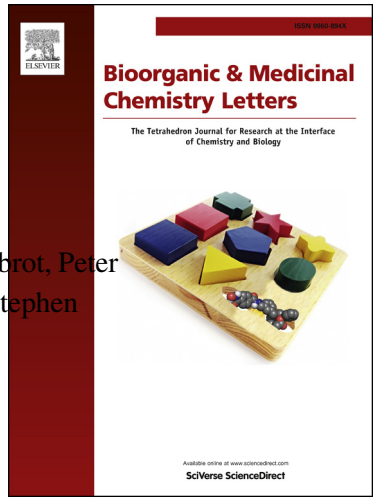

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4-Aminoindazolyl-dihydrofuro[3,4-*d*]pyrimidines as non-covalent inhibitors of mutant epidermal growth factor receptor tyrosine kinase

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ABSTRACT

The treatment of epidermal growth factor receptor (EGFR)-driven non-small cell lung cancers with the T790M resistance mutation remains a significant unmet medical need. We report the identification of 4-aminoindazolyl-dihydrofuro[3,4-*d*]pyrimidines as non-covalent inhibitors of EGFR, with excellent activity against the T790M resistance double mutants and initial single activating mutants. Using an optimization strategy focused on structure-based design and improving PK properties through metabolite identification, we obtained advanced leads with high oral exposure.

Non-small cell lung cancers (NSCLC) bearing activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) are sensitive to treatment with the tyrosine kinase inhibitors (TKIs) erlotinib, gefitinib, and afatinib.[1-5] These activating mutations, most commonly the point mutation L858R or deletions within exon 19 (e.g. residues 746-750), increase EGFR-driven cell proliferation and survival.[6-8] Unfortunately, successful treatment of EGFR-mutated NSCLC with first or second generation TKIs[9] is followed by an acquired resistance, with approximately 60% of that resistance arising from a secondary mutation within the EGFR kinase domain, substitution of the gatekeeper residue Thr790 with methionine (T790M).[10-15] Advances in third-generation EGFR inhibitors have demonstrated the ability to overcome this T790M-mediated resistance. Third-generation inhibitors AZD9291 and CO1686 both form a covalent bond with Cys797 in the active site and have activity against the resistance mutations (T790M/L858R, TMLR; T790M/del(746-750), TMdel) as well as the two initial activating mutations.[16-21] However, recent reports of the C797S mutation emerging in the clinic conferring resistance to AZD9291 support the continued need for a T790M inhibitor that does not depend on a covalent interaction with Cys797.[22, 23] We report here the identification of 4-aminoindazolyl-dihydrofuro[3,4-*d*]pyrimidines as non-covalent inhibitors of the T790M containing EGFR mutants as well as the initial single activating mutants.

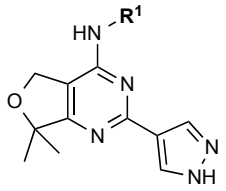
A high-throughput screen of our internal library identified 4-aminoindazolyl-dihydrofuro[3,4-*d*]pyrimidine **1** as a potent TMLR inhibitor with a K_i of 2 nM (Table 1) and moderate activity in a cell-based assay[24] (H1975 pEGFR IC_{50} = 0.60 μM). While possessing excellent biochemical activity for an initial hit, several parameters required optimization. Compound **1** is moderately stable in human liver microsomes (HLMs) and human hepatocytes (HHeps) and is stable in rat liver microsomes and hepatocytes (RLMs and RHeps). However, it is rapidly cleared in rats in vivo (Cl = 86 mL/min/kg) and exhibits low oral bioavailability (F = 13%, Table 2). The high clearance of **1** raised concerns of underprediction of clearance by in vitro metabolic stability data for this chemical series. Permeability was moderate as measured in Madin-Darby canine kidney (MDCK) cells, with minimal active efflux. We hypothesized that this low oral exposure was a result of pre-systemic metabolism. Therefore, our optimization program focused on identification of inhibitors with retained potency and improved oral PK properties.

