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Facile and efficient synthesis and biological evaluation of 4-anilinoquinazoline derivatives as EGFR inhibitors

Zheng Wang,[a] Xue Wu,[c] Li Wang,[a] Jiao Zhang,[a] Jianli Liu,[b] Zhongxing Song,[a] Zhishu Tang[a]*

[a] *Shaanxi Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Shaanxi Province Key Laboratory of New Drugs and Chinese Medicine Foundation Research, Shaanxi Rheumatism and Tumor Center of TCM Engineering Technology Research,Shaanxi University of Chinese Medicine, Xian Yang 712083, China*
[b] *Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, College of Life Science, Northwest University, Xi'an 710069, China*
[c] *Department of Burns and Cutaneous Surgery, Xijing Hospital, Xi'an, 710032,China*

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ABSTRACT

Series of 4-anilinoquinazoline derivatives were conveniently and efficiently synthesized and their antitumor activities were evaluated by MTT assay in three human cancer cell lines: H1975, HepG2 and SMMC-7721. New compounds **19a-19h** were designed and synthesized to seek for powerful EGFR inhibitors and to explore whether methyl group at C-2 position of quinazoline ring has a positive effect on EGFR inhibition. All the compounds of **19a-19h** were found potent against all three cell lines and five compounds (**19c**, **19d**, and **19f-19h**) were found more potent against H1975 than gefitinib. SAR studies revealed that methyl group at C-2 position of quinazoline ring could significantly improve the antitumor potency of 4-anilinoquinazolines. The same conclusion was also drawn according to the results of Western blotting analysis. Among all the tested compounds, **19g** exhibited extremely potent against H1975 with an IC_{50} value of 0.11μM, remarkably lower than that of gefitinib (1.23μM). The results of western blotting analysis showed that compounds **19c** and **19g** could notably inhibit the expression of phosphorylated EGFR, especially **19g**, almost inhibited completely.

[1]The epidermal growth factor receptor (EGFR, erbB1) is a member of the erbB family of receptors including erbB2/HER2, erbB3/HER3, and erbB4/HER4.[1, 2] It is over-expressed in a large number of human solid tumors, and is associated with cancer cell proliferation, apoptosis, angiogenesis, and metastasis.[3,4] Therefore, EGFR as a target of anticancer drugs has received much attention in the past decade. A series of EGFR inhibitors with different molecular scaffolds have been discovered. Among them, 4-anilinoquinazolines play a key role and have been extensively studied. As shown in **Figure 1**, PD153035, named 6, 7-dimethoxy-4-anilinoquinazoline, was the first EGFR inhibitor reported in 1994.[5] Though finally it was abandoned due to the depressing water solubility, the chemical structure of PD153035 was reserved to do structure modification. Gefitinib and erlotinib have been used to treat non-small-cell lung cancer (NSCLC).[6-11] Canertinib is the first irreversible kinase inhibitor to enter clinical trial. [12]

These agents all belong to the 4-anilinoquinazoline class of inhibitors and the key features between the receptor and this template have been revealed as follows [13,14], (1) the quinazoline moiety fits into the ATP binding pocket of the kinase domain, (2) the N-1 of the quinazoline ring interacts with the backbone NH of Met-769 via a hydrogen bond, and water mediated hydrogen bonding is observed between the N-3 of the quinazoline ring and the Thr-766 side chain, (3) the aniline moiety lies in a deep and hydrophobic pocket, and (4) the substitution at C-6 or C-7 of the quinazoline ring conveys a more favorable pharmacokinetic profile and improves the physical properties.

