopropanenitrile (6a). Compound 6a was prepared according to General Procedure C using deprotected 5a (250.4 mg, 0.85 mmol, 1 equiv), cyanooacetic acid (110.75 mg, 1.28 mmol, 1.50 equiv), HOBt (172.42 mg, 1.28 mmol, 1.50 equiv), DIPEA (747.91 µL, 4.25 mmol, 5.00 equiv), and EDC·HCl (249.60 mg, 1.28 mmol, 1.50 equiv).

Yield: 243.10 mg (79%). The product was used without further purification.

LC–MS: $R_t = 3.30$ min, [M + H] = 362 g/mol.

(R)-3-(3-(4-Amino-3-(3-(trifluoromethyl)phenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-oxopropanenitrile (6a).
nitrile (6b). Compound 6b was prepared according to General Procedure C using deprotected 5b (287.8 mg, 0.79 mmol, 1 equiv), cyanoacetic acid (103.41 mg, 1.19 mmol, 1.50 equiv), HOBr (160.98 mg, 1.19 mmol, 1.50 equiv), DIMEA (698.31 μL, 3.97 mmol, 5.00 equiv), and EDC-HCl (233.05 mg, 1.19 mmol, 1.50 equiv). Yield: 202.50 mg (59%). The product was used without further purification.

LC–MS: $R_t = 4.35$ min, $[M + H]^+ = 430$ g/mol.

(R)-3-(3-(4-Amino-3-(naphthalen-1-yl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-oxopropanenitrile (6c). Compound 6c was prepared according to General Procedure C using deprotected 5c (300.90 mg, 0.87 mmol, 1 equiv), cyanoacetic acid (113.75 mg, 1.31 mmol, 1.50 equiv), HOBr (177.08 mg, 1.31 mmol, 1.50 equiv), DIMEA (768.12 μL, 4.37 mmol, 5.00 equiv), and EDC-HCl (256.34 mg, 1.31 mmol, 1.50 equiv). Yield: 211.80 mg (59%). The product was used without further purification.

LC–MS: $R_t = 3.96$ min, $[M + H]^+ = 412$ g/mol.

(R)-3-(3-(4-Amino-3-(1H-pyrrolo[2,3-b]pyridin-5-yl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-oxopropanenitrile (6d). Compound 6d was prepared according to General Procedure C using deprotected 5d (421.70 mg, 1.26 mmol, 1 equiv), cyanoacetic acid (164.19 mg, 1.89 mmol, 1.50 equiv), HOBr (255.62 mg, 1.89 mmol, 1.50 equiv), DIMEA (1.11 mL, 6.31 mmol, 5.00 equiv), and EDC-HCl (370.04 mg, 1.89 mmol, 1.50 equiv). Yield: 71.50 mg (14%). The product was used without further purification.

LC–MS: $R_t = 2.75$ min, $[M + H]^+ = 402$ g/mol.

(R)-3-(3-(4-Amino-3-(1-methyl-1H-indol-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-oxopropanenitrile (6e). Compound 6e was prepared according to General Procedure C using deprotected 5e (175.00 mg, 0.50 mmol, 1 equiv), cyanoacetic acid (65.58 mg, 0.76 mmol, 1.50 equiv), HOBr (102.10 mg, 0.76 mmol, 1.50 equiv), DIMEA (442.87 μL, 2.52 mmol, 5.00 equiv), and EDC-HCl (147.80 mg, 0.76 mmol, 1.50 equiv). Yield: 129.10 mg (62%). The product was used without further purification.

LC–MS: $R_t = 3.60$ min, $[M + H]^+ = 415$ g/mol.

(R,E)-2-(3-(4-Amino-3-phenyl-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-carbonyl)-3-cyclopropylacronitrile (1b). Compound 1b was prepared according to General Procedure D using 6b (181.60 mg, 0.42 mmol, 1 equiv), cyclopropanecarbaldehyde (48.37 μL, 0.63 mmol, 1.50 equiv), and piperidine (46.06 μL, 0.47 mmol, 1.10 equiv). The crude product was purified by flash column chromatography (DCM/(DCM/MeOH (90/10, v/v)) (75/25, v/v) + 1% NH3 as additive). Yield: 34.3 mg (17%).