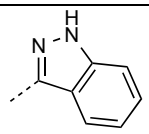
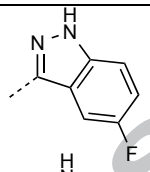
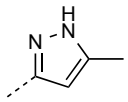
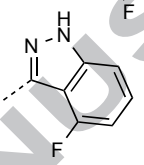
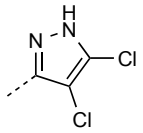
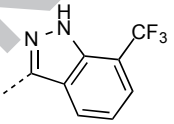
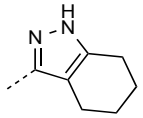
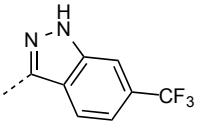
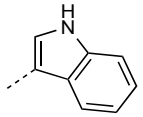
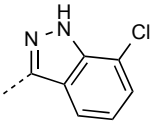
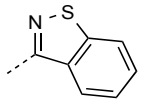
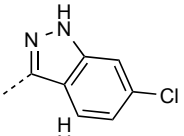
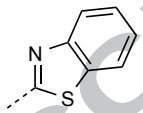
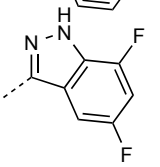
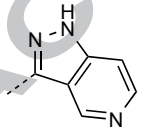
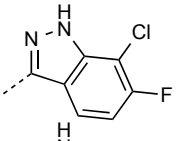
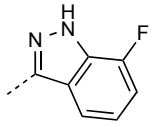
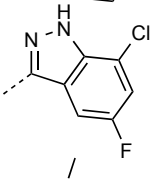
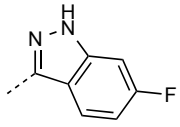
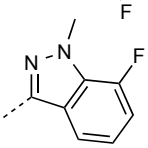
Compounds described in Tables 1-4 were prepared as shown in Scheme 1.[25] Non-commercial 3-aminoindazoles were obtained through reaction of ortho-fluorobenzonitriles with hydrazine. SnAr reaction of these aminoindazoles with 2,4-dichloro-7,7-dimethyl-5,7-dihydrofuro[3,4-d]pyrimidine[26] afforded the penultimate intermediates. Suzuki coupling with aryl boronates provided the final test compounds **1**-**28**.

Metabolite identification studies carried out using plasma samples from rats dosed intravenously with **1** indicated that oxidation of the indazole ring was a primary circulating metabolite. We were also aware of the high potential of indazole systems to be subject to N-glucuronidation. We therefore set out

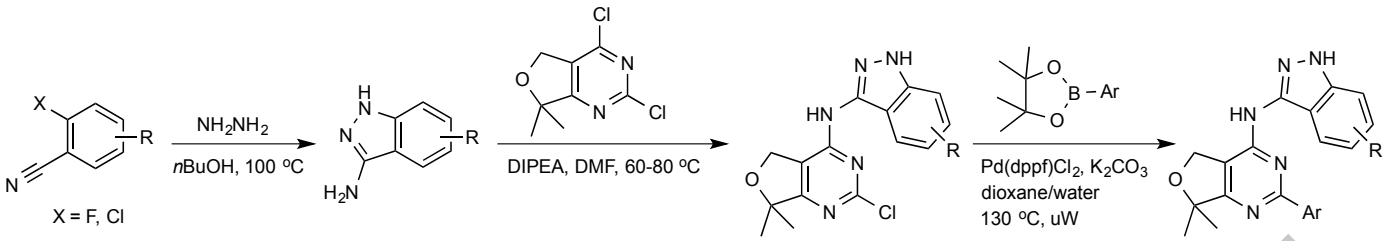
to identify indazole ring modifications or replacements that might mitigate these metabolic liabilities with compounds described in Table 1. In light of the underprediction of in vivo clearance by in vitro systems for **1**, we considered low turnover in LMs and Heps as a minimum criterion for achieving low to moderate in vivo clearance.