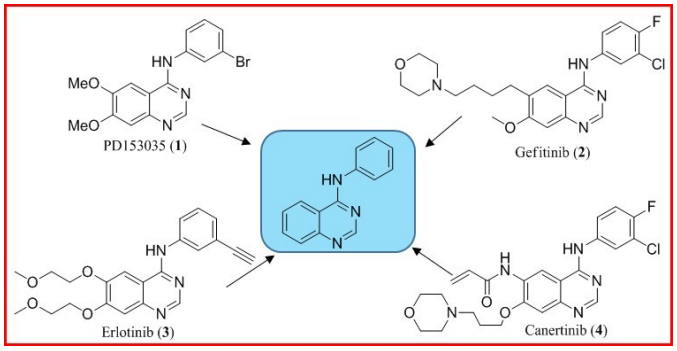


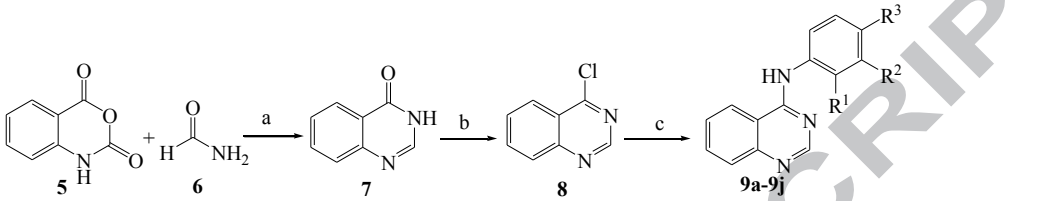
Figure1. Chemical structure of tyrosine kinase inhibitors

It was reported that 4-substitution was assumed to be optimal as aniline, with other linkers being less effective. However, the structure activity relationship (SAR) of 4-arylamino was reported barely. Therefore, nine 4-anilinoquinazolines with different anilines substituted at 4-position were synthesized and the SAR was discussed. In our ongoing to seek for more potential substituent to enhance the antitumor activity of 4-

[1] *Corresponding author: Tel.: +86 (0)29 38182201; e-mail address: zst6565@126.com

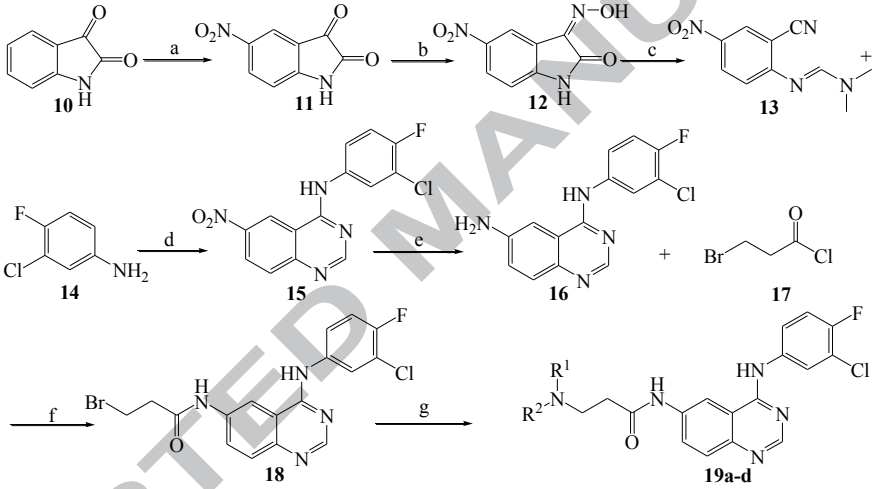
anilinoquinazolines, we focus on Carmi's work which reported 3-aminopropanamide at C-6 position of 4-anilinoquinazoline can covalently interact with a conserved cysteine residue in the kinase domain of EGFR.[15] In addition, it was reported methyl group at C-2 position of quinazoline ring could improve the antitumor activity according to the SAR of AZixa (MPC-6827), which was discovered as proapoptotic molecules and mitotic inhibitor with potency at low concentration in multiple tumor cell lines.[16] Therefore, novel 4-anilinoquinazoline derivatives **19a-19h** were synthesized with methyl group at C-2 position and 3-aminopropanamide at C-6 position. And fortunately, compound **19g** was found the most potent against H1975 with an IC_{50} value of 0.11μM, greatly lower than that of gefitinib with an IC_{50} value of 1.23μM.

