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Drug Design

Pyrimido[4,5-*d*]pyrimidin-4(1*H*)-one Derivatives as Selective Inhibitors of EGFR Threonine^{790} to Methionine^{790} (T790M) Mutants**

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The epidermal growth factor receptor (EGFR, erbB1, HER1) has been well-validated as a molecular target in anticancer drug discovery. In non-small-cell lung cancer patients (NSCLCs) harboring active mutations in the EGFR tyrosine kinase domain (L858R and del E746-A750),[1–4] the first generation inhibitors, gefinitib and erlotinib, have achieved significant clinical benefits but emerging acquired resistance to them has become a major clinical challenge. The "gatekeeper" T790M mutation (threonine^{790}→methionine^{790}) of EGFR, by which the binding of ATP with the kinase is favored,[5] is one of the primary mechanisms for resistance and plays a role in the circa 50% of NSCLC patients who acquired clinical resistance.[6,7] Although the Cys797-chelating irreversible EGFR inhibitors displayed promising potential to overcome EGFR^{T790M} related resistance in animal models, their non-selective inhibition against wild-type EGFR (EGFR^{WT}) and/or other kinases results in a relatively low maximal-tolerated-dose (MTD) and poor clinical outcomes in human patients.[8–11] Inhibitors selectively targeting EGFR^{T790M} mutants are an attractive strategy for the clinical management of NSCLC patients with acquired resistance.

However, because EGFR^{WT} and the EGFR^{T790M} mutants share highly similar three-dimensional structures and have almost identical binding affinities with ATP,[5] nearly all of the reported irreversible EGFR inhibitors displayed equal potencies against the T790M mutants and the wild-type enzyme, highlighting the challenge in the search for EGFR^{T790M} mutant-selective inhibitors. Only recently, WZ4002 was reported as a new irreversible EGFR inhibitor displaying moderate selectivity on EGFR^{T790M} mutants over the wild-type kinase.[12] A phase I clinical trial was recently initiated on another moderately mutant-selective EGFR inhibitor CO-1686 (K_d(EGFR^{WT})/K_d(EGFR$^{L858R/T790M}$) = 25, NCT01526928) whose chemical structure was not disclosed,[13] and PKC412 was reported as a novel reversible T790M mutant-selective EGFR inhibitor with promising in vivo efficacy.[14] Herein, we wish to report the successful discovery of novel pyrimido[4,5-*d*]pyrimidin-4(1*H*)-one-based EGFR^{T790M} inhibitors with more than 100-fold selectivity over the wild-type kinase.

We have successfully designed compounds **1** and **2** (Scheme 1) as novel EGFR^{T790M} inhibitors with low nanomolar IC_{50} and K_d values. However, these compounds only displayed four-fold selectivity on EGFR^{T790M} mutants over EGFR^{WT}.[15a,b] The use of conformational constraint is a general strategy with which to improve ligand selectivity for a molecular target, and accordingly a series of pyrimido[4,5-*d*]pyrimidin-4(1*H*)-one derivatives **3a**–**3h** with more rigid conformations based on the structure-activity relationship (SAR) studies of compounds **1** and **2** (Scheme 1)[15a,b] were designed. The compounds were readily synthesized by using the similar procedures to that of **3a** (Scheme 2; Supporting Information, Scheme S1). Briefly, a direct nucleophilic coupling of commercially available ethyl 2,4-dichloropyrimidine-5-carboxylate (**4**) with *tert*-butyl-3-aminophenylcarbamate (**5**) produced ethyl 4-[{3-[(*tert*-butoxycarbonyl)amino]phenyl}amino]-2-chloropyrimidine-5-carboxylate (**6**).[16] Hydrolysis of compound **6** with 1M NaOH in a H_2O/THF mixed solution yielded the carboxylic acid (**7**). The condensation of **7** and **8a** in the presence of HATU and DIPEA in dry CH_2Cl_2 gave the intermediate **9a**. Compound **9a** was coupled with aniline by nucleophilic substitution followed by deprotection with 50% trifluoroacetic acid in CH_2Cl_2 to yield the key precursor **10a**. The new inhibitor **3a** was finally obtained by acryloylation of **10a** with acryloyl chloride.