Table 1. Indazole modification



Cmpd	R^1	EGFR (TMLR) Ki (nM)	HLM / RLM Cl_{hep} (mL/min/kg)[a]	Cmpd	R^1	EGFR (TMLR) Ki (nM)	HLM/RLM Cl_{hep} (mL/min/kg)[a]
1		2	7 / 14	**11**		8	7 / 17
2		844	4 / 21	**12**		17	6 / 17
3		389	4 / 19	**13**		134	5 / 24
4		>2000	8 / 16	**14**		>2000	7 / 22
5		17	12 / 34	**15**		2	5 / 23
6		747	18 / 36	**16**		16	13 / 20
7		>2000	4 / 8	**17**		5	9 / 21
8		>2000	12 / 14	**18**		4	7 / 16
9		3	7 / 19	**19**		16	7 / 22
10		2	11 / 21	**20**		57	11 / 23

[a]Human and rat liver microsome predicted hepatic clearance.[27]



Scheme 1. Synthesis of 4-aminoindazolyl-dihydrofuro[3,4-*d*]pyrimidines

Table 2. in vitro properties and PK parameters

Cmpd	HHep / RHep Cl_{hep} (mL/min/kg)[a]	MDCK AB Papp (x10^{-6} cm/sec)[b]	MDCK BA/AB efflux ratio	Rat plasma Cl (mL/min/kg)	Rat Vss (L/kg)	Rat PO[b] %F	Rat PO[d] AUC_{total} (uM*hr)	Rat PO[d] AUC_{free} (uM*hr)
1	11 / 9	6.7	2.4	86[e]	6.0	13%	0.37	0.05
11	12 / 13			81[f]	6.8			
13	11 / 16	8.5	1.3	27[f]	2.4	5%	0.36	0.03
14	9 / 14	10.3	1.5	91[f]	1.2			
15	14 / 18	9.5	1.6	74[f]	3.6			
18	7 / 16	7.3	1.1	60[f]	5.5			
20	15 / 18			60[f]	1.5			
27	15 / 17	12.9	0.9	38[f]	2.3	117%	7.5	0.23
28	17 / 18	8.4	0.7	36[f]	3.3	62%	3.4	0.09

[a]Human and rat hepatocyte predicted hepatic clearance.[27]
[b]Apparent permeability (Papp) from the apical (A) to basolateral (B) direction in MDCK cells.
[c]ratio of Papp BA to Papp AB in MDCK cells.
[d]PO dosed at 5.0 mg/kg as a suspension in MCT.
[e]IV dosed as a solution in 10/40/50 ethanol/PEG400/citrate pH 5 buffer
[f]IV dosed as a solution in 50/35/15 Cremophor EL/NMP/Miglyol®

We determined that the truncated pyrazole **2** lost most of the activity of the indazole, and could not be recovered through the use of dichloro-substitution as a phenyl isostere (compound **3**). Saturation of the phenyl ring, which would be desirable to reduce planarity and improve overall physiochemical properties was also not tolerated (compound **4**). Indole **5** was slightly less active than the indazole (17 nM vs. 2 nM), however benzoisothiazole **6** lost nearly 400-fold activity. These data suggest that both hydrogen bond donor and acceptor of the indazole are important for binding, with a more significant contribution from the hydrogen-bond donor. Benzothiazole **7** improved HLM and RLM stability, but was completely inactive.

We attempted to modulate metabolism of the indazole ring through the introduction of an additional heteroatom to reduce electron density, however pyrazolopyrimidine **8** lost all activity. Substitution of the indazole ring with electron-withdrawing substituents was more fruitful; compounds **9**, **10**, **11**, and **12** showed that fluoro substitution was tolerated with minimal loss of activity, with substitution at the 6- or 7- position affording compounds equipotent with starting point **1**.

