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Design, synthesis and biological evaluation of biphenylurea derivatives as VEGFR-2 kinase inhibitors (II)

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ABSTRACT

Inhibition of VEGFR-2 signaling pathway is one of the most promising approaches for the treatment of cancer. In this paper, we reported the design, synthesis, and biological evaluation of a series of biphenylurea derivatives as VEGFR-2 inhibitors. Among these compounds, **39** exhibited potent inhibitory activity against VEGFR-2 both *in vitro* and *in vivo*. The antiangiogenesis activity of **39** was further confirmed by both tube formation assay and chick chorioallantoic membrane assay.

1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is a normal and indispensable process in growth and development as well as in wound healing and female reproductive cycling [1,2]. Pathological angiogenesis has been correlated with a variety of diseases, such as retinopathies, diabetes, rheumatoid arthritis, psoriasis, and cancers [3,4]. Tumor angiogenesis is vital for tumor growth as it is a fundamental step in the growth and metastasis of cancer [5], making angiogenesis inhibition a promising therapeutic strategy against cancer.

VEGFs and their receptors (especially VEGFR-2) are very important in the direct regulation of angiogenesis, and the overexpression of VEGFR-2 has been closely implicated in the angiogenesis of solid tumors [6]. Neutralization of the circulating VEGF by antibodies and inhibition of VEGF receptor tyrosine kinases with small molecules have been found to effectively inhibit angiogenesis, tumor progression and dissemination [7]. Enormous efforts have been made on the development of drugs targeting VEGFR-2, leading to several launched anticancer drugs, such as sunitinib [8,9], sorafenib [10], vandetanib [11], pazopanib [12] and axitinib [13]. Nevertheless, intolerable adverse effects that cause dose reductions and treatment discontinuations may potentially negate the life-prolonging effects of these drugs [14], indicating an unmet need for safer VEGFR-2 inhibitors with high efficacy. As a part of our continuing program on VEGFR-2 inhibitors, we reported here the discovery and structural optimization of biphenylurea derivatives as new VEGFR-2 inhibitors. Sorafenib and pazopanib are now being used in clinic for the treatment of advanced renal cell carcinoma (RCC). A close look of their binding modes may provide some useful information; in general, both molecules bind to the ATP binding site, penetrated into the extended hydrophobic pocket and filled the RDP (regulatory domain pocket) region, locking the protein in a "DFG-out" conformation (Fig. 1); residing in the ATP binding site, Cys919 forms H-bonds with the pyridine nitrogen and amide N–H of sorafenib and the anilinopyrimidine scaffold of pazopanib. Glu885 is served as a H-bond donor to both molecules; Asp1046, however, only interacted with sorafenib [15].

Direct H-bonds with Glu885 and Asp1046 were considered a significant opportunity for better selectivity, and greater molecular size in the RDP site would cause a reduction of efficacy [15]. We

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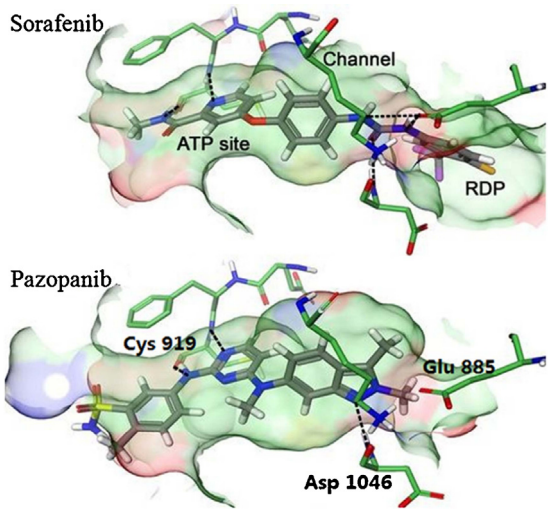


Fig. 1. Binding models of sorafenib (PDB ID: 4ASD) and pazopanib with VEGFR-2. Reproduced with the permission from Ref. [15].

envisioned that an additional hydrogen bond to the Asp1046 by grafting the biphenylurea moiety from sorafenib to the anilinopyrimidine structure of pazopanib would hopefully provide a new scaffold with high selectivity. Tuning the size of the 3-chloro-4-(trifluoromethyl)benzene ring may yield better efficacy (Fig. 2). To our delight, compound **1** inhibited VEGFR-2 at the enzymatic level with an IC_{50} value of 69 nmol/L. Inspired by this promising result, we then synthesized a series of biphenylurea analogs of **1** to investigate the structure–activity relationships (SARs).