Compounds **9a-9j** were prepared via a convenient and efficient way using isatoic anhydride as starting material. As shown in **Scheme 1**, quinazolin-*4(3H)*-one **7** was prepared via heating isatoic anhydride in formamide at 135~140 °C for 10h. Chlorination of **7** using thionyl chloride yielded the desired intermediated **8**. The coupling of **8** with anilines generated the corresponding target compounds **9a-9j**.



9a: R^1=H, R^2=H, R^3=H; 9b: R^1=H, R^2=Cl, R^3=F; 9c: R^1=H, R^2=NO_2, R^3=H;
9d: R^1=H, R^2=MeO, R^3=H; 9e: R^1=H, R^2=Br, R^3=H; 9f: R^1=H, R^2=Cl, R^3=H;
9g: R^1=H, R^2=F, R^3=H; 9h: R^1=F, R^2=H, R^3=H; 9i: R^1=H, R^2=H, R^3=F;
9j: R^1=Cl, R^2=H, R^3=Cl

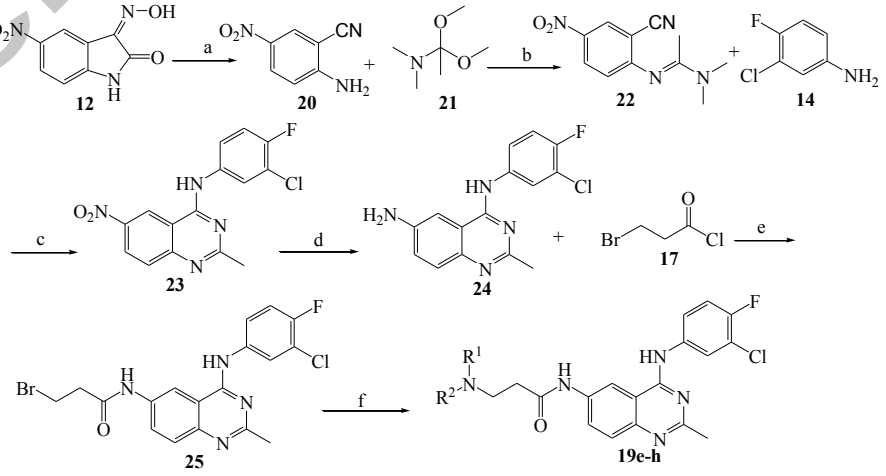
Scheme1. Reagents and conditions: (a) 135~140°C, 10h; (b) $SOCl_2$, DMF, 70°C, 5h; (c) aniline derivatives, isopropanol, reflux, 6h



19a: R^1R^2NH=morpholine **19b:** R^1R^2NH=piperidine

19c: R^1R^2NH=dimethylamine **19d:** R^1R^2NH=methanamine

Scheme2. Reagents and conditions: (a) 95% H_2SO_4, $NaNO_3$, 5°C, 3h; (b) $HONH_3Cl$, H_2O, sodium acetate, reflux, 30min; (c) $POCl_3$, DMF, Na_2CO_3, H_2O, 0°C – rt-70°C, 4h; (d) acetic acid, reflux, 3h; (e) ethyl acetate, $SnCl_2$, 75°C, 2h; (f) THF, 0°C – rt, TEA, 2h; (g) amine, KI, ethanol, reflux.



19e: R^1R^2NH=morpholine **19f:** R^1R^2NH=piperidine

19g: R^1R^2NH=dimethylamine **19h:** R^1R^2NH=methanamine

Scheme3. Reagents and conditions: (a) DMF, Na_2CO_3, 140°C, 1h; (b) toluene, acetic acid, 105°C, 1h; (c) acetic acid, reflux, 3.5h; (d) ethyl acetate, $SnCl_2$, 75°C, 3h; (e) THF, 0°C –rt, TEA, 3h; (f) amine, KI, ethanol, reflux.

