Kinase Inhibitors

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Lung Cancer: EGFR Inhibitors with Low Nanomolar Activity against a Therapy-Resistant L858R/T790M/C797S Mutant

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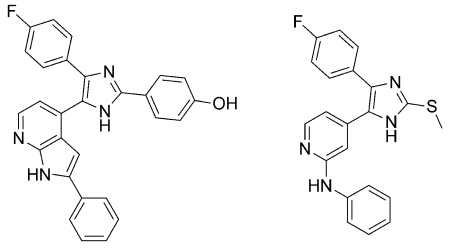
Abstract: *The treatment of non-small-cell lung cancer (NSCLC) with epidermal growth factor receptor (EGFR) inhibitors is made challenging by acquired resistance caused by somatic mutations. Third-generation EGFR inhibitors have been designed to overcome resistance through covalent binding to the Cys 797 residue of the enzyme, and these inhibitors are effective against most clinically relevant EGFR mutants. However, the high dependence of these recent EGFR inhibitors on this particular interaction means that additional mutation of Cys 797 results in poor inhibitory activity, which leads to tumor relapse in initially responding patients. A new generation of irreversible and reversible mutant EGFR inhibitors was developed with strong noncovalent binding properties, and these compounds show high inhibitory activities against the cysteine-mutated L858R/T790M/C797S EGFR.*

Non-small cell lung cancer (NSCLC) is among the most deadly cancer types worldwide.[1] Even after early diagnosis, most patients that receive surgery or platinum-based chemotherapy suffer from the development of drug resistance and tumor progression. As a member of the ERBB family, the receptor tyrosine kinase EGFR has been identified as one of the most promising drug targets for the treatment of NSCLC.[2] However, at least one so-called activating mutation in the tumor genome must be present for significant patient response.[3] The two most frequently observed activating mutations are the single amino acid exchange L858R, caused by a somatic mutation in exon 21, and the delE746-A750 deletion in exon 19.[4] While treatment of NSCLC patients harboring these activating mutations with first-generation EGFR inhibitors generally leads to rapid and massive tumor shrinkage,[5] patients harboring the wild-type (wt) EGFR show little response to small-molecule anti-EGF drugs.[6] The two approved amino quinazoline tyrosine kinase inhibitors (TKIs) Gefitinib and Erlotinib show very potent and selective inhibition profiles with respect to these activating mutations. However, almost all patients that initially show a dramatic response to an anti-EGF therapy develop acquired drug resistance through different mechanisms. In approximately 50% of initially responding patients, a secondary somatic mutation, the gate-keeper mutation T790M, is the underlying cause of drug resistance.[7] The mutation at the gate-keeper Thr 790 residue is a single amino acid exchange in the catalytic domain of EGFR.[8] Third-generation EGFR inhibitors (Rociletinib, Osimertinib, and WZ4002) have aimed to overcome T790M resistance through covalent inhibition of EGFR by binding to a cysteine side chain (Cys 797) through Michael addition with a suitable electrophile attached to the inhibitor molecule.[9] Recently, several studies have revealed the development of resistance to these new third-generation TKIs.[10] A recent study exposed the occurrence of the tertiary point mutation C797S in 40% of the patients treated with AZD9291 (a current mutant-selective FDA-approved third-generation EGFR inhibitor).[11] Loss of the covalent interaction results in a marked decrease in inhibition, and selection pressure then leads to resistance development These new findings suggest that the development of EGFR inhibitors with new structural features as well as unique selectivity profiles for different EGFR mutations is necessary to study and overcome resistance against third-generation EGFR inhibitors.

Based on selectivity screening of the highly potent reversible p38 inhibitor **1**, we identified EGFR inhibition as an off-target effect of this compound (Figure 1).[12] High potency, as well as moderate physicochemical properties and cellular activity against p38, lead us to pick this compound as a first lead structure for further improvements in terms of the inhibition of EGFR mutants. In general pyridinyl imidazoles can potentially inhibit cytochrome P450 (CYP) enzymes, however this toxic side effect is not linked to the entire



	wt EGFR IC_{50} [nM]
1	1000
2	287

Figure 1. Lead structures from screening data. IC_{50} values [nM] in a wt EGFR enzyme assay.