2. Experimental

The synthesis of analogs was illustrated in Scheme 1. 2,3 (or 4,6)-Dichloropyrimidine (**2** and **3**) were coupled with substituted *p*-nitrophenol to give intermediates **4–7** which were subsequently reduced to corresponding amine **8–11**. Ureas **12–19** were prepared through a well-established method [16]. All the derivatives (**1**, **20–40**) were prepared through an acid-catalyzed S_NAr reaction of urea intermediates with substituted anilines in the final step. All derivatives listed in Tables 1–3 were evaluated for their enzymatic activities against VEGFR-2 and cellular activities against adenocarcinomic human alveolar basal epithelial cells (A549) and human microvascular endothelial cell (HMEC-1).

3. Results and discussion

We first explored the effect of variation in rings B and C of compound **1** (Table 1). Incorporation of F atom on an aromatic ring could increase lipophilicity, mask a potential metabolic site and offer a polarized C–F bond that may participate in binding interaction [17]. Regorafenib, for example, was invented as a fluoro derivative of sorafenib with better activities. The derivatives with fluoro substitution on the B ring (**20** and **21**) were synthesized. Unfortunately, no improvement of activity was observed. We also altered the substitution pattern of the

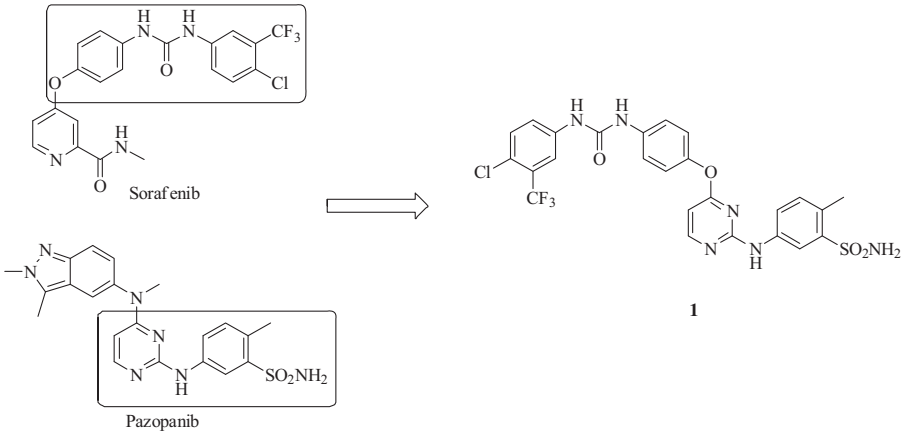
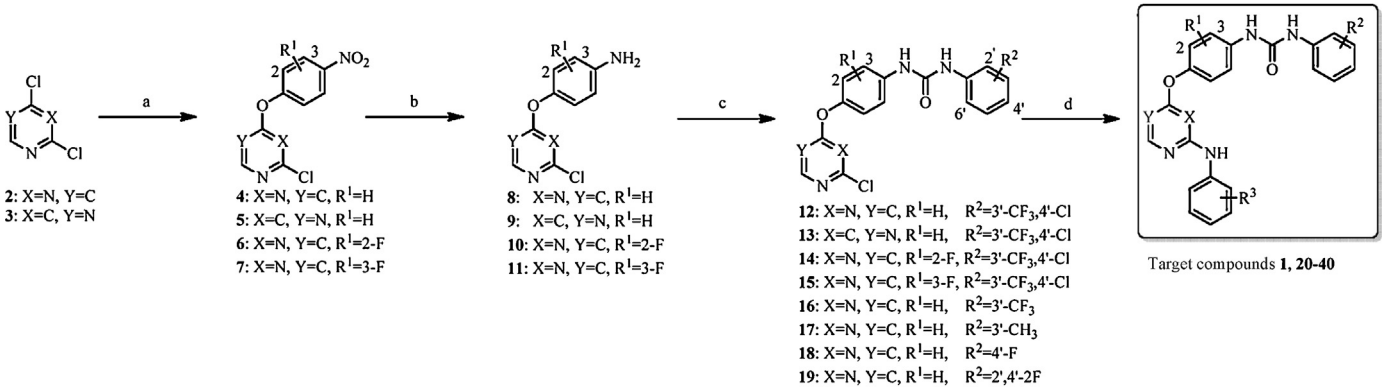


Fig. 2. Design of hybrid compound **1**.



