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Design, Synthesis, and Biological Evaluation of Novel Conformationally Constrained Inhibitors Targeting EGFR

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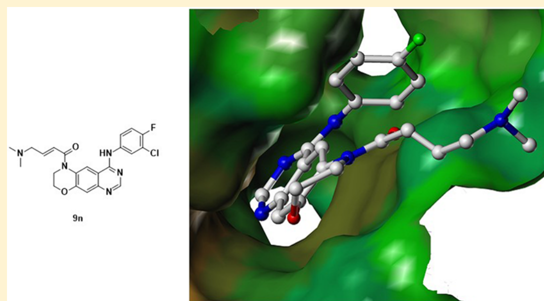
Supporting Information

ABSTRACT: This letter describes the construction of conformationally constrained quinazoline analogues. Structure−activity relationship studies led to the identification of the lead compound **9n**. Compound **9n** exhibits effective in vitro activity against A431[WT,overexpression] and H1975[L858R/T790M] cancer cell lines but is significantly less effective against EGFR negative cancer cell lines (SW620, A549, and K562). Compound **9n** was also assessed for potency in enzymatic assays and in vivo antitumor studies. The results indicated that **9n** is a potent kinase inhibitor against both wild-type and T790M mutant EGFR kinase. Meanwhile, an oral administration of **9n** at a dose of 200 mg/kg produced a considerable antitumor effect in a A431 xenograft model, as compared to gefitinib. A preliminary pharmacokinetic study of **9n** also indicates it has good pharmacokinetic properties, and therefore, it is a good starting point for further development.



KEYWORDS: *Anticancer, kinase inhibitor, EGFR, conformationally constrained*

The clinical success of a number of kinase-directed drugs indicates that kinase represent therapeutically relevant targets. The epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases (RTKs), which plays an important role in the regulation of cell growth, differentiation, and survival.[1] Because of their multidimensional role in the progression of cancer, EGFR and its family members have emerged as attractive targets for anticancer therapy.[2] Accordingly, targeting EGFR has been intensely pursued, with the approval of gefitinib, erlotinib, and lapatinib for use in the clinic.[3−7] As is common with many therapies, challenges with respect to treatment resistance emerge over time. This situation is certainly true of EGFR inhibitor therapy. Clinical studies have demonstrated the occurrence of resistance to gefitinib with the T790M mutation accounting for 50% of the clinically observed resistant mutations.

In order to overcome the T790M mutation related drug resistance, a variety of irreversible inhibitors were developed. These compounds contain a Michael acceptor moiety designed to form a covalent bond with the conserved cysteine residue (Cys797) at the lip of the EGFR ATP binding site.[8,9] So far, canertinib,[10,11] neratinib,[12,13] dacomitinib,[14] and afatinib[15] have been developed as second generation irreversible inhibitors with limited clinical efficacy. These drugs demonstrate the utility of quinazoline derivatives as an attractive scaffold for the development of EGFR inhibitors. To date, many studies have been targeted at finding new structures based on quinazolines that are potent EGFR inhibitors.[16−18] All of these inhibitors share a common feature: upon the binding to EGFR, a hydrogen bond forms between the N1 atom of the compound and the backbone NH of Met769 in the hinge region.

To investigate the potential use of a novel scaffold as an EGFR ATP binding site inhibitor, a series of conformationally constrained quinazoline derivatives were synthesized (consisting of 34 structurally characterized compounds with purity >98%). (For general synthetic procedures refer to Schemes 1 and 2 and Supporting Information.) From this set of new compounds, a specific analogue was identified that inhibits the growth of both gefitinib-resistant (H1975[T790M/L858R]) and gefitinib-sensitive (A431[WT,overexpression]) cell lines but is not effective against EGFR-negative (A549, K562, and SW620) cells.

