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Synthesis and biological evaluation of crown ether fused quinazoline analogues as potent EGFR inhibitors

Shaojing Hu [b,*], Guojian Xie [a], Don X. Zhang [a], Charles Davis [a], Wei Long [b], Yunyan Hu [b], Fei Wang [b], Xinshan Kang [b], Fenlai Tan [b], Lieming Ding [b], Yinxiang Wang [b,*]

[a] Betapharma USA, 31 Business Park Dr. Branford, CT 06405, USA
[b] Zhejiang Betapharma, 589 Hongfong Rd., YuhangDistrict, Zhejiang 311100, PR China

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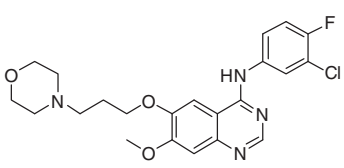
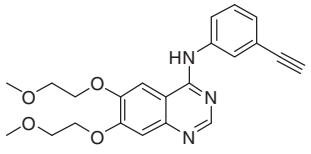
ABSTRACT

Crown ether fused anilinoquinazoline analogues were synthesized as novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors. Representative compounds showed potent and selective EGFR inhibitory activities in an in vitro EGFR kinase assay and an EGFR-mediated intracellular tyrosine phosphorylation assay. The synthesis and preliminary biological, physical, and pharmacokinetic evaluation of these fused quinazoline compounds is reported.

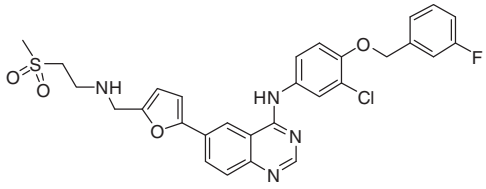
The epidermal growth factor receptor (EGFR), as a transmembrane glycoprotein, belongs to the erbB family of closely related cell membrane receptors that includes EGFR (erbB-1 or HER1), erbB-2 (HER2), erbB-3 (HER3), and erbB-4 (HER4).[1] Expression, overexpression, or dysregulated function of EGFR is observed in many human solid tumors, including breast, ovarian, non–small-cell lung (NSCLC), colorectal, and head and neck cancers.[2–5] In support of its important role in tumor biology, EGFR activation may assist tumor growth by increasing cell proliferation, motility,[6] adhesion, and invasive capacity[7] and by blocking apoptosis.[8] EGFR-dependent aberrant signaling, such as overexpression and dysregulation, is associated with indices of poorer prognosis in patients and is associated with metastasis, late-stage disease, and resistance to chemotherapy, hormonal therapy, and radiotherapy.[2,4,9–12] Small molecule EGFR inhibitors have been shown to be effective antitumor agents.[1] For example, two closely related anilinoquinazoline-containing EGFR inhibitors, gefitinib (Iressa™, **1**)[2] and erlotinib (Tarceva™, **2**)[3] have efficacy against several types of cancers in human clinical trials and were approved for the treatment of NSCLC and colon cancers. The dual EGFR/HER2 inhibitors lapatinib (Tykerb™, also known as GW-572016, **3**) was recently approved for the treatment of HER2-positive metastatic breast cancer.[4] Many more EGFR inhibitors including antibodies are either still under evaluation in clinical trials or been approved for the treatment of cancer.[1]



Iressa, **1**



Tarceva, **2**



Lapatinib, **3**

There are numerous crystal structures of EGFR available, including the co-crystal structures of EGFR with erlotinib or gefitinib, which have provided a rich set of structural information for our drug discovery effort. The structure–activity relationship (SAR) of these EGFR inhibitors are mostly well understood.[13] As shown in Figure 1, binding of erlotinib depends on several key interactions with the ATP binding pocket of EGFR, including the critical H-bond with the hinge region using its quinazoline core, the filling up of the so-called 'back pocket' by the ethynylphenyl substitution, and the interactions in the tail region. There is also a hydrophobic

* Corresponding authors.
E-mail address: shaojing.hu@betapharma.com.cn (S. Hu).