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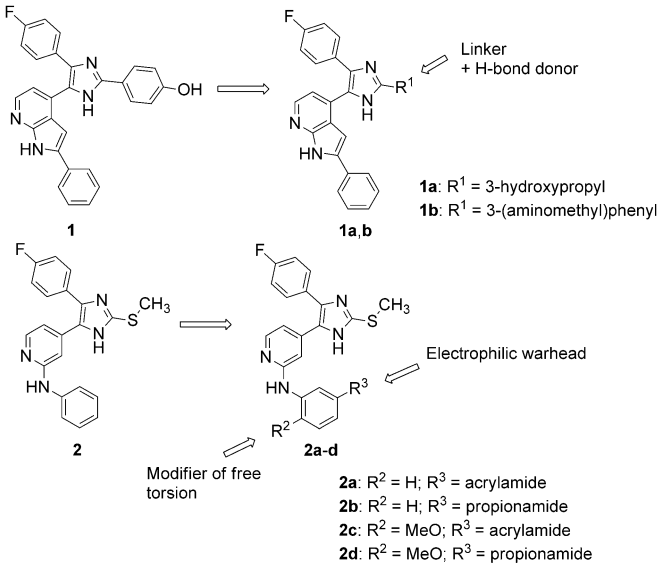
structural class. In the past, we have successfully designed several inhibitors against different kinases (e.g., p38)[13] based on this scaffold with low hepatotoxicity/CYP interaction. We examined approximately 2000 proprietary compounds that were structurally similar or related to **1** in an activity screen against wt EGFR and identified **2** as a promising lead structure for further investigation. In order to minimize the vulnerability of the compounds toward Cys 797 mutations, we applied a two-pronged approach. On the one hand, we designed reversibly binding inhibitors with the ability to overcome T790M resistance. On the other hand, we synthesized covalent irreversible inhibitors with strong reversible binding properties. The irreversible binding was not utilized to overcome T790M resistance, but rather to tune target residence time and activity. Both approaches yielded compounds with high activity against the L858R/T790M/C797S mutant that is resistant to the covalent attack by irreversible third-generation EGFR inhibitors (Scheme 1).



Scheme 1. A rational approach to the design of two compound series derived from **1** and **2**, supported by computer-aided drug design.

Driven by molecular docking of several derivatives of **1** with a T790M EGFR mutant, we identified **1a** and **1b**, which should form beneficial hydrogen bonds to Asn 842 (Figure 2 A), thereby giving higher inhibitory activity. Furthermore, we identified position 3 of the aniline moiety of **2** as an appropriate position to attach a Michael acceptor to target Cys 797 covalently, yielding **2a**. Compound **2c** was decorated with an additional methoxy group in position 6 to provide gentle steric hindrance against the free rotation around the N–C bond, thereby directing the electrophilic warhead toward Cys 797. We synthesized the reversibly binding analogues **2b** and **2d** for comparison. Molecular modelling indicated bidentate hinge binding for both **1a** and **2c** to Met 793 (Figure 2). For **1a**, an additional hydrogen bond to Asn 842 can be observed. In this modelling study, an intramolecular hydrogen bond between the carbonyl O atom of