HRMS (ESI-MS) calcd: 481.1838 g/mol for C23H23F3N4O, found: $[M + H]^+ = 482.1904$ g/mol.

$^1$H NMR (500 MHz, DMSO-d6) $\delta$ 8.29 (s, 1H), 7.97 (d, $J = 6.4$ Hz, 2H), 7.80 (dt, $J = 15.5$, 7.6 Hz, 2H), 6.85 (s, 2H), 6.51 (d, $J = 10.9$ Hz, 1H), 4.86 (td, $J = 9.3$, 4.6 Hz, 1H), 4.19 (d, $J = 12.2$ Hz, 1H), 3.91 (d, $J = 13.3$ Hz, 1H), 3.65 (s, 1H), 3.37–3.35 (m, 1H), 2.38–2.25 (m, 1H), 2.19 (dd, $J = 8.5$, 4.1 Hz, 1H), 2.04–1.96 (m, 1H), 1.91–1.80 (m, 1H), 1.77–1.63 (m, 1H), 1.17 (dt, $J = 13.5$, 6.5 Hz, 2H), 0.91–0.74 (m, 2H).

$^{13}$C NMR (126 MHz, DMSO-d6) $\delta$ 162.0–161.0 (m), 158.1–157.6 (m), 155.9–155.1 (m), 133.9–133.2 (m), 132.1–131.5 (m), 129.9, 125.3–124.6 (m), 124.6–124.1 (m), 115.4–115.0 (m), 106.4–106.1 (m), 52.5–51.5 (m), 29.2–28.3 (m), 21.4–22.6 (m), 14.7, 9.3.

$^{15}$F NMR (500 MHz, DMSO-d6) $\delta$ 162.39, 161.8, 160.8–160.6 (m), 159.1–158.8 (m), 157.7, 155.5, 153.5, 141.8, 133.3, 131.1, 129.7, 129.0, 128.0 (s), 128.0, 126.6, 126.0, 125.3, 125.1, 115.2, 106.2, 99.2, 51.9, 28.8, 23.2–23.0 (m), 14.7, 13.6–13.5 (m), 9.4.

$^{15}$N NMR (126 MHz, DMSO-d6) $\delta$ 162.39, 161.8, 160.8–160.6 (m), 159.1–158.8 (m), 157.7, 155.5, 153.5, 141.8, 133.3, 131.1, 129.7, 129.0, 128.0 (s), 128.0, 126.6, 126.0, 125.3, 125.1, 115.2, 106.2, 99.2, 51.9, 28.8, 23.2–23.0 (m), 14.7, 13.6–13.5 (m), 9.4.

$^{19}$F NMR (500 MHz, DMSO-d6) $\delta$ 11.69 (s, 1H), 8.49 (d, $J = 7.5$, 1.9 Hz, 1H), 8.27 (s, 1H), 8.19 (d, $J = 1.7$ Hz, 1H), 7.57–7.51 (m, 1H), 6.72 (s, 2H), 6.59–6.54 (m, 1H), 6.50 (d, $J = 10.9$ Hz, 1H), 4.85 (dt, $J = 13.4$, 4.5 Hz, 1H), 4.20 (d, $J = 10.6$ Hz, 1H), 3.94 (dd, $J = 15.6$, 10.8 Hz, 1H), 3.66 (s, 1H), 3.30 (dd, $J = 23.2$, 12.7 Hz, 1H), 2.33 (ddd, $J = 13.9$, 12.2, 4.1 Hz, 1H), 2.23–2.14 (m, 1H), 2.07–1.98 (m, 1H), 1.85 (dd, $J = 7.0$, 0.9 Hz).
ACN, acetonitrile; CRI, covalent-reversible inhibitor; DIAD, diisopropyl azodicarboxylate; DIPEA, N,N-diisopropylethylamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EGFR, epidermal growth factor receptor; EtoAc, ethyl acetate; EtOH, ethanol; HOBut, hydroxybutyrate; $k_{inact}$, inactivation constant; MeOH, methanol; $R_T$, retention time; SD, standard deviation; TCEP, tris(2-carboxyethyl)phosphine

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