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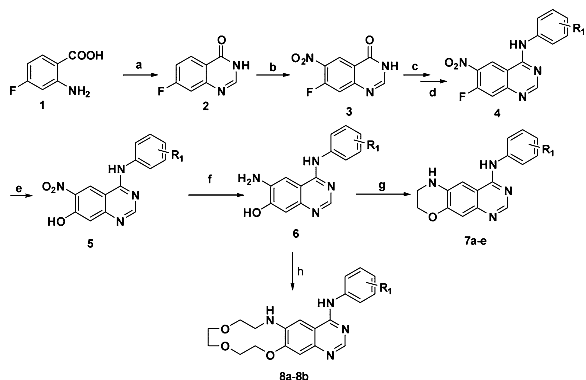
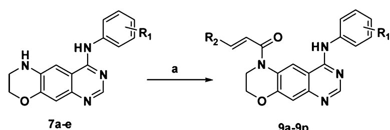
Scheme 1. Synthesis of Compounds 7a–e and 8a–b



Scheme 2. Synthesis of Compounds 9a–9p



The screening result (Table S1, Supporting Information) of the synthesized analogues **7a–7o** and **8a–8b** did not show obvious inhibition on the proliferation of A431 human epithelial carcinoma cells with overexpressed $EGFR^{WT}$, but surprisingly, some of the synthesized analogues suppressed the enzymatic activity of $EGFR^{WT}$, which might be due to the complex genomic background and the strong binding of $EGFR^{WT}$ with ATP.[19] For instance, **7b** inhibited the enzymatic activities of $EGFR^{WT}$ with an IC_{50} value of 1.29 nM, but its activity on A431 cancer cells was approximately 20 000 times less potent (IC_{50} value was more than 30 μM). The results indicated that a further modification of the scaffold might prove to be an effective strategy for identifying more potent inhibitors. The impact of the R_3 group was first studied by replacement with other acrylamide groups. Interestingly, the inhibitory potency against $A431^{WT,overexpression}$ and wild-type EGFR was significantly increased by over 20-fold (Table S2, Supporting Information). For instance, when the hydrogen on the R_3 substitution was replaced with acrylamide, the resulting compounds **9a–9e** are almost 5 times more potent than **7a–7e**. However, when the substituted acrylamide was replaced with a slightly hydrophobic moiety (**9f–9h**), the potencies against $A431^{WT,overexpression}$ and wild-type EGFR kinase were almost completely abolished. Further investigation also revealed that the potency loss of **9f–9h** was partially restored by introducing a *N*-morpholino moiety (**9i–9l**) at the position of the original phenyl moiety. The result suggests that the substituted acrylamide at the R_3 position can be replaced with a more hydrophilic group in order to improve the potency against the A431 and wild-type EGFR kinases. This hypothesis was verified by the in vitro screening result of **9m–9p**. The inhibitory activities against $A431^{WT,overexpression}$ and $EGFR^{WT}$ were improved, with IC_{50} values almost equal to the reference irreversible inhibitor, canertinib. We further profiled the synthesized analogues **9a–9p** against NSCLC cell line H1975. The H1975 cell line bears the drug resistant mutation in $EGFR^{[L858R/T790M]}$ and is a typical EGFR-driven cell lines. Several compounds (**8c–8d** and **9n–9p**) also displayed strong antiproliferative effects on gefitinib-resistant H1975 cells, with the IC_{50} value equal to or more potent than that of canertinib (Table S2, Supporting Information). Three closely related analogues, **9n–9p**, were identified from the screen that possessed approximately equal IC_{50} values against $A431^{WT,expression}$ and $H1975^{[L858R/T790M]}$ as the reference inhibitors (Figure 1 and Tables S2 and S3, Supporting Information).