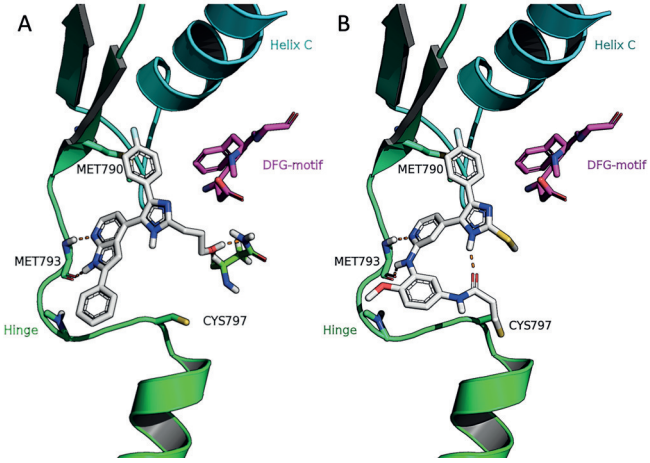


Figure 2. Molecular docking of **1a** (A) and **2c** (B) in a T790M X-ray structure (PDB ID: 2JIU) employing glide-induced fit (A) and glide covalent docking (B) plugins. Proposed H-bonds are indicated as orange dashed lines.

the acrylamide and the imidazole NH can be seen for **2c** (Figure 2 B). This hydrogen bond might lock the inhibitor molecule in a conformation that has a perfect orientation of the Michael acceptor toward Cys 797, thus enabling covalent binding.

We first tested **1a**, **1b**, and **2a–d** in an enzyme assay against EGFR wt, L858R, and L858R/T790M (Table 1). We

Table 1: Kinase inhibitory activities of the new EGFR inhibitors. * indicates IC_{50} values below the resolution limit of the assay.

	IC_{50} [nM] against EGFR		
	wt	L858R	L858R/T790M
1a	<0.5*	<0.5*	6.6±0.1
1b	<0.5*	<0.5*	160±61
2a	<0.5*	<0.5*	<0.5*
2b	7.1±0.8	1.3±0.2	211±2.2
2c	<0.5*	<0.5*	<0.5*
2d	0.65±0.01	<0.5*	34±0.2
Staurosporine	17.1	4.2	<0.5*
WZ4002	1.4±0.02	1.1±0.62	<0.5*
AZD9291	<0.5*	<0.5*	<0.5*

obtained IC_{50} values below the resolution limit of 0.5 nM with **1a** and **1b** with wt and L858R. However, both compounds showed a slight decrease in activity in the double-mutant assay, with IC_{50} values of 6.6 and 160 nM. Nevertheless, **1a** is a highly potent single-digit nanomolar reversible T790M inhibitor. Our second approach to synthesize covalent EGFR inhibitors yielded compounds **2a** and **2c**. Both compounds showed extraordinarily high activities in all three kinase assays. The reversible counterpart **2d**, which is decorated with an additional methoxy group, proved to be a highly active reversible EGFR inhibitor, with IC_{50} values of 0.65 nM, less than 0.5 nM, and 34 nM against wt, L858R and L858R/T790M, respectively. The high activity of **2d** indicates that the covalent interaction is only a minor contributor to the IC_{50} value of **2c**, which is in distinct contrast to reported third-generation irreversible EGFR inhibitors.

Kinetic studies revealed low K_d values in the single-digit nanomolar range. There were no significant differences in the K_d values for **2c** used undiluted (dark gray bars) and 30-fold diluted (light gray bars, Figure 3). By contrast, the reversible

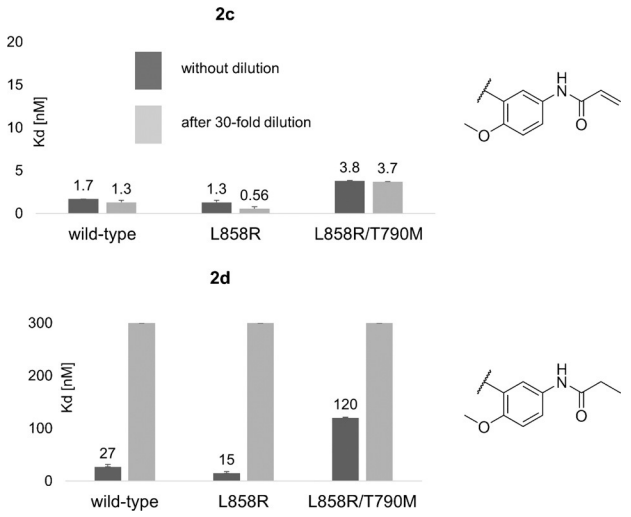


Figure 3. Binding kinetics of **2c** and **2d** (scanKINETIC). K_d values displayed as 300 nM were actually measured to be > 300 nM.

counterpart **2d** showed a tremendous K_d shift after dilution, thus supporting the strong assumption that **2c** is an irreversible inhibitor. Another experiment without dilution but with an equilibration time of 6 hours (see the Supporting Information) showed 3 to 5-fold lower K_d values. This outcome indicates slow association kinetics of these compounds, meaning equilibrium formation is not complete after one hour of incubation.