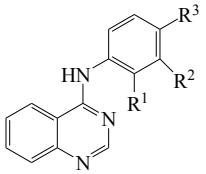
The target compounds **19a-19d** were prepared as shown in **Scheme 2**. It was a facile, novel and efficient strategy to synthesize 4-anilinoquinazolines using isatin **10** as starting material. Nitration of **10** using sodium nitrate in concentrated

sulfuric acid at 5°C gave **11**. Compound **11** underwent condensation with hydroxylamine hydrochloride followed by heating in the solution of DMF and $POCl_3$ to form **13**.[17] Compound **13** reacted with 3-chloro-4-fluoro-benzenamin to generate **15**. The nitro group of **15** was reduced by $SnCl_2 \cdot 2H_2O$ in ethyl acetate to produce **16**. Acylation of compound **16** with 3-bromopropanoyl chloride gave the corresponding intermediated **18**. Finally, reaction of compound **18** with different secondary amines in the presence of KI generated the target compounds **19a-19d**.[18-20]

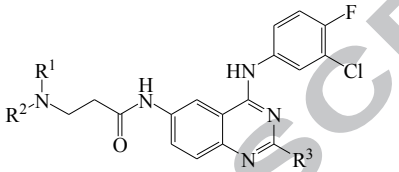
The synthesis of target compounds **19e-19h** was shown in **Scheme 3**. The starting material was the intermediate **12** synthesized in **Scheme 2**. The 2-amino-5-nitrobenzonitrile **20** was prepared by heating compound **12** in the presence of Na_2CO_3 in DMF at 140°C for 1h. Reaction of compound **20** with N, N-dimethylacetamide dimethyl acetal generated the key intermediate **22**. The following steps to synthesize the final compounds **19e-19h** was similar to the preparation of compounds **19a-19d** from compound **13**.

The effect of the target compounds on cell proliferation was evaluated by MTT assay in three human cancer cell lines, H1975 [21], HepG2 [22] and SMMC7721 [23], which over expresses EGFR. The marketed drug gefitinib, an EGFR inhibitor, was used as a positive control for the assay. The biologic results are shown in **Table 1**.

Table1. Inhibitory effect of target compounds **9a-9j** and **19a-19h** on the growth of the three tumor cell lines



9a-9i



19a-19h

Compd.	Structure			IC_{50}(μmol/L)[a]		
	R^1	R^2	R^3	H1975[b]	HepG2[c]	SMMC7721[d]
9a[17]	H	H	H	35.62±1.36	65.81±4.82	35.62±1.21
9b[17]	H	Cl	F	4.51±1.22	>100	50.1±3.30
9c[24]	H	NO_2	H	61.25±2.72	51.41±3.08	58.98±2.77
9d[17]	H	MeO	H	31.32±1.66	24.18±1.79	15.62±1.64
9e[17]	H	Br	H	10.56±1.44	78.62±3.85	24.65±1.06
9f[25]	H	Cl	H	10.38±1.22	23.47±1.24	22.04±2.21
9g[25]	H	F	H	7.58±1.07	13.23±1.82	14.98±1.52
9h[17]	F	H	H	10.64±1.36	69.34±1.94	93.9±4.79
9i[17]	H	H	F	23.51±1.89	28.96±2.27	22.04±1.97
9j[17]	Cl	H	Cl	7.12±1.26	32.51±2.15	9.86±1.52
19a	R^1R^2NH=morpholine		H	3.01±0.50	16.33±1.35	19.55±1.98
19b	R^1R^2NH =piperidine		H	1.85±0.15	8.59±1.39	12.57±1.08
19c	R^1R^2NH =dimethylamine		H	0.36±0.08	9.36±1.05	9.54±1.05
19d	R^1R^2NH =methanamine		H	0.68±0.09	14.78±1.69	20.87±1.19
19e	R^1R^2NH =morpholine		Me	1.14±0.06	1.98±0.21	3.26±0.63
19f	R^1R^2NH =piperidine		Me	0.87±0.09	3.21±0.58	2.84±0.66
19g	R^1R^2NH =dimethylamine		Me	0.11±0.03	1.86±0.12	1.04±0.07
19h	R^1R^2NH =methanamine		Me	0.48±0.09	1.13±0.11	1.98±0.41
Gefitinib				1.23±0.30	7.52±0.95	4.16±0.77