Unfortunately, mono-fluoro substitution at any position failed to provide an improvement in HLM or RLM stability. Larger groups such as chloro or trifluoromethyl were moderately tolerated only at the 7-position (**13** and **15**), with larger drops in potency observed for substitution at the 6-position (**14** and **16**). No improvement in HLM or RLM stability was observed with either chloro or trifluoromethyl substitution. Di-substitution with electron withdrawing groups was evaluated (compounds **17**-**19**) and while activity was retained, clearance predicted by HLM and RLM remained in the moderate to high range.

Several compounds were profiled in vivo in rat PK studies to determine if their in vitro profiles correlated with in vivo findings (Table 2). Compounds with single fluoro or chloro substitution (**11** and **15**) were observed to have high clearance similar to the starting point **1** and above expected clearance based on liver microsome and hepatocyte incubations. Di-substituted compound **18** resulted in only a slight reduction in clearance (Cl = 60 mL/min/kg). While 7-trifluoromethyl substitution (**13**) afforded a reduction in plasma clearance to 27 mL/min/kg, unfortunately, oral bioavailability remained low (F = 5%). Conversely, 6-trifluoromethyl substitution (**14**) resulted in high clearance.

Metabolite ID studies were carried out in rat and human hepatocytes for compounds **11**, **15** and **13**. Phase I and phase II metabolites were observed for all compounds, with glucuronidation as a major metabolite in both rat and human hepatocytes (site of glucuronidation not determined). Oxidative metabolism occurred largely on the dihydrofuryl moiety, with oxidation on the indazole ring to only a minor degree, indicating that the strategy of introducing electron withdrawing substituents had been successful in reducing oxidative metabolism on the indazole ring. While the 7-trifluoromethyl substitution was not sufficient to entirely block glucuronidation, the decreased in vivo clearance of **13** relative to 6-trifluoromethyl **14** suggests that the steric hindrance of the 7-trifluoromethyl group may be able to reduce the rate of indazole N-glucuronidation. To avoid indazole N-glucuronidation altogether, the indazole N-methylated compound was prepared (**20**), but found to have a 19-fold reduction in activity, and remained only moderately stable in both rat and human LM and Heps, with high clearance in vivo (Cl = 60 mL/min/kg) (Tables 1 and 2). The potential decrease in glucuronidation may be counteracted by the introduction of a metabolic soft-spot (potential for oxidative N-demethylation). In general, all compounds tested displayed higher in vivo clearance than predicted by in vitro microsomal or hepatocyte systems; however, good passive permeability was maintained.

To guide further optimization of this series, we sought an X-ray structure with TMLR, resulting in the structure with **15** shown in Figure 1. The pyrazole ring functions as the hinge binding element, adopting a position consistent with hydrogen-bonds with main chain atoms of Gln791 (C=O) and Met793 (NH). The dihydrofuropyrimidine core is situated in the center of the active site, with the gem-dimethyl groups occupying the

lipophilic ribose pocket and lipophilic contacts between the pyrimidine ring and the side chain of Val726. Intriguingly, the methionine gatekeeper is not in the extended conformation observed for other inhibitors as previously published (see supplemental Figure S1).[28] Instead, the Met790 sidechain is folded down to form a three-dimensional lipophilic pocket that is filled by the indazole, with the aromatic ring sandwiched between the sidechains of Met790 and Lys745. This is reminiscent of the binding site observed for erlotinib in wild-type EGFR, where the shorter Thr790 side chain forms the boundary of a similar pocket (see supplemental Figure S2).[29] Finally, the indazole N1 nitrogen is positioned consistent with a hydrogen-bonding interaction with Glu762. While the indazole N2 nitrogen does not make any direct interactions with the protein, it resides in a polar environment and there is the possibility of water-mediated interactions not visible at this resolution (2.8 Å). In light of this binding mode and the tight lipophilic pocket occupied by the indazole phenyl ring, the limited tolerance observed for substitution or addition of polar heteroatoms on the indazole ring is easily rationalized.