Scheme 1. Synthesis of compounds **1,** and **20–40**. Reagents and conditions: (a) substituted *p*-nitrophenol, aq. sodium hydroxide, acetone, 0–80 °C; (b) Fe, aq. ammonium chloride, ethanol, 70 °C; (c) substituted anilines, triphosgene, triethyl amine, methylene chloride, r.t.; and (d) substituted anilines, 36% HCl, isopropanol, sealed tube, 100 °C.

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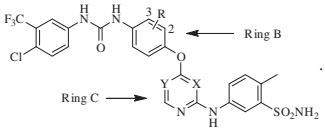
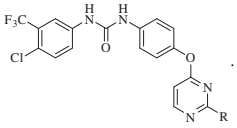
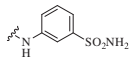
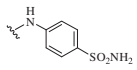
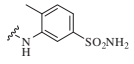
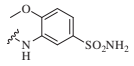
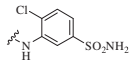
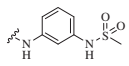
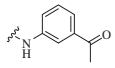
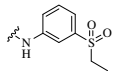
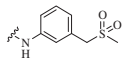
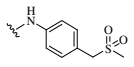
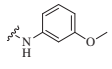
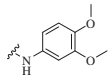
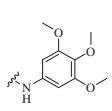
Table 1
SAR of ring B and ring C.



Compd.	*R*	*X*	*Y*	IC_{50} (μmol/L)		
				VEGFR-2	A549	HMEC-1
1	H	N	C	0.069 ± 0.033	5.12 ± 1.08	5.92 ± 1.35
20	2-F	N	C	0.072 ± 0.007	>100	>100
21	3-F	N	C	0.079 ± 0.004	19.22 ± 15.68	18.33 ± 14.63
22	H	C	N	0.123 ± 0.058	10.46 ± 3.84	11.02 ± 1.45
Su11248	–	–	–	0.002 ± 0.001	0.10 ± 0.01	0.12 ± 0.03

Table 2
SAR of 2-substituents on the pyrimidine ring.



Compd.	*R*	IC_{50} (μmol/L)		
		VEGFR-2	A549	HMEC-1
23		0.039 ± 0.006	>100	>100
24		0.069 ± 0.033	>100	>100
25		0.004 ± 0.001	>100	>100
26		0.064 ± 0.016	>100	>100
27		0.007 ± 0.001	>100	>100
28		0.007 ± 0.008	>100	>100
29		0.014 ± 0.001	>100	>100
30		0.006 ± 0.010	7.92 ± 2.34	7.87 ± 2.01
31		0.004 ± 0.002	>100	>100
32		2.269 ± 1.852	>100	>100
33		0.048 ± 0.024	>100	>100
34		0.007 ± 0.002	2.88 ± 0.89	2.81 ± 1.02
35		0.025 ± 0.004	>100	>100
36		0.005 ± 0.006	8.40 ± 3.039	46.28 ± 2.78
Su11248	–	0.002 ± 0.001	0.10 ± 0.01	0.12 ± 0.03

pyrimidine ring to move the nitrogen closer to the amino acid residues in the hinge region, hoping for an extra interaction. Compound **22**, the 4,6-disubstitued pyrimidine analog of **1**, was synthesized and examined. The result indicated that **22** displayed a 1.8-fold reduction in enzymatic activity (IC_{50}: 123 nmol/L). These facts implied that unmodified rings B and C in compound **1** were favorable for maintaining activity.