The binding affinities of the compounds with EGFR^{WT} and its T790M mutants were determined with an active-site-dependent competition binding assay conducted by Ambit Bioscience, San Diego, USA. The kinase inhibitory activities of the compounds were also evaluated by the well-established

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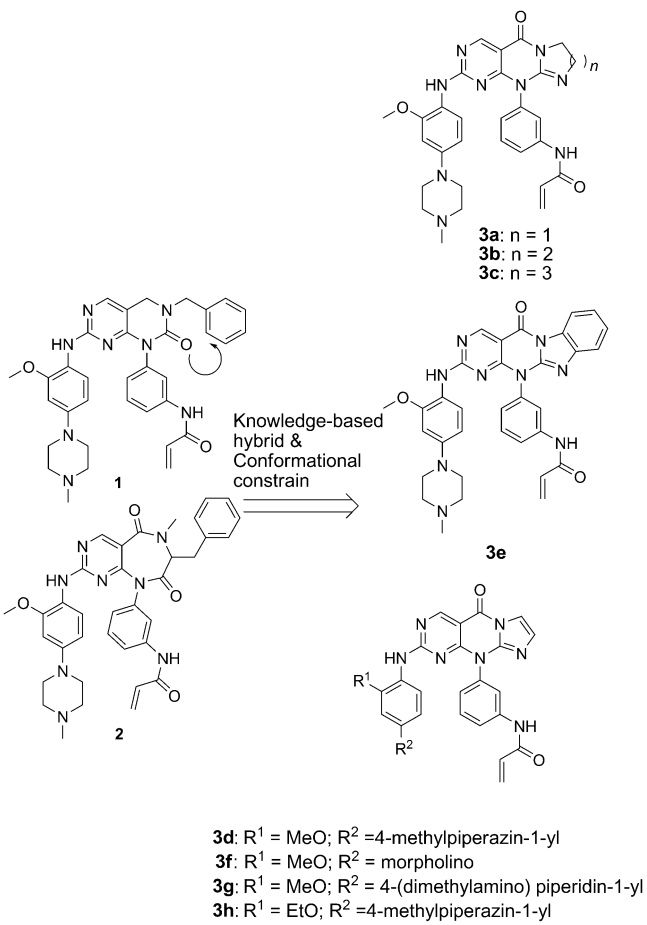
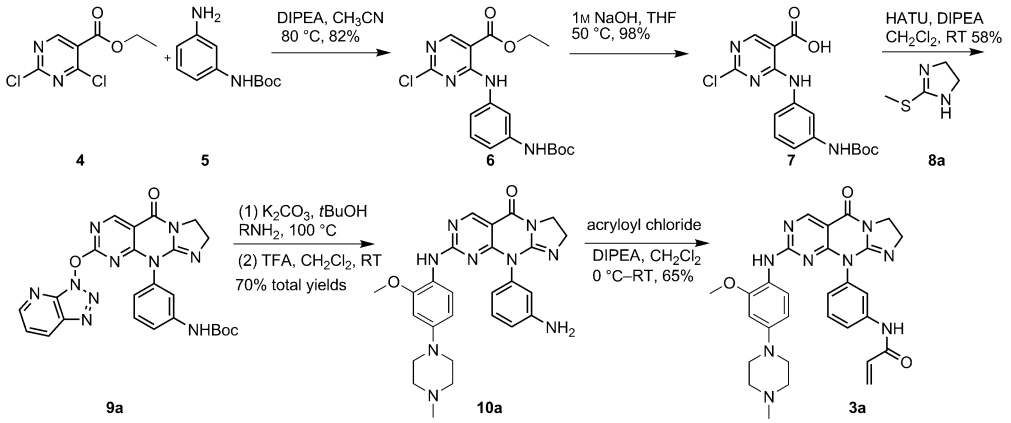


Scheme 1. Knowledge-based design of pyrimido[4,5-*d*]pyrimidin-4(1*H*)-one derivatives **3a**–**3h** as new EGFR^{T790M} inhibitors.