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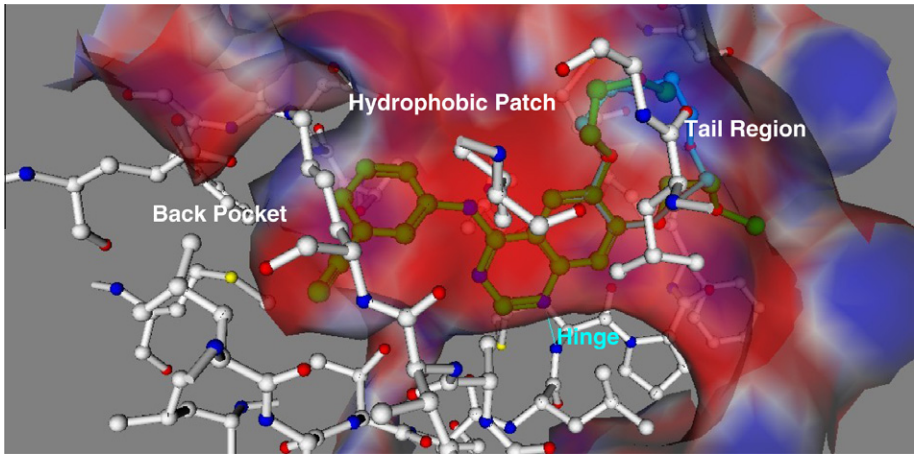
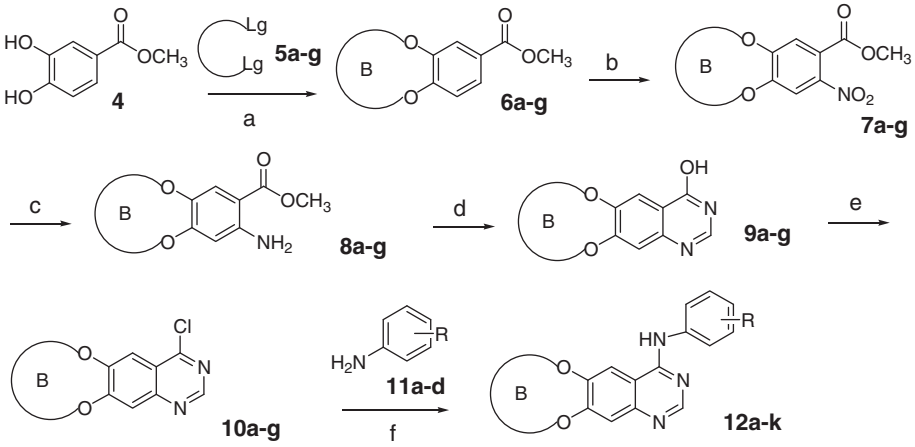


Figure 1. Crystal structure of erlotinib (gold) in EGFR (1M17 pdb). Compound **12k** (cyan) was modeled in for comparison. The protein surface of the ATP binding pocket of EGFR was shown, with red representing the hydrophobic areas and blue the hydrophilic areas.

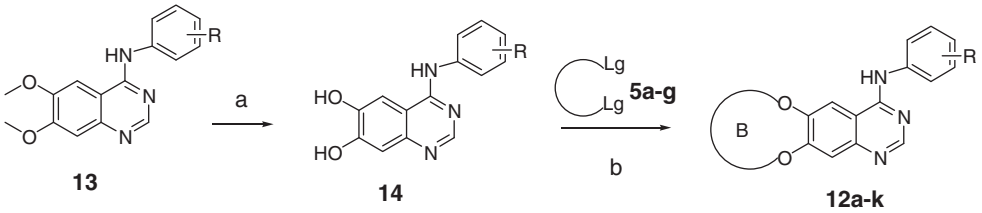


Scheme 1. General synthetic approach to fused quinazolines. Reagents and conditions: (a) K_2CO_3/DMF, 90 °C, 3 h, 24–45%; (b) HNO_3/H_2SO_4, HOAc, 72%; (c) H_2, Pd/C, 96%; (d) $HCONH_2/HCO_2NH_4$, 165 °C, 80%; (e) $POCl_3$, 77%; (f) *i*-PrOH/DMF,72%.

patch a little further away from the hinge, which may also provide additional opportunities for ligand optimization. In particular, the tail region is able to accommodate a variety of functional groups without affecting the activities of the compounds too much. The various substituents at the 6 and 7 positions of the quinazolines ring which occupies the tail region are able to significantly modulate the properties of the compounds, and such differences between gefitinib, erlotinib and lapatinib contribute in part to the distinct profiles of these three EGFR inhibitors. Therefore this is a major area to further optimize good lead molecules.

