Design, synthesis, and biological evaluation of tetrahydroisoquinoline-based diaryl urea derivatives for suppressing VEGFR-2 signaling

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A novel structural series of tetrahydroisoquinoline-based compounds that incorporate the diaryl urea moiety was designed, synthesized, and biologically evaluated as suppressors of VEFGR-2 signaling. As a consequence, compounds 9k and 9s exhibited comparable or superior cytotoxic activity to that of gefitinib against the tested three cell lines, including A549, MCF-7, and PC-3. Importantly, both of them downregulated the expression of VEGFR-2, and inhibited VEGFR-2 phosphorylation at the concentration of 0.5 or 1.0 μmol/l. Besides, they suppressed human umbilical vein endothelial cell tube formation at the concentration of 4.0 μmol/l. Considering their capability of down-regulating VEGFR-2 expression and inhibiting VEGFR-2 phosphorylation, 9k and 9s may serve as suppressors of angiogenesis for further investigation. ***Anti-Cancer Drugs*** **00:000–000 Copyright © 2018 Wolters Kluwer Health, Inc. All rights reserved.**

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Introduction

Tumor angiogenesis plays a crucial role in malignant cell proliferation and metastasis [1–3], which serves as a major contributor to cancer-related death. Therefore, inhibition of angiogenesis has been given increasing importance in the treatment of cancers [4,5]. As a well-established and specific angiogenic factor, VEGF regulates the angiogenesis and vascular migration and is overexpressed in a variety of malignant tumors, thereby being closely related to the growth, metastasis, and poor prognosis of tumors. VEGFR, belonging to receptor tyrosine kinase family, mediates many biochemical and physiological processes for neovascularization through binding to VEGF [6,7]. VEGFR mainly comprises three receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4). Among them, VEGFR-2 has been identified as the predominant mediator of tumor angiogenesis. Upon responding to the stimulus of VEGF, VEGFR-2 triggers the proliferation of vascular endothelial cell, thereby facilitating the blood vessel growth, increasing the vascular permeability and promoting tumor development [8–10]. So far, numerous antiangiogenic drugs (Fig. 1) that target VEGFR-2 signaling have been marketed, including sorafenib [11], regorafenib, and linifanib [12]. In structure, all of them contain a diaryl urea moiety, highlighting its importance as a structural element in inhibitors of VEGFR-2 signaling.

Tetrahydroisoquinoline alkaloids, as a class of naturally occurring bioactive ingredients from medicinal plants, were capable to suppress tumor proliferation through different mechanisms, such as alkylation, regulation of growth-related receptors and antiapoptotic genes, as well as inhibition of angiogenesis [13–16]. Choquette *et al.* [17] found that some tetrahydroisoquinolines can inhibit the formation of nascent microcapillaries and prevent tumor cells from absorbing nutrients by selectively suppressing VEGFR-2 signaling. Our group previously designed and synthesized a series of tetrahydroisoquinoline derivatives, among which compounds **17d** (IC_{50} = 2.6 nmol/l) and **17e** (IC_{50} = 0.89 nmol/l) (Fig. 2) exhibited remarkable antiproliferative activity against MCF-7 cells [18].

In view of the importance of diary urea as structural element of compounds that suppress VEGFR-2 signaling and the antitumor activity of tetrahydroisoquinoline alkaloids, we have therefore employed pharmacorphore-combination strategy for designing suppressors of VEFGR-2 signaling. On the basis of the tetrahydroisoquinolines discovered in our previous study, a series of structurally novel hybrid molecules were obtained by replacing (*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylic acyl moiety of them with diaryl urea fragments. Besides, the substituents on the phenyl attached to the tetrahydroisoquinoline carbon and the terminal aryl of the diaryl fragment were investigated.