Next, we investigated the effect of substitution pattern of the aniline at pyrimidine ring on the activity. At first, a set of five sulfonamides including four *meta*-sulfonamides (**23**, **25**–**27**) and one *para*-sulfonamide (**24**) were explored. Although these derivatives exhibited high inhibitory potency against VEGFR-2 with IC_{50} values ranging from 4 nmol/L to 69 nmol/L, they were inactive on cancer cell proliferation assay. Then, the synthesis of a series of derivatives with various substituted anilines on pyrimidine ring led to **34** (3,4-dimethyl substitution) which exhibited potent activities in both enzymatic and cellular assays (IC_{50} values of 7 nmol/L, 2.88 μmol/L and 2.81 μmol/L for VEGFR-2, A549 and HMEC-1, respectively). Interestingly, unsubstituted aniline derivative **36** showed high potency against VEGFR-2 and A549 (IC_{50}: 5 nmol/L and 8.4 μmol/L, respectively) but low potency against HMEC-1 (IC_{50}: 46.28 μmol/L). Taken together, the aniline group at the 2-position of pyrimidine was tolerable to modification. 3,4-dimethoxy substituted analog was proven to be the best in this series of compounds at both enzymatic and cellular levels, and therefore further investigation was focused on the 3,4-dimethoxy substituted analogs.

Finally, a set of analogs of **34** was prepared to explore the influence of the substituent on ring A, and this exploration led to analogs **37**–**40** as shown in Table 3. Among them, **39** exhibited high enzymatic inhibitory activity with an IC_{50} value of 0.4 nmol/L, which was one of the most potent VEGFR-2 inhibitors as far as we knew. The privileged compound **39** was further examined in HUVEC proliferation assay and it exhibited high inhibitory potency with an IC_{50} value of 20.36 nmol/L.

Compound **39** was then profiled against a panel of tyrosine kinases. The result demonstrated that **39** inhibited a distinctive, narrow range of kinases at pharmacologically relevant concentrations (as shown in Table 4). In addition to its high inhibitory potency on VEGFR-2, **39** also exhibited great potency against VEGFR-1, PDGFR-α and PDGFR-β with IC_{50} values of 3.4, 4.4 and 3.6 nmol/L, respectively. Simultaneous inhibition of these distinct proangiogenic receptors may enhance the antitumor efficacy of **39** and overcome resistance to VEGF- and VEGFR-2-pathway targeted agents. Moreover, **39** showed high selectivity over RET (1574-fold, IC_{50}: 629.7 nmol/L) and excellent selectivity (>25,000-fold) over ErbB2, ErbB4, EGFR, EGFR (T790M/L858R), ABL, EPH-A2, FGFR-1, FGFR-2 and IGF-1R.

Subsequently, inhibition of VEGF stimulated of cell proliferation of HUVEC by **39** (positive control: AZD2171 [18]) was tested. As shown in Fig. 3, the VEGF-induced phosphorylation of VEGFR2

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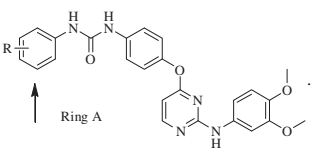
Table 3
SAR of ring A.



Compd.	*R*	IC_{50} (μmol/L)		
		VEGFR-2	A549	HMEC-1
37	3-CF_3	0.014 ± 0.001	5.83 ± 0.45	5.07 ± 0.78
38	3-CH_3	0.020 ± 0.004	4.06 ± 1.48	4.24 ± 1.65
39	4-F	0.0004 ± 0.0002	2.65 ± 1.30	2.93 ± 1.45
40	2,4-difluoro	0.0016 ± 0.0001	1.37 ± 0.45	1.38 ± 1.16
Su11248	–	0.002 ± 0.001	0.10 ± 0.01	0.12 ± 0.03

Table 4
Kinase-selectivity profiling of compound **39**.

Enzyme	IC_{50} (nmol/L)	Enzyme	IC_{50} (nmol/L)
VEGFR-1	3.4	EGFR	>10,000
VEGFR-2	0.4	EGFR(T790M/L858R)	>10,000
VEGFR-3	215.8	ABL	>10,000
RET	629.7	EPH-A2	>10,000
PDGFR-α	4.4	FGFR-1	>10,000
PDGFR-β	3.6	FGFR-2	>10,000
ErbB2	>10,000	IGF-1R	>10,000
ErbB4	>10,000		

(p-VEGFR-2) was substantially decreased in the presence of **39** in a dose-dependent manner. Meanwhile, Akt and Erk, the downstream effector of VEGF/VEGFR, were also attenuated in a dose-dependent manner.