Our computer model study provides us much needed information to design new EGFR inhibitors. We have focused our development on the modification of tail region by attaching cyclic systems to the quinazoline ring. Therefore we synthesized a series of structural modified quinazolines compounds by fused with numerous ring systems, aimed at identifying potent, selective, and bioavailable EGFR inhibitors as anti-cancer agents. Herein we report their synthesis and preliminary biological evaluation.

A general approach to synthesize the designed crown ether fused quinazolines compounds **12a**–**k** is shown in Scheme 1, starting from commercially available methyl 2,3-dihydroxybenzoate **4**. The key intermediates, methyl benzoates **6a**–**g** with different ring system were synthesized from the reaction of methyl 2,3-dihydroxybenzoate **4** with various α,ω-dihalogens or α,ω-tosylates **5a**–**g** in the presence of abase such as potassium carbonate at elevated temperature. The standard nitration procedure is employed for the nitration of methyl benzoates **6a**–**g**, which was reduced by hydrogenation in methanol to give anilines **8a**–**g**. Compounds

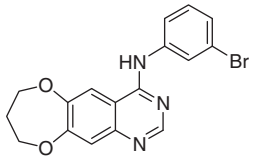
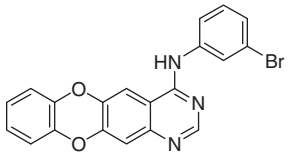
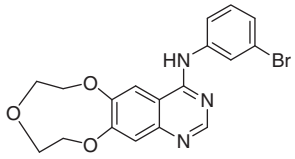
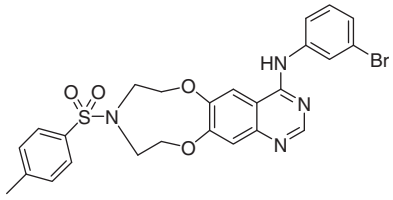
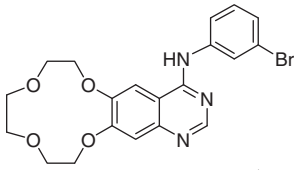
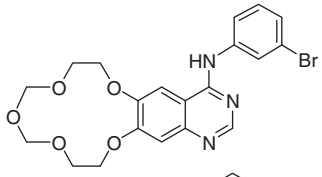
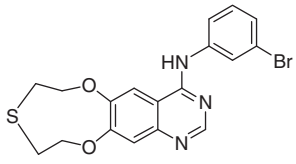
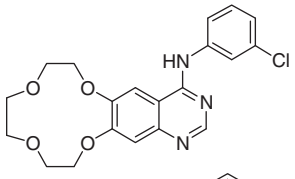
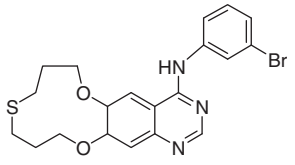


Scheme 2. Alternative synthetic approach to fused quinazolines. Reagents: (a) BBr_3, THF, 63%; (b) K_2CO_3/DMF, 20–36%.

8a–g were treated with formamide and ammonium formate to provide substituted quinazolin-4-ones **9a–g**. Using 5 equiv of $POCl_3$ yielded the key intermediates **10a–g**. Coupling **10a–g** with substituted anilines **11a–d** in a solvent such as isopropanol, diglyme, or butoxyethanol gave the target compounds, the crown ether fused quinazolines **12a–k** in six steps of 11–20% yield.