The two most potent inhibitors, **1a** and **2c**, showed very strong reversible binding to the EGFR active site, so we tested them in an EGFR assay against the triple mutant L858R/T790M/C797S, which plays a crucial role in the development of drug resistance in lung cancer patients who had previously been treated with third-generation inhibitors (Table 2). As expected, the third-generation compound WZ4002 exhibited very low potency against this mutant kinase (> 7 μM), since most of its binding affinity relies on covalent bond formation, which cannot take place without Cys797. Our most promising compounds showed IC_{50} values of 90 and 20 nM, which implies that this new generation of EGFR inhibitors with a very strong reversible binding pattern can tolerate cysteine mutation better than the irreversible third-generation EGFR inhibitors.

We also tested the most promising compounds in a set of tumor-relevant cancer cell lines with different EGFR mutations in a direct comparison to the most recent gold standard treatments (Table 3). Inhibitor **1a** revealed no significant activity in the cell lines. Surprisingly, lower IC_{50} values were observed in the mutant cells, with a slight selectivity over the wt cells. Compound **2c** is a very potent inhibitor of the clinically relevant cell lines H1975 (18 nM) and LXFA PC-9 (5 nM); these values were in the same range or better than the most potent and recently FDA-approved third-generation inhibitor AZD9291. Furthermore, **2c** showed high selectivity (> 700 fold) towards the double-mutant cell line LXFA NCl-H1975 over the wt cell line LXFA 526. A kinome scan of **2c**, which included 410 kinases, demonstrated a good selectivity profile with a selectivity score of 0.039 (Table 4). Additionally, this tyrosine kinase inhibitor was a very potent inhibitor of all clinically relevant EGFR mutants. An interesting off-target hit was the tyrosine kinase BRK (2% residual activity), which has been identified in different cancer types, including NSCLC.[14]

Table 3: Inhibitory activities in different NSCLC cell proliferation assays.

	IC_{50} [μM] in different NSCLC cell lines		
	LXFA 526 wt	LXFA PC-9 Exon 19 del	LXFA NCl-H1975 L858R/T790M
1a	3.568	1.050	2.488
2c	14.034	0.005	0.018
Gefitinib	12.838	0.037	12.672
WZ4002	5.847	0.138	0.056
AZD9291	2.976	0.019	0.011

Compounds **1a** and **1b** were synthesized by using the optimized Stille cross-coupling reported by Selig et al. (Figure 4).[12] The imidazoles **3a** and **3b** were obtained through Radziszewski imidazole synthesis. The nitrile of **3b** was reduced to an amine followed by triple BOC protection to give **4b**. These protected imidazoles were stannylated using *n*BuLi and $Sn(Bu)_3Cl$. Stille cross-coupling with the previosly synthesized 4-chloro-1-(methoxymethyl)-2-phenyl-1*H*-pyrrolo[2,3-b]pyridine using a 1:3 mixture of $Pd(OAc)_2$/X-

Table 2: Kinase inhibitory activities (single point) of the most potent new compounds in a triple-mutant EGFR enzyme assay.