[a]IC_{50}, compound concentration required to inhibit tumor cell proliferation by 50%; [b]Human lung cancer cell line (H1975); [c]Human hematoma carcinoma cell line (HepG2); [d]Human hematoma carcinoma cell line (SMMC7721).

The results indicated that most compounds showed moderate to good activity with IC_{50} values in the μM range, except compound **9b** against HepG2 with an IC_{50} value more than 100μM. Eight compounds of **19a-19h** were found more potent against all three tumor cell lines than compounds of **9a-9j**. It was indicated that 3-aminopropanamide at C-6 position significantly improved the potency of 4-anilinoquinazoline.

We designed compounds **9a-9j** in order to identify a suitable 4-arylamino group of 4-anilinoquinazolines. Though all the compounds exhibited moderate potent against all three cell lines, compound **9b** was found more potent against H1975. The biologic results of compounds **9e-9g** with different halogen atoms at the same position**,** indicated fluorine was more suitable than chlorine and bromine to improve the antitumor activity of 4-anilinoquinazolines. The IC_{50} values of compounds **9g-9i** with fluorine at different positions of aniline ring, demonstrated that substituent at C-3 aniline position exhibited more potent than substituent at C-2 and C-4 aniline positions. There was a slight increase in inhibitor potency for 2, 4-dichlorobenzyl compound **9j**. It was found that larger groups such as methoxyl group (**9d**) and nitro group (**9c**) at C-3 aniline position exhibited reduced potency against tumor cell proliferation.

Compounds **19a-19h** were synthesized to seek for more potent EGFR inhibitors and to investigate whether methyl group at C-2 position of quinazoline ring can improve the antitumor activity of 4-anilinoquinazoline. By comparing the biological results of compounds **19a-19h** with the inhibitor potency of compound **9b**, it was concluded that 3-aminopropanamide at C-6 position could greatly improve the antitumor potency of 4-anilinoquinazolines. Replacing the morpholine of **19a** with other amines, including piperazine (**19b**), dimethyllamine (**19c**), and methanamine (**19d**), caused increase of the inhibitor potency, especially of compounds **19c** and **19d** against H1975. A similar trend was also reported in the series **19e-19h** with methyl group appending at C-2 position of quinazoline ring. Comparing the antitumor potency of compounds **19a-19d** with compounds **19e-19h**, it was revealed that methyl group at C-2 position of quinazoline ring could significantly improve the antitumor potency. All the compounds of **19a-19h** showed excellent inhibitor potency that was comparable or even better than that of positive control gefitinib, especially compound **19c** (0.36μM) and **19g (**0.11μM).