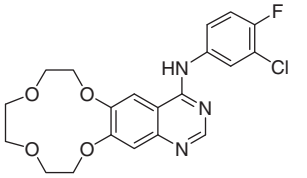
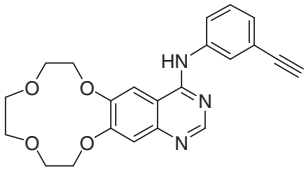
An alternative approach to synthesize the desired compounds **12a–k** is shown in Scheme 2. The known 4-anilinoquinazoline class of compounds, (6,7-dimethoxy-quinazolin-4-yl)phenyl-amines

Table 1
Structure and EGFR protein kinase inhibitory activity of the fused quinazolines **12a–k**

Compound	Structure	EGFR IC_{50}[a] (nM)	Tyrphospor-IC_{50}[b] (nM)
12a		85	N/D[c]
12b		120	N/D[c]
12c		55	N/D[c]
12d		150	N/D[c]
12e		5	50
12f		5	50
12g		7	>1000
12h		2	55
12i		8	>1000

(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	EGFR IC_{50}[a] (nM)	Tyrphospor-IC_{50}[b] (nM)
12j		2	45
12k		2	45

[a] In vitro kinase assay according to Ref. 15.
[b] EGFR-mediated intracellular tyrosine phosphorylation assay according to Ref. 14.
[c] Not determined (N/D).

13,[13c] were treated with boron tribromide to give the (6,7-dihydroxy-quinazolin-4-yl)-phenylamines **14**. The final ring formation was completed by the reaction of intermediate **14** with α,ω-dihalogen or α,ω- tosylates **5a–g** in the presence of base such as potassium carbonate to provide crown ether fused quinazolines **12a–k** in total 20–36% yield.

Table 1 summarizes the structures and EGFR inhibitory activities for the series of fused quinazolines **12a–k**. SAR studies of the series were first focused on the variation of different ring size. A ring size of 12 or higher is clearly more preferred than 7 or 9 member ring (IC_{50} = 2–7 nM vs >50 nM). Heteroatoms other than oxygen in the ring system slightly reduced EGFR potency (oxygen with sulfur, IC_{50} = 2–5 nM and 7–9 nM, respectively). However introduction of a sulfur atom in the ring system almost completely lost inhibition activity in the cell assay (compound **12g** and compound **12i**). A nitrogen atom capped with a sulfonyl group embedded in the ring (or something similar) drastically reduced EGFR potency (compound **12d** vs compound **12c**, IC_{50} = 150 vs 55 nM). As for aniline, which occupied the 'back pocket' or hydrophobic pocket of EGFR, like gefitinib and erlotinib, small non-polar substituents at the anilines, such as chloro, bromo, or ethynyl, are well tolerated and more preferred at the meta-position, as in compound**s 12h–k** (IC_{50} = 2–8 nM).

Compounds **12e–12k** with $IC_{50} \leqslant 5$ nM in the in vitro kinase assay were selected for further evaluation in an EGFR-mediated intracellular tyrosine phosphorylation assay in human epidermoid A431 carcinoma cell line .[14]

Fused quinazolines with oxygen substituted heterocycles and ring size higher than 12 members are shown to be the most potent EGFR inhibitors in this series. Representative compounds **12e**,**12f**, **12h**, **12j** and **12k** showed potent inhibition of EGFR kinase in an A431 cell with IC_{50} values in 45–55 nM. Perhaps because of lack cell permeability, **12g** and **12i** are not potent at this assay.

Compound **12k** and other selected compounds were profiled in a panel of kinase assays. No inhibition of Abl, Arg and c-Src tyrosine kinases was observed even at concentrations higher than 1000 nM by **12k** (unpublished data).

Preliminary pharmacokinetic studies were conducted in rats with selected compound **12k** (Table 2). Pharmacokinetic parameters were determined after single oral (35 mg/kg) and intravenous (35 mg/kg) dosing. Compound **12k** demonstrated good oral bioavailability (52%) and exposure level (C_{max} and AUC) in rats. Therefore **12k** was selected for the advanced preliminary pharmacological study. After oral administration, **12k** inhibits the growth of a broad range of human solid tumor xenografts in a dose-dependent manner (range 50–120 mg/kg, po once daily). Figure 2 shows the effects of compound **12k** on the tumor size of A431 xenograft.