	IC_{50} [nM] against EGFR L858R/T790M/C797S
1a	21
1b	336
2c	90
2d	167
Staurosporine	1
WZ4002	7294

Table 4: Inhibitory activities of **2c** in mutant EGFR enzyme assays

	Residual activity in % with **2c** [200 nM]
EGF-R d746-750	7
EGF-R d747-749/A750P	7
EGF-R d747-752/P753S	5
EGF-R d752-759	9
EGF-R G719C	3
EGF-R G719S	7
EGF-R L861Q	4
EGF-R T790M	5

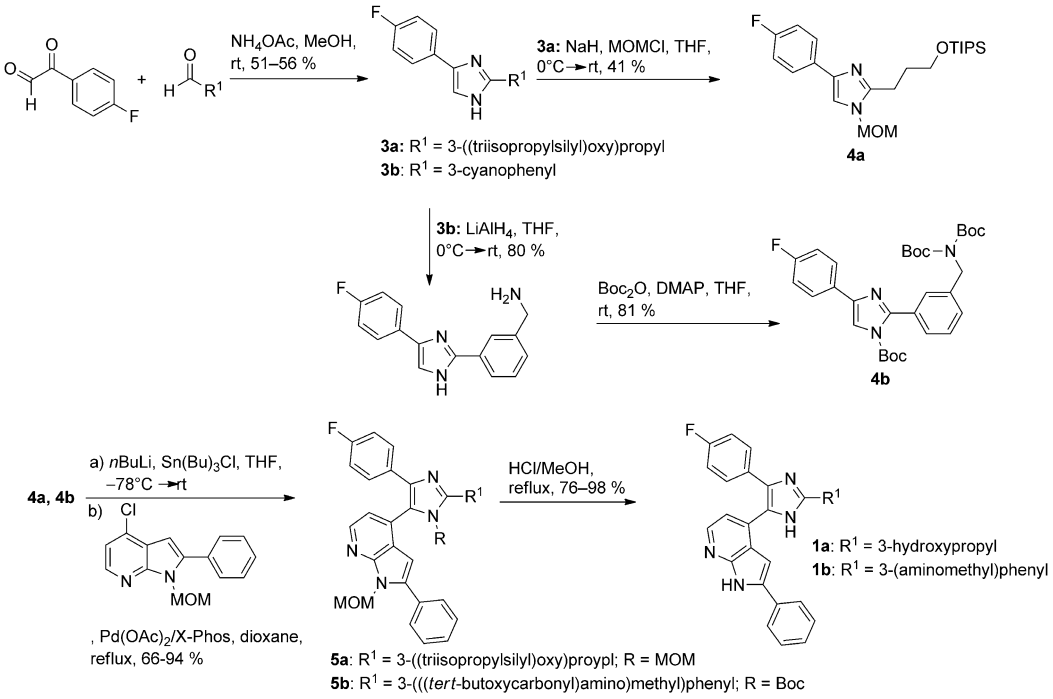


Figure 4. Synthesis of compounds **1a** and **1b**. Boc = *tert*-butoxycarbonyl, DMAP = 4-(dimethylamino)pyridine, MOM = methoxymethyl, TIPS = triisopropylsilyl.

Phos in refluxing dioxane resulted in compounds **5a** and **5b**. Deprotection of the coupled products in HCl/MeOH under reflux led to the desired compounds **1a** and **1b**.

We synthesized compounds **2a–d** by utilizing a modular route, which allowed derivatization in the last step before deprotection (Figure 5). The commercially available 4-fluoroacetophenone was alpha brominated using bromine in CH_2Cl_2, and the resulting α-bromoketone **6** was cyclized with *S*-methylthiopseudourea to yield imidazole **7** in moderate yields. Subsequent SEM protection of the deprotonated imidazoles initially yielded a 60:40 mixture of regioisomers. The regioisomeric mixture was converted into the sterically favored isomer **8** through heating with a catalytic amount of SEM-Cl in acetonitrile to give pure **8** in excellent yields.[15] High yielding electrophilic bromination of the imidazole core was performed under very controlled conditions using *N*-bromosuccinimide in acetonitrile. To minimize proto-deboronation, we used optimized reaction conditions for the Suzuki cross-coupling of **9** and applied portion-wise addition of the boronic acid ester. We achieved excellent yields of **10** with this procedure. Deacetylation of **10** was performed under basic conditions and elevated temperatures. We recently optimized a Buchwald amination for the synthesis of a series of dual JNK3/p38 inhibitors[16] and were able to use this procedure to yield **12a–d** in good to excellent yields using predecorated bromoani-