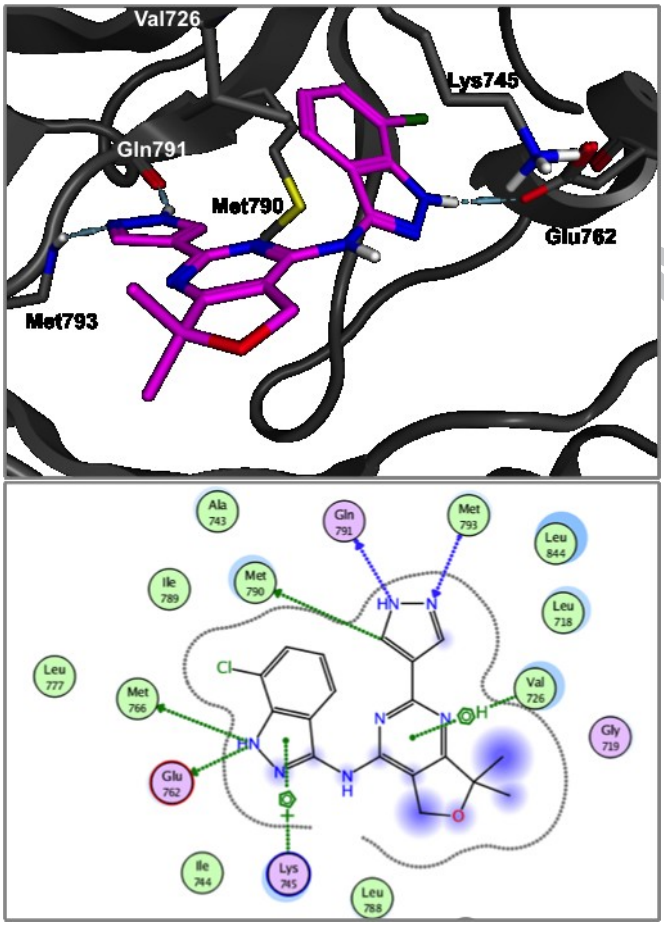
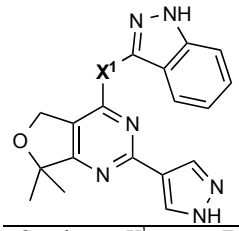


Figure 1. a) X-ray structure of **15** complexed with TMLR; b) ligand-protein interaction map. PDB accession 5EDQ.

Given that the NH linker of **15** does not appear to be making any interactions, we attempted to remove that possible site where phase II conjugation could occur. Conformational analysis in Macromodel v9.9 (Schrödinger, LLC, New York, NY) suggested that there was not a replacement for the –NH– linkage that gave the same conformation between rings, although several provided angles within 20-30° of the crystal conformation and 0.5-2.3 kcal/mol of their global minimum conformations (see Supplemental Table S1). As shown in Table 3, N-methylation or replacement with an ether or ketone linkage (compounds **21**-**23**) all resulted in a substantial reduction in potency, consistent with the calculations, and with no concomitant improvement in LM or Hep stability.

Table 3. Linker modification



Cmpd	X^1	EGFR (TMLR) Ki (nM)	HLM/RLM Cl_{hep} (mL/min/kg)[a]	HHep/RHep Cl_{hep} (mL/min/kg)[b]
21	NCH_3	519	16 / 26	
22	O	85	10 / 32	16 / 33
23	C=O	104	13 / 36	

[a]Human and rat liver microsome predicted hepatic clearance.[27]
[b]Human and rat hepatocyte predicted hepatic clearance.[27]