In summary, a series of fused anilino-quinazoline analogues showed modest to potent EGFR inhibition, with IC_{50} values ranging from 2 nM to 150 nM. SAR studies of the series revealed that oxygen containing heterocycles with ring size higher than 12 member are the favorable fused anilinoquinazolines, and the preferred substituent on the 7-anilino is a halogen such as chlorine, bromine or ethynyl group at the meta-position. A subset of the selected compounds proved to be active in an EGFR-mediated intracellular tyrosine phosphorylation assay in human tumor cell line A431. Compound **12k** is shown as a potent inhibitor of EGFR with excellent selectivity against Abl, Arg and other kinases, exhibits a favorable pharmacokinetic profile in the preclinical studies, and inhibits the growth of a broad range of human solid tumor xenografts in a dose-dependent manner (range 50–100 mg/kg, po once daily). **12k**

Table 2
Pharmacokinetic properties of the selected compound **12k**

F (%) (rat)	52
C_{max} (po)	6.12 μg/mL
AUC (po)	49.4 μg/mL
$T_{1/2}$ (po)	2.8 h
Cl	21 mL/min/kg
Vss	3.9 L/kg
MRT	6.9 h

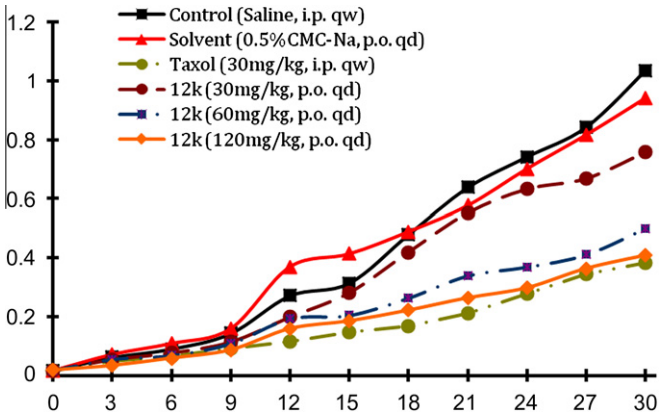


Figure 2. Effects of Compound **12k** on the tumor size of A431 xenografts. On tumors achieving volumes of approximately 20 mm^3, tumor-bearing mice were randomized into treatment groups (n = 10/group) and po qd Icotinib at doses of 30, 60, and 120 mg/kg in a vehicle of 0.5% CMC-Na solution for 30, 18, 18, 27 days, Taxol ip qw at dose of 30 mg/kg as positive control.

has been further moved into clinical evaluation as a single agent for the treatment of NSCLC patients.[16] Recent publications has highlighted that Icotinib, **12k**, provides similar efficacy to gefitinib, but with better tolerability, in NSCLC patients previously treated with one or two chemotherapy agents.[16b]

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14. *EGFR-mediated intracellular tyrosine phosphorylation assay*: A431 cells (1.5×10^5) were grown in 12-well plates in DMEM medium supplemented with 10% FCS and antibiotics for 2 days in a 5% CO_2 incubator at 37 °C. Before treated with an inhibitor, the cells were grown in serum-free medium for 18 h. Cells were then treated with the inhibitor at 0, 10, 50, 250 and 1000 nM and incubated for another 2.5 h at 37 °C. Upon stimulation with 100 ng/ml of EGF for 5 min, cells were lysated by SDS sample buffer including 1 mM vanadate. After boiling for 4 min, cell lysates were separated by 10% SDS–PAGE gel and transferred onto a nitrocellulose membrane. The membrane was probed by anti-phosphotyrosine antibodies (pY99 and 4G10) and then HRP-labeled secondary antibody. The signal of tyrosine-phosphorylated proteins were visualized by ECL and quantified by a Densitometer (Molecular Dynamics). The blot was then striped and re-probed by anti-EGFR antibody as an internal control to show whether or not the compound affect the overall level of EGFR.protein.
15. In vitro kinase assay: EGFR protein (2.4 ng/μl, 14.5 units/μg, Sigma) was mixed with GST-Crk (32 ng/μl) in 25 μl kinase reaction buffer containing 1 μM cold ATP and 1 μCi 32P-γ-ATP. The mix was incubated with BPI-2009 at 0, 0.5, 2.5, 12.5 and 62.5 nM on ice for 10 min, then switched to 30 °C for another 20 min. After quenching by SDS sample buffer at 100 °C for 4 min, the protein mix was separated by 10% SDS–PAGE gel. The dried gel was then exposed to Phosphorimager for quantification. The phosphorylated Crk was plotted against the concentration of the compound.
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