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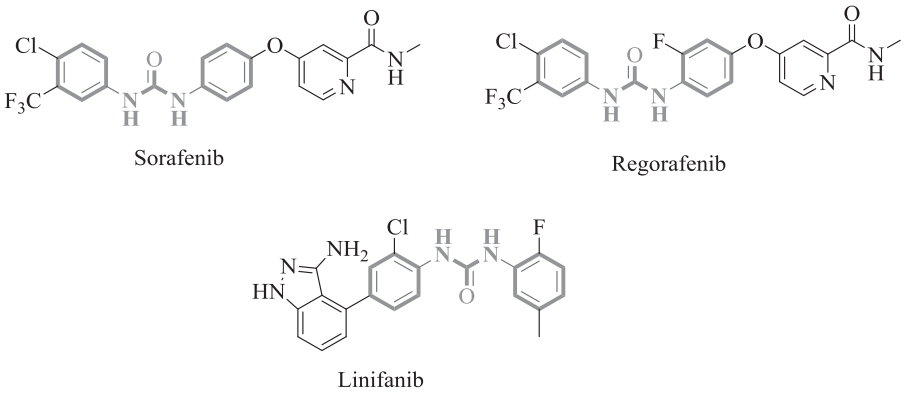
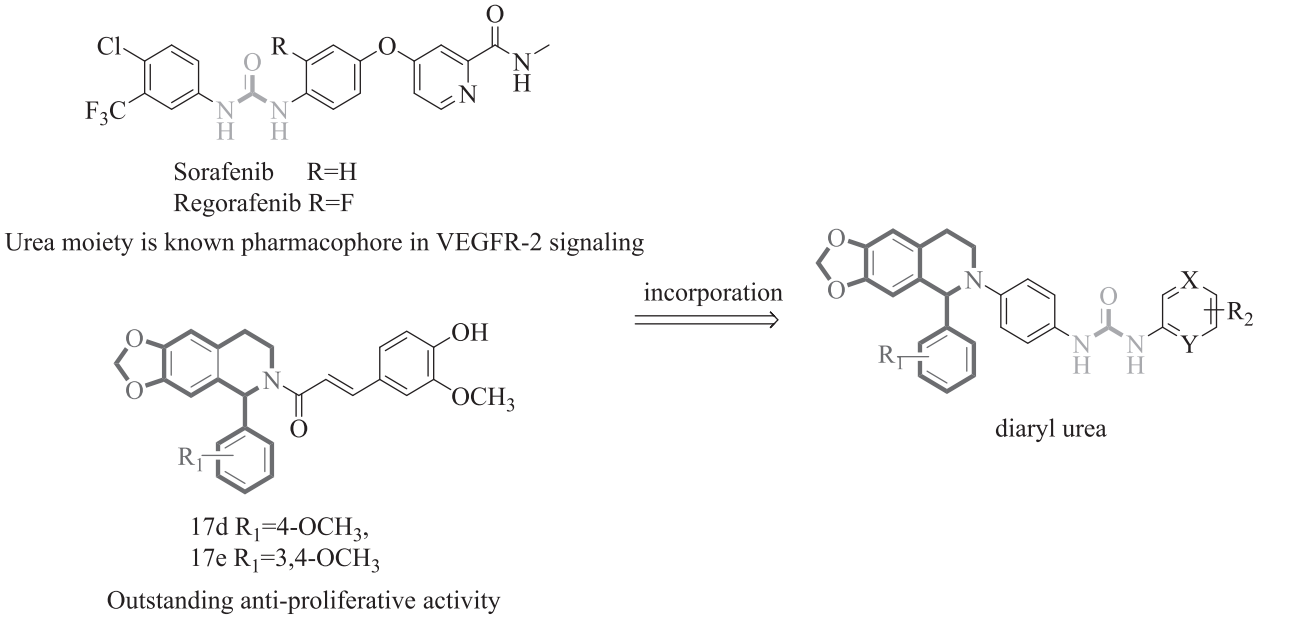
Fig. 1



The structures of sorafenib, regorafenib, and linifanib.

Fig. 2



The design ideas of novel diaryl urea compounds.

Materials and methods

Experimental section

General information

The reagents and solvents for reaction were purchased from common commercial suppliers. If necessary, purification was carried out before use. Melting points are determined on melting point apparatus (RDCSY-I) and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on 600 and 150 MHz instruments (Bruker, Fallanden, Switzerland), respectively, with tetramethylsilane as internal standard. MS spectra were measured with a Hewlett-Packard 1100 LC/MSD spectrometer (Agilent, Waldbronn, Germany).