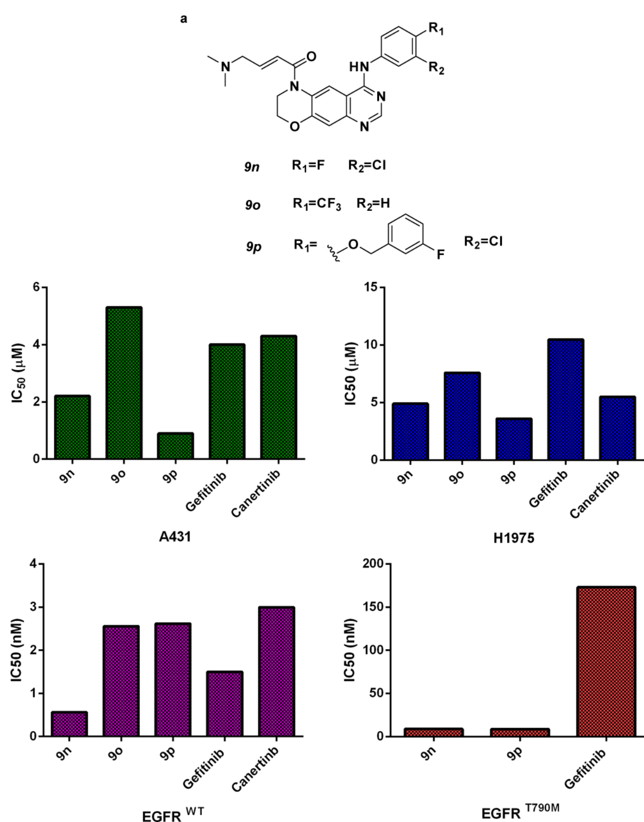


Figure 1. Compounds **9n–9p** are novel EGFR inhibitors, suppress the growth of $EGFR^{WT}/EGFR^{L858R/T790M}$ containing cancer cell lines, and inhibit the enzymatic activities of $EGFR^{WT}/EGFR^{T790M}$ kinase. (a) Chemical structures of **9n–9p**. IC_{50} values for A431 (top left), H1975 (top, right), $EGFR^{WT}$ (bottom, left), and $EGFR^{T790M}$ (bottom, right). Growth inhibitory was assessed using the MTT.

To better understand how our conformationally constrained compounds interact with the ATP-binding cleft of EGFR and how this relates to their potency for $EGFR^{T790M}$, we determined a putative binding mode of one representative compound **9n** within the active binding pocket of the $EGFR^{T790M}$ (Figure 2). The morpholine fused scaffold is located in the ATP-binding pocket. Two highly conserved hydrogen bonds are formed between the quinazoline core and the hinge region. The molecule is oriented such that 6-substituted acrylamide branch extends toward the solvent, and the 4-aniline moiety is directed into the backpocket of EGFR kinase. The docking result is shown to form a covalent bond with Cys797 as expected, with a measured distance of 2.54 Å. The prediction is consistent with observed result from Western blot studies (Figure 3). In addition, a salt bridge was formed between the carboxyl group of Asp800 and the nitrogen atom of the dimethylamino group. Furthermore, the protonated dimethylamino group might facilitate the nucleophilic attack of the sulfhydryl group of Cys797.[20] The prediction is also consistent with our observations that a hydrophobic substituent (e.g., phenyl) led to a decrease in the potency in comparison with the dimethylamino group.

Compound **9n** and canertinib inhibit the autophosphorylation of EGFR in no wash (left) and wash out (right), while gefitinib

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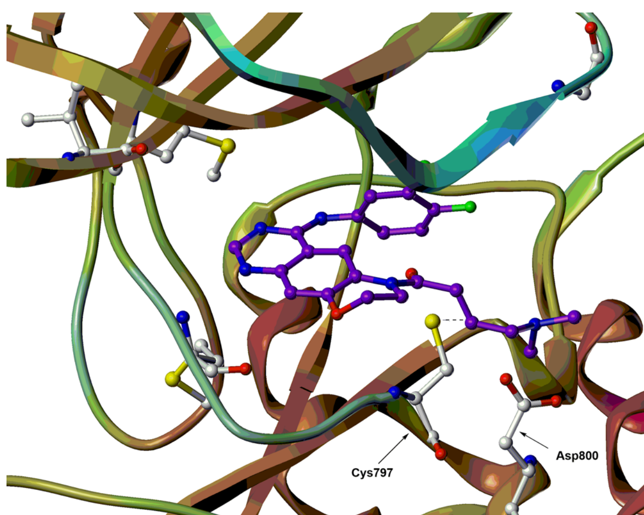

Figure 2. Putative binding mode of **9n** within the active pocket of the EGFR^{T790M} (PDB code: 4I24).