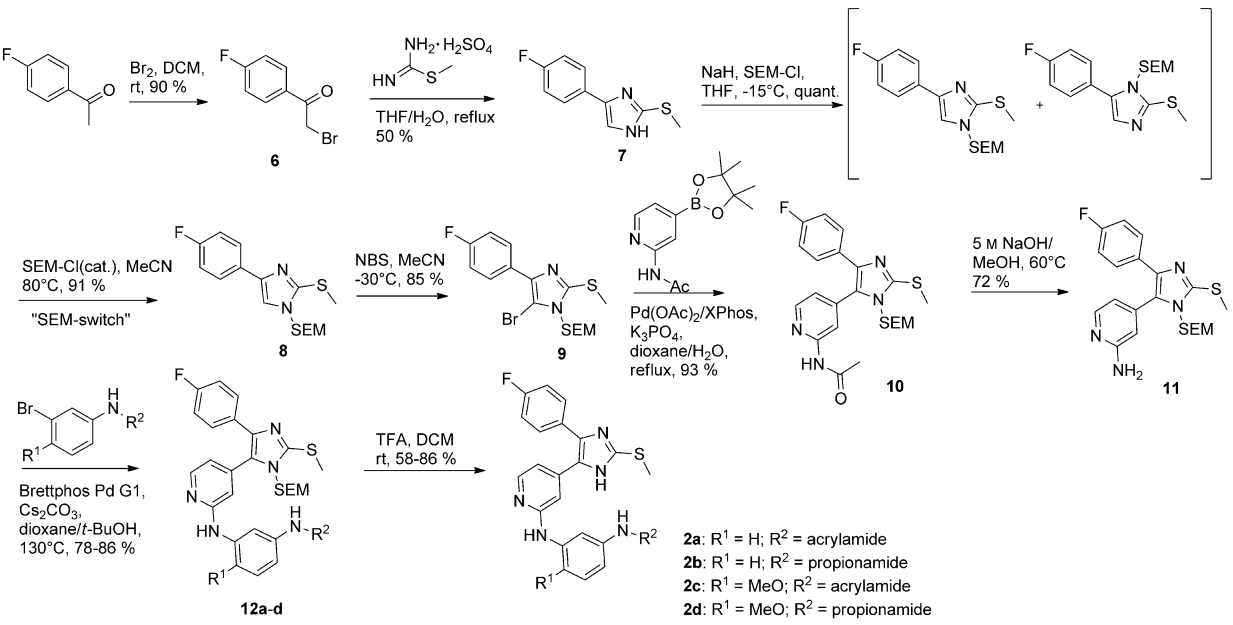


Figure 5. Synthesis of compounds **2a–2d**. DCM = Dichloromethane, NBS = *N*-Bromosuccinimide, SEM = 2-(trimethylsilyl)ethoxymethyl, TFA = Trifluoroacetic acid.

lines for the cross-coupling. SEM removal was performed under mild conditions using TFA in CH_2Cl_2 at room temperature to yield the final compounds **2a–d**.

In summary, we have successfully developed a highly potent, reversible EGFR L858R/T790M inhibitor with potency in the single-digit nM range that is not reliant on a covalent bond formation for strong binding to the active site. Furthermore, this compound is a strong inhibitor of the recently discovered triple mutant that occurs in patients after treatment with third-generation inhibitors like Osimertinib. Furthermore, we developed an irreversible L858R/T790M EGFR inhibitor with potency in the pM range that is equipotent and more selective for wild type EGFR inhibition in tumor cell lines compared to Osimertinib. Moreover, this compound also displays a strong reversible binding component and is able to inhibit the EGFR triple mutant in the low nM range. We have shown that covalent binding can be a useful tool to tune the potency and target-residence time, although this interaction is not crucial for compound potency in general. The attractive properties of these compounds should allow them to serve as lead compounds for the further development of highly potent L858R/T790M/C797S inhibitors.

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