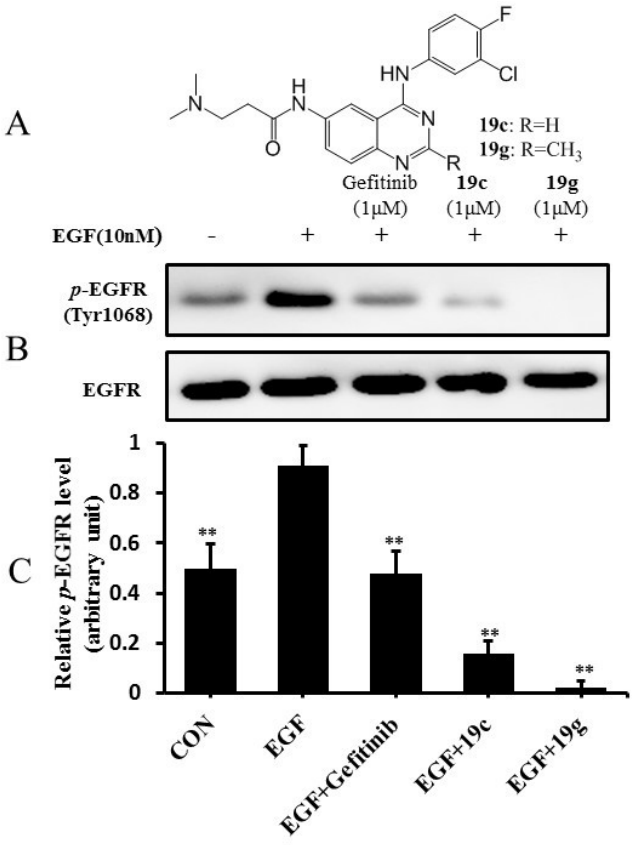


Figure2. Inhibitory effect of compound**19c** and **19g** upon EGFR autophosphorylation. (A) The chemical structure of compound **19c** and **19g**. (B) H1975 cells treated with or without gefitinib, **19c** and **19g**. The change of p-EGFR was analyzed by Western blotting. (C) The result of Western blotting was analyzed by Image J. The X-axis represents different groups and the Y-axis represents the ratio of the gray value of p-EGFR to that of EGFR. ***p*<0.01

In order to determine the EGFR inhibitor potency of **19a-19h**, compounds **19c** and **19g** were examined to determin their abilities to inhibit EGFR autophosphorlation in the H1975 cell line. Results of Western blotting analysis are shown in **Figure 2**. Both the tested compounds **19c** and **19g** exhibited more potent inhibitory potency than that of gefitinib at the same concentration. The column height of the last group incubated with 1μM of compound **19g** was lower than that of the group incubated with **19c**, indicating that methyl group at C-2 position of quinazoline ring had a positive effect on phosphorylated EGFR inhibition. Furthermore, compound **19g** showed encouraging inhibitory potency against EGFR at 1μM which almost could completely inhibit the expression of phosphorylated EGFR.

In conclusion, series of 4-anilinoquinazoline derivatives were facilely and efficiently synthesized and their EGFR inhibitor activity was evaluated by MTT assay in three human tumor cell lines. Compounds **9a-9j** were synthesized to elucidate the structure activity relationship of 4-arylamino. Novel compounds **19a-19h** were synthesized to seek for more powerful EGFR inhibitors and to explore whether methyl group at C-2 position of quinazoline ring could improve the inhibitor potency of 4-anilinoquinazolines. Fortunately, compound **19g** was found extremely potent against H1975 cells with an IC_{50} value of 0.11μM according to the MTT assay. By comparing the IC_{50} values of compounds **19a-19h**, it was concluded that methyl group at C-2 position of quinazoline ring had a positive effect on tumor cell proliferation. And the same conclusion was also drawn according to the results of Western blotting analysis.

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Graphical Abstract

Facile and efficient synthesis and biological evaluation of 4-anilinoquinazoline derivatives as EGFR inhibitors

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Zheng Wang,[a] Xue Wu,[c] Li Wang,[a] Jiao Zhang,[a] Jianli Liu,

[a] *Shaanxi Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Shaanxi Province Key Laboratory of New Drugs and Chinese Medicine Foundation Research, Shaanxi Rheumatism and Tumor Center of TCM Engineering Technology Research,Shaanxi University of Chinese Medicine, Xian Yang 712083, China*

[b]*Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, College of Life Science, Northwest University, Xi'an 710069, China*

[c]*Department of Burns and Cutaneous Surgery, Xijing Hospital, Xi'an, 710032,China*