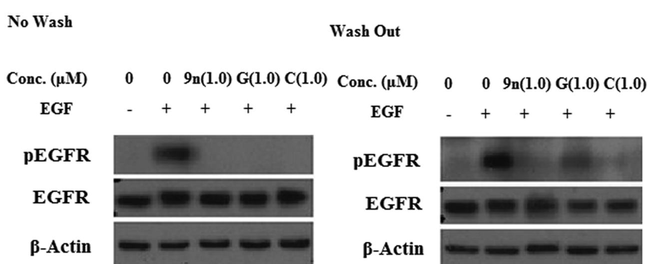

Figure 3. Inhibition of EGFR autophosphorylation in A431 by Western blot assay. G, gefitinib; C, canertinib.

has no effects in the wash out group. The result is the evidence of irreversible binding mode, suggesting **9n** is acting the same as irreversible inhibitor canertinib.

The in vitro data shown in Figure 1 demonstrates that the new synthesized analogues **9n**–**9p** strongly inhibit the EGFR^{WT} kinase. Because many tissues use wild-type EGFR for normal cellular processes, the potential for toxicity from irreversible EGFR inhibitors is a concern. Therefore, the growth inhibitory of the compounds against various cancer cell lines was evaluated to control the potential toxicity. As shown in Table 1, the potent

Table 1. In Vitro Growth Inhibitory Activities against Various Cancer Cell Lines

cancer type	cell line	characteristic	9n (μM)	9p (μM)	gefitinib (μM)
epidemal	A431	EGFR^{WT,overexpression}	2.2	0.9	4.0
NSCLC	H1975	EGFR$^{L858R/T790M}$	4.9	3.61	10.48
NSCLC	A549	EGFR$^{WT,\kappa\text{-Ras mutation}}$	>10	>10	>10
colon	SW620	EGFR negative	25.4	6.2	28.20
malignant myeloid	K562	EGFR negative	>10	>10	>10

EGFR inhibitor **9n** did not display obvious inhibition on the growth of A549, SW620, and K562 cancer cells, all of which have low levels of EGFR expression. The results indicate that the cytotoxic effects of **9n** are minimal. Meanwhile, in the human ether-a-go-go-related gene (hERG) potassium channel patch clamp assay,[21] **9n** has an IC_{50} >10 μM, which indicates it has a low potential for cardiac toxicity (Table S4, Supporting Information).

Furthermore, **9n** and **9p** have a good pharmacokinetic in vitro ADME profiles: the inhibition of cytochrome P450 was assessed in recombinant human cytochrome P450 isoforms (1A2, 2C9, 2D6, and 3A4), and the IC_{50} values were higher than 10 μM, suggesting a low potential for **9n** and **9p** to be involved in any drug–drug interactions. The intrinsic clearance found in liver microsomes predicts good to excellent in vivo clearance (Table S5, Supporting Information).

A pharmacokinetic study of **9n** and **9p** was performed in BALB/c mice. As shown in Table 2, **9n** demonstrated desirable

Table 2. Pharmacokinetic Parameters for 9n[a]

route	AUC(0–24 h) (μM·min)	$t_{1/2}$ (min)	t_{max} (min)	C_{max} (μM)	F%
i.v.	25.4	762	2	0.29	
p.o.	133	548	45	0.36	26.1

[a]The pharmacokinetic parameters are obtained after a single i.v. (3 mg/kg) or oral (10 mg/kg) administration. The data are obtained from 12 mice in each treatment group.

results with a half-life (9.1 h) and oral bioavailability (26.1%), while **9p** displayed an unsatisfactory pharmacokinetic prolife (F = 6.42%) (Table S6, Supporting Information).

We further determined the effectiveness of **9n** in vivo by using a nude mouse xenograft model harboring EGFR^{WT,expression}. We chose **9n** for the in vivo studies because, in vitro, it is effective against EGFR expressing cancer cell lines and has a good ADME prolife. Gefitinib (200 mg/kg) was used as a reference drug to validate the models. In the A431 tumor model, the mice were treated via oral gavage once daily with either **9n** or gefitinib once the tumor had grown to a volume of 50–150 mm^2. Oral administration of **9n** and gefitinib at 200 mg/kg/day inhibited tumor growth at 66.8% and 35.9%, respectively. No mortality or significant weight loss was observed during the treatment (Figure 4).