Eventually, the anti-angiogenic effect of **39** was also examined. *In vitro* tube formation assay of HUVECs showed that HUVECs formed a complete network structure within 6 h. After treatment of **39**, the tube formation was suppressed in a dose-dependent manner (Fig. 4A). Further examination of the

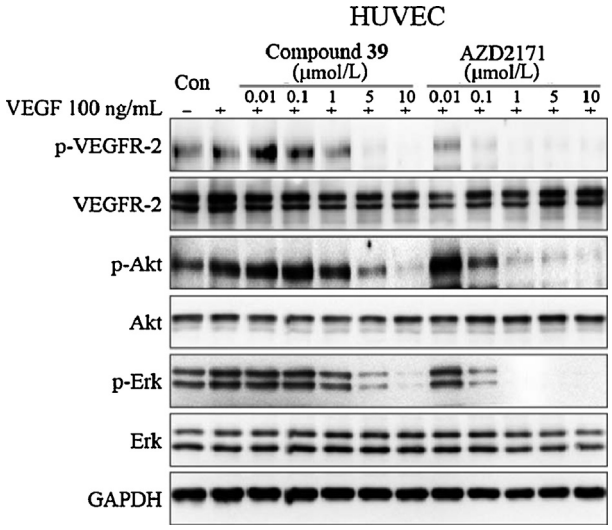


Fig. 3. Inhibition of VEGFR-2 phosphorylation and related signaling pathways by compound **39**. HUVEC cells were starved for 12 h and treated with indicated concentration of compounds for 2 h and then stimulated by 100 ng/mL VEGF for 15 min. Cells were harvested and subjected to Western blot analysis.

antiangiogenic effect of **39** was performed, using chick chorioallantoic membrane (CAM) assay, a method to evaluate *in vivo* neovascularization. The results (Fig. 4B) demonstrated that vascularization in chick embryos was significantly inhibited when the embryos were treated with **39** (1 and 10 nmol per egg). These results proved that **39** possessed potent antiangiogenic activities both *in vitro* and *in vivo*.

4. Conclusion

In summary, we designed and synthesized a series of compounds as VEGFR2 inhibitors. A highly potent VEGFR-2 inhibitor **39** was identified with an IC_{50} value of 0.4 nmol/L. Kinase profiling study indicated that **39** also inhibited three distinct proangiogenic receptors, VEGFR-1, PDGFR-α and PDGFR-β with high selectivity over other kinases. More importantly, **39**

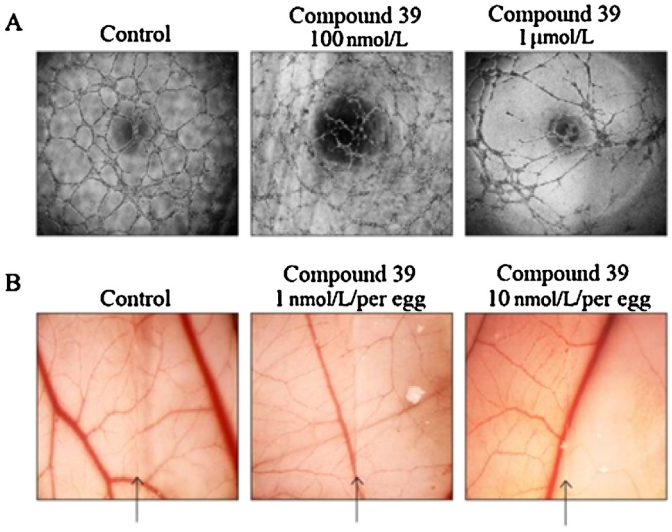


Fig. 4. Antiangiogenesis activities of compound **39**. A. Compound **39** inhibited *in vitro* tube formation of HUVECs. HUVECs containing with or without indicated concentration of compound were seeded in 96-well plates which were coated with Matrigel. After 6 h, the tube was examined with an inverted phase contrast microscope. B. Compound **39** showed anti-angiogenesis in CAM model. Glasscover-slip saturated with or without compound **39** was placed on the right side of per field. Arrows indicated the edge line of the coverglass.

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effectively inhibited angiogenesis in both *in vitro* HUVEC tube formation assay and *in vivo* CAM assay. Therefore, a new VEGFR-2 inhibitor scaffold and an understanding of their SARs offer promising opportunities to develop more effective anticancer agents.

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