General procedure for synthesis of compounds

*4-(5-(3,4-dimethoxyphenyl)-7,8-dihydro-[1,3]dioxolo[4,5-g] isoquinolin-6(5H)-yl)aniline (**8a**)*

5-(3,4-dimethoxyphenyl)-5,6,7,8-tetrahydro-[1,3]dioxolo[4,5-*g*]isoquinoline (**5a**) was prepared with our previously reported method [18]. A mixture of Compound **5a** (2.87 g, 9.2 mmol), *p*-fluoronitrobenzene

(1.26 g, 11.4 mmol), and K_2CO_3 (1.6 g, 11.6 mmol) in dimethylsulfoxide (DMSO) (20 ml) was stirred for 6 h at 80°C and cooled to room temperature. Afterward, the reaction mixture was diluted with aqueous solution and extracted with CH_2Cl_2. The organic layer was washed with brine, dried with anhydrous sodium sulfate, filtered, and evaporated in vacuum. The crude product was purified by column chromatography (petroleum ether/EtOAc = 4 : 1 ~ 2 : 1) to yield the compound **7a**. To the stirred solution of compound **7a** in 90% ethanol (80 ml), Fe (1.2 g, 21.4 mmol) and NH_4Cl (1.75 g, 32.7 mmol) were added. The mixture was refluxed for 3 h and filtrated to give the crude product after cooling to room temperature, which was purified by column chromatography to give compound **8a** as a yellow liquid.

Compound **8b** was synthesized according to the synthetic procedure given above (see details in supporting data, Supplementary Data, Supplemental digital content 1, *http://links.lww.com/ACD/A276*).

*1-(4-(5-(3,4-dimethoxyphenyl)-7,8-dihydro-[1,3]dioxolo[4,5-g] isoquinolin-6(5H)-yl)phenyl)-3-(4-methoxyphenyl)urea (**9a**)*

A solution of 4-dimethoxybenzoic acid (0.42 g, 2.74 mmol), DPPA (0.6 ml, 2.8 mmol), and Et_3N (0.4 ml, 2.9 mmol) in benzene (30 ml) was stirred under 40°C for 45 min, and then refluxed for 2 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved directly with $CHCl_3$ (10 ml) for next step. Then a solution of **8a** (1.10 g, 2.72 mmol) in $CHCl_3$ (10 ml) was added into the aforementioned mixture. The resulting mixture was then stirred on oil bath at 65°C for 4 h. After the reaction was completed, the solvent was removed under reduced pressure, and the residue was dissolved with CH_2Cl_2 (30 ml), washed with brine (50 ml × 2), dried over anhydrous Na_2SO_4, filtered, and concentrated to afford the crude product, which was further purified by silica gel flash chromatography (petroleum ether/EtOAc = 6 : 1 ~ 4 : 1) to furnish 0.3 g of **9a** as a white solid.

Compounds **9b ~ 9s** were synthesized according to the synthetic procedure given above (see details in supporting data, Supplementary Data, Supplemental digital content 1, *http://links.lww.com/ACD/A276*).

Methyl thiazolyl tetrazolium assay

The antiproliferative activity of compounds against A549, MCF-7, and PC-3 cell lines, as well as human umbilical vein endothelial cells (HUVECs) was evaluated by methyl thiazolyl tetrazolium assay (MTT) assay. Exponentially growing cells were harvested and plated in 96-well plates at a concentration of 5×10^3 cells/well. The cells in wells were treated with title compounds, respectively, at various concentrations for 48 h. Then, 22 ml of MTT (5 mg/ml) was added to each well and incubated for 4 h at 37°C. Supernatant was discarded, and DMSO was added to each well. Absorbance values were determined at 570 nm. The IC_{50} values were calculated according to inhibition ratios.

$$\text{Inhibition rate}\,(\%) = \frac{\text{OD control} - \text{OD administration}}{\text{OD control}} \times 100\,\%.$$

Immunofluorescence assay

Cells were washed with PBS and fixated in paraformaldehyde (4%). Samples were permeabilized and blocked in 0.1% Triton X-100/10% goat serum in PBS for 1 h at room temperature and incubated with primary antibodies (goat-anti-human VEGFR-2, rabbit-anti-human Phospho-VEGFR-2 (Tyr1175) overnight at 4°C. After washing with PBS, samples were incubated with secondary antibody (mouse anti-goat IgG-Cy3, mouse anti-rabbit IgG-FITC) for 30 min and DAPI for 5 min at 37°C. After discarding DAPI and washing with PBS, photographs were taken under a fluorescence microscope (Nikon Eclipse, TS100-FDH1; Nikon Corporation, Tokyo, Japan).

Tube formation assay

Overall, 75 μl of growth factor-reduced Matrigel (Corning Incorporated, Shanghai, China) was loaded into prechilled 96-well tissue plates, and plates were placed at 37°C for 60 min. HUVECs ($6 \sim 8 \times 10^4$ cells) were added into each well and cultured in endothelial cell medium on the gel for 4 ~ 6 h. Different concentrations of test compound and sofafenib were then added and incubated for 8 h. The capillary networks were photographed by a microscope.