In summary, a series of conformationally constrained quinazoline derivatives have been designed and synthesized and shown utility as EGFR inhibitors. The most potent compounds **9n** and **9p** strongly inhibited the enzymatic activities of wild-type EGFR kinase as well as clinical resistant EGFR^{T790M} mutant kinase. The kinase inhibitory efficiency of the compounds were further validated by Western Blot analysis for the activation of EGFR. Further in vitro assay demonstrated that **9n** and **9p** are effective against H1975 nonsmall cell lung cancer cells bearing EGFR$^{[L858R/T790M]}$, with potencies better than gefitinib. Compounds **9n** and **9p** showed minimal cytotoxicity to K562 and SW620. A hERG assay demonstrated their unlikely cardiac toxicity, indicating that these analogues might possess a high safety index. An in vivo antitumor assay demonstrates that an oral once daily dose of **9n** at 200 mg/kg produces considerable tumor inhibition in the A431 xenograft model, as compared to gefitinib. The pharmacokinetic studies indicate that **9n** possesses good pharmacokinetic properties. In conclusion, our studies identified a new lead compound containing a novel scaffold ideally suited for the development of therapeutically relevant EGFR inhibitor effective against EGFR^{T790M} mutations. Further detailed studies of the mechanisms of action for **9n** are currently underway.

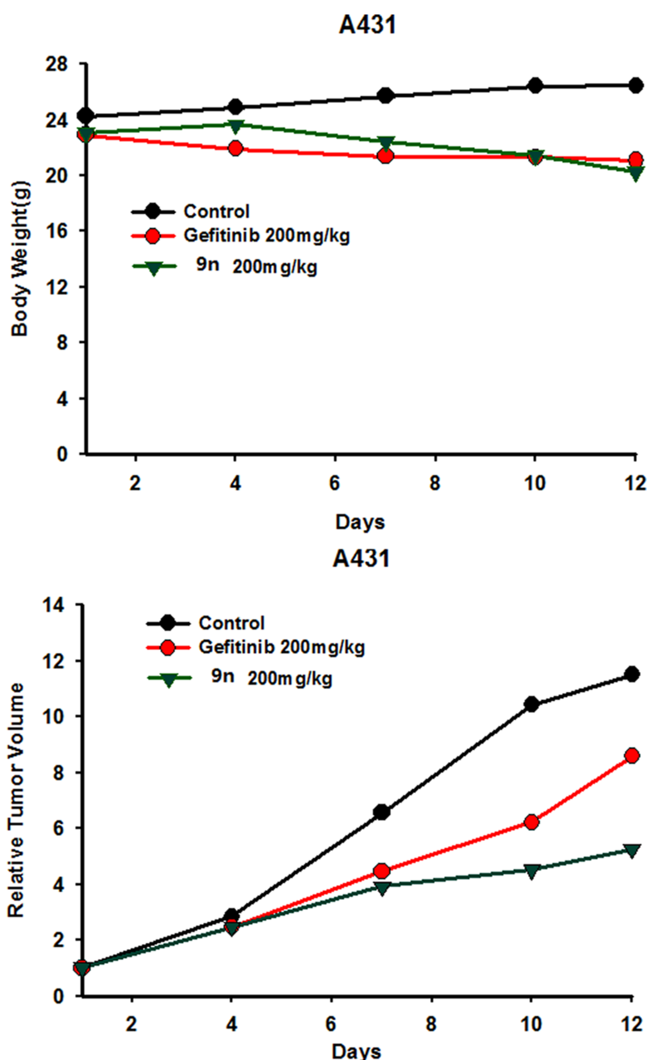

Figure 4. In vivo antitumor effects of compound **9n**. (top) Body weight curves for the A431 xenograft model. (bottom) Tumor growth curves for the A431 xenograft model. Animals were randomized into groups (n = 6/group) and administered **9n**, gefitinib, and vehicle once daily at the indicated dose level when the tumors reached the determined size, and the animal weight and tumor volume were monitored twice weekly. Data points represent the mean tumor mass (SEM of 6 mice). Relative tumor growth is calculated by mean tumor mass on day 4, 7, 10, or 12 divided by mean tumor mass on day 0. A p-value ($p < 0.05$) indicates a statistically significant reduction in relative tumor growth of the treated groups compared with the control group.

■ ASSOCIATED CONTENT

Supporting Information

Details of the synthesis and characterization of all compounds and intermediates together with protocols for biological experiments, some biological data, and computational methods used for molecular docking This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

[⊥]J.W. and W.C. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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