FRET-based Z′-Lyte assay (Table 1).[15a,b] All four of the rigid compounds (**3a**–**3d**) bound effectively to EGFR^{T790M} and EGFR $^{L858R/T790M}$ with K_d values in the low-nanomolar range. The compounds also potently inhibited the kinase functions with IC_{50} values between 1.52 nM and 13.87 nM. Although the conformational rigidity of the compounds apparently had no effect on the binding affinities and kinase inhibitory activities of the compounds on EGFR^{T790M} and EGFR$^{L858R/T790M}$ mutants, it dramatically impacted their effects on wild-type EGFR. For instance, compounds **3a**–**3d** bound to EGFR $^{L858R/T790M}$ with K_d values of 3.6, 1.1, 0.88, and 1.8 nM, respectively, and they also inhibited the enzymatic function of EGFR$^{L858R/T790M}$ with similar IC_{50} values (7.03, 2.09, 1.69, and 1.52 nM, respectively). However, their binding affinities with EGFR^{WT} were 210, 72, 17 and 140 nM, respectively; that is, 58.3, 65.5, 19.3, and 77.8-fold greater than the K_d values with EGFR$^{L858R/T790M}$. The most rigid compounds (**3a** and **3d**) were indeed significantly more selective than the other compounds. The tetracyclic compound **3e** displayed almost equal potencies and selectivity to that of **3d** on EGFR^{T790M} mutants, but its poor solubility impeded further investigation. Although compound **3f**, with an *N*- morpholino moiety, was 2–3 times more selective than **3d**, its potency was 3–5-fold lower. Compound **3h** was more selective but slightly less potent than compound **3d**, while compound **3g** with a 4-(dimethylamino)piperidin-1-yl substituent displayed improved selectivity and equal potency to that of **3d** (Table 1). Further kinase profiling investigations against a panel of 456 kinases revealed that **3g** displayed excellent selectivity on EGFR^{T790M} mutants at 100 nM, about 77- and 32-times greater than its K_d values with EGFR^{T790M} and EGFR$^{L858R/T790M}$, respectively. Only four of the 395 non-mutate kinases, CAMK2A, DAPK2, DAPK3, and HIPK2, manifested binding with compound **3g** (inhibition rate $\geq 65\,\%$, or Ctrl % $\leq 35\,\%$), and the S(35) selectivity score of **3g** was 0.01. To the best of our knowledge, compound **3g** is one of the most selective EGFR^{T790M} inhibitors reported to date (Supporting Information, Figures S7, S8).

The strong EGFR^{T790M} kinase inhibition of the new inhibitors was further confirmed by examination of the effects of the representative compounds **3d** and **3g** on the activation of EGFR and the downstream signals in gefitinib-resistant H1975 NSCLC cells harboring EGFR$^{L858R/T790M}$. It was shown that compounds **3d** and **3g** dose-dependently inhibited the phosphorylation of EGFR and decreased the protein levels of downstream *p*-Akt, *p*-Erk, while gefitinib showed no activity (Figure 1). Further studies revealed that the compounds also potently inhibited EGFR activation in NCI-H820 NSCLC cells with EGFR$^{del\ E746\text{-}E749/T790M}$, but had no obvious effect in a panel of NSCLC cells with wild-type EGFR (that is, NCI-H1299, A549, 95D, NCI-H446, NCI-H358, NCI-H661, NCI-



Scheme 2. Synthesis of compound **3a**. DIPEA = diisopropylethylamine, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid.

Table 1: Binding and kinase inhibitory activities of the new $EGFR^{T790M}$ inhibitors.

No.	EGFR Binding affinity (K_d, [nM])[a]					Kinase inhibition (IC_{50} [nM])[b]	
	WT	T790M	L858R/T790M	WT:T790M	WT:DM	T790M	L858R/T790M
3a	210	1.8	3.6	116.7	58.3	13.87 ± 5.29	7.03 ± 2.85
3b	72	0.88	1.1	81.8	65.5	3.22 ± 0.49	2.09 ± 0.41
3c	17	0.43	0.88	39.5	19.3	3.36 ± 0.95	1.69 ± 0.51
3d	140	1.4	1.8	100	77.8	4.50 ± 1.33	1.52 ± 0.81
3e	110	0.69	1.1	159.4	100	3.57 ± 2.47	1.82 ± 0.20
3f	950	4	6.1	237.5	155.7	14.84 ± 0.31	7.00 ± 0.19
3g	310	1.3	2.6	238.5	119.2	4.55 ± 0.25	2.18 ± 0.1
3h	420	1.3	3.1	323.1	135.5	8.41 ± 0.40	3.85 ± 0.27
1	1.2	0.29	0.3	4.1	4.0	0.67	0.93

[a] Binding constant values (K_d) were determined from Ambit KINOMEscan. The data are means from two independent experiments. [b] EGFR kinase assays were performed using the FRET-based Z′-Lyte assay according to the manufacturer's instructions. The compounds were incubated with the kinase reaction mixture for 1.5 h before measurement. The data are means from at least three independent experiments. WT = wild-type. DM = L858R/T790M double mutations.