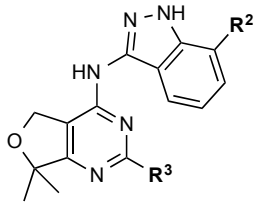
We therefore turned our attention to the pyrazole hinge binder. Triazole **24** lost nearly all TMLR activity; despite providing the required complement of hydrogen-bonding partners, lone-pair repulsion between the triazole and pyrimidine nitrogens may prevent the desired co-planar conformation. Thiazole **25** is well tolerated (TMLR Ki = 5.6 nM), suggesting that an aromatic CH hydrogen bond donor is sufficient to retain activity.[30] Pyrazole regioisomer **26** also offers a CH hydrogen bond donor but is much less active at 175 nM, possibly due to the low-energy tautomer placing the hydrogen bond acceptor at the wrong location. However, this pyrazole regioisomer exhibits significantly improved stability in both HLMs and RLMs. N-methylated pyrazole **27** enforces the desired tautomer and regains 5-fold activity. An X-ray structure of **27** with TMLR confirms the expected binding mode and pyrazole positioning consistent with an aromatic CH hydrogen bond with Gln791 (C=O) (See supplemental Figure S3).

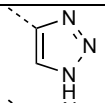
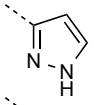
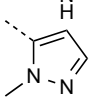
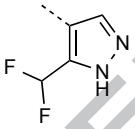
Compound **27** has reduced HLM stability relative to **26**, but retains the good stability in RLMs seen for the des-methyl pyrazole. When tested in vivo in rats, **27** was found to have moderate clearance (Cl = 38 mL/min/kg) and excellent oral bioavailability (F ~ 100%) with an oral AUC 20-fold higher than that of compound **1** (unbound AUC 5-fold higher than compound **1**, Table 2). These results implicate glucuronidation of the pyrazole hinge-binder as a likely driver of the low oral bioavailability observed for compounds **1** and **13**. While **27** has an appealing in vivo exposure profile, the 34 nM Ki translated into only marginal activity in a cell-based assay (H1975 pEGFR IC_{50} = 3 μM).

A combination of difluoromethyl pyrazole ring substitution for electronic and steric deactivation together with an electron-withdrawing substituent on the indazole ring afforded compound **28**. This compound exhibits biochemical activity similar to the starting point **1**. The indazole and pyrazole substitution are expected to have an impact on both oxidative and conjugative metabolism; while **28** has only moderate stability in human and rat microsomes in vitro, gratifyingly, when tested in vivo **28** exhibits significantly reduced clearance (Cl = 36 mL/min/kg), high oral bioavailability (F = 62%) and satisfactory oral AUC. Metabolite identification experiments conducted on rat urine and bile samples from animals dosed intravenously with **28** confirmed that oxidative and glucuronide metabolites are predominant excretion products, with no **28** excreted unchanged.

This observation confirms the primary role of metabolism as the main elimination pathway, as opposed to active transport or direct excretion of the parent molecule. Interestingly, the increased bioavailability for **28** relative to **13** is not predicted based on similar in vitro metabolic stability or MDCK permeability for these two compounds (Table 2). This may be due to contribution of non-hepatic metabolism (e.g., glucuronidation in the gut) or a difference in absorption due to dissolution or solubility. While the two compounds have similar kinetic solubility (3-5 μM), thermodynamic solubility was not measured. Compound **28** also exhibits moderate activity in a cell-based assay (H1975 pEGFR IC_{50} = 0.37 μM)[24], slightly improved relative to the starting point **1**, thereby achieving our initial goal of an improved pK profile without sacrificing activity.

Table 4. Hinge binder modification



Cmpd	R^2	R^3	EGFR (TMLR) Ki (nM)	HLM/RLM Cl_{hep} (mL/min/kg)[a]
24	H		732	9 / 7
25	H		5.6	13 / 24
26	H		175	3 / 9
27	H		34.3	10 / 9
28	Cl		9.0	10 / 22

[a]Human and rat liver microsome predicted hepatic clearance.[27]

Several key compounds from this chemical series were tested against all relevant EGFR mutants and the wild-type protein (wtEGFR) as shown in Table 5. All compounds tested displayed an intriguing pan-inhibitor profile, strongly inhibiting both single activating mutations and the T790M double mutants. The biochemical activity of compounds in Table 5 compares favorably to other pan-mutant inhibitors currently in clinical trials, including AZD9291, suggesting the potential of this series of inhibitors to provide a clinical benefit without a requisite covalent interaction with Cys797. The ability of these compounds to inhibit both Thr790- and Met790-containing proteins may be rationalized by their binding mode. As shown in Figures 1 and S2, the indazole ring makes favorable lipophilic contacts with the Met790 sidechain that would not be available with the Thr790 sidechain. Similar to structures with different classes of inhibitors (e.g. Supplemental Figure S1), an apo TMLR structure places the Met790 sidechain in an extended conformation incompatible with inhibitor binding (see Supplemental Figure S4). The possible energy penalty associated with Met790 sidechain folding to accommodate the inhibitors may explain the similar Ki values across both Thr790 and Met790 proteins.

While these compounds were generally equipotent against the wild-type enzyme and the activating mutants in a biochemical assay, the activating mutants' decreased affinity for ATP is expected to afford selectivity in a cell based setting, similar to that observed for erlotinib.[31,32] However, the T790M resistance mutation restores ATP affinity to a similar level as wtEGFR, and increased biochemical selectivity for TMLR and TMdel relative to wtEGFR would be desirable to prevent wtEGFR-related toxicities.[33] Biochemical selectivity against a larger panel of kinases was also determined for several Table 5 compounds. Compound **1** displayed good selectivity, inhibiting only 14 out of 296 kinases at > 80% when tested at 100 nM, approximately 100x the EGFR Ki. Tested at a higher concentration of 1.0 μM against an abbreviated panel, compound **28** inhibited 4 out of 16 kinases at > 80%. See Supplementary Tables S3-4 for kinase panel data.

Table 5. Biochemical activity against wild-type EGFR and EGFR mutants

Cmpd	wt-EGFR Ki (nM)	EGFR (L858R) Ki (nM)	EGFR (del746-750) Ki (nM)	EGFR (TMLR) Ki (nM)	EGFR (TMdel) Ki (nM)
1	0.6	0.5	0.2	2.0	1.6
15	1.5	1.3	0.4	2.1	1.3
27	1.1	< 0.5	< 0.5	34.3	21.3
28	6.5	3.4	1.7	9.0	2.5
erlotinib	0.1	0.3	0.1	123	95
AZD-9291	3.5			3.2	2.2

In conclusion, beginning with a potent high-throughput screening hit, we identified a novel series of non-covalent 4-aminoindazolyl-dihydrofuro[3,4-*d*]pyrimidines as inhibitors of EGFR. An optimization strategy driven by metabolite identification, PK, and structure-based design provided advanced compounds with improved oral PK properties. Their excellent activity against the resistance T790M double mutants as well as the single activating mutants renders these molecules promising leads for the treatment of EGFR-mutant NSCLC.

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Supplementary Material

Supplementary material associated with this article may be found, in the online version, at XXX. PDB accession codes are as follows: **15**/TMLR, 5EDQ; **27**/TMLR, 5EDR; apo-TMLR, 5EDP.

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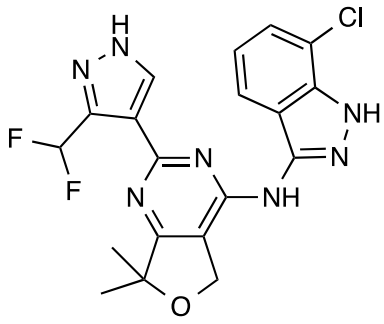
Graphical abstract



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EGFR(del746-750)
EGFR(T790M/L85
EGFR(T790M/del7