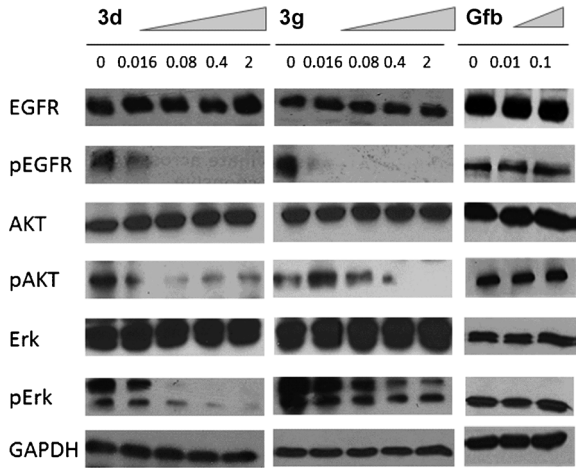


Figure 1. Inhibition by compounds **3d** and **3g** of the activation of EGFR and downstream signaling in H1975 NSCLC cells harboring $EGFR^{L858R/T790M}$. In contrast, gefitinib (Gfb) has no effect (right).

Table 2: The anti-proliferative activities of **3d** and **3g** against cells with different EGFR.[a]

Cells	EGFR status	Anti-proliferation (IC_{50}, [μM])	
		3d	**3g**
H1975	L858R/T790M	0.143 ± 0.026	0.086 ± 0.018
H322	WT	> 30	> 30
A549	WT	8.85 ± 0.76	> 30
H1299	WT	> 30	> 30
H1703	WT	20.15 ± 9.92	> 30
H661	WT	20.83 ± 2.95	> 30
95D	WT	> 30	> 30
H358	WT	6.25 ± 1.15	> 30
HCC827	del E746-A750	0.039 ± 0.013	0.049 ± 0.027
HL-7702	WT	9.96 ± 5.08	> 30
HLF-1	WT	3.96 ± 0.11	10.47 ± 1.46

[a] The anti-proliferative activities of the compounds were evaluated using MTS assay. The data were means from at least four independent experiments.

H1703, NCI-H322 cancer cells; Supporting Information, Figure S2).

The cell-growth inhibitory effects of the compounds were also investigated against a panel of NSCLC cells with different EGFR status (Table 2; Supporting Information, Table S1). Compounds **3d** and **3g** potently inhibited the growth of H1975 NSCLC cells harboring the EGFR T790M mutation with $IC_{50} = 0.143$ and 0.086 μM, respectively, but were 60–400-fold less potent to NSCLC cells with wild-type EGFR. These results correlated exactly with the selective inhibition by **3d** and **3g** of $EGFR^{T790M}$ mutants. Compounds **3d** and **3g** were also significantly less cytotoxic to normal HL-7702 (normal human liver cells) and HLF-1 (human lung fibroblast cells) harboring $EGFR^{WT}$. Further investigation revealed that the compounds dose-dependently arrested the H1975 cells in the G1 phase (Supporting Information, Figure S3), caused cell apoptosis (Supporting Information, Figure S4), and strongly inhibited tumorigenesis (Supporting Information, Figure S5) and metastasis of H1975 cells in vitro (Supporting Information, Figure S6). Thus they may be used as promising lead compounds for further drug discovery with a view to overcoming the clinically acquired resistance against gefitinib.

In summary, we have described the discovery of a new series of pyrimido[4,5-*d*]pyrimidin-4(1*H*)-one derivatives that are highly selective and potent inhibitors targeting the resistance of related $EGFR^{T790M}$ mutants. The extraordinary selectivity of the compounds over wild-type EGFR and other kinases makes them attractive lead compounds for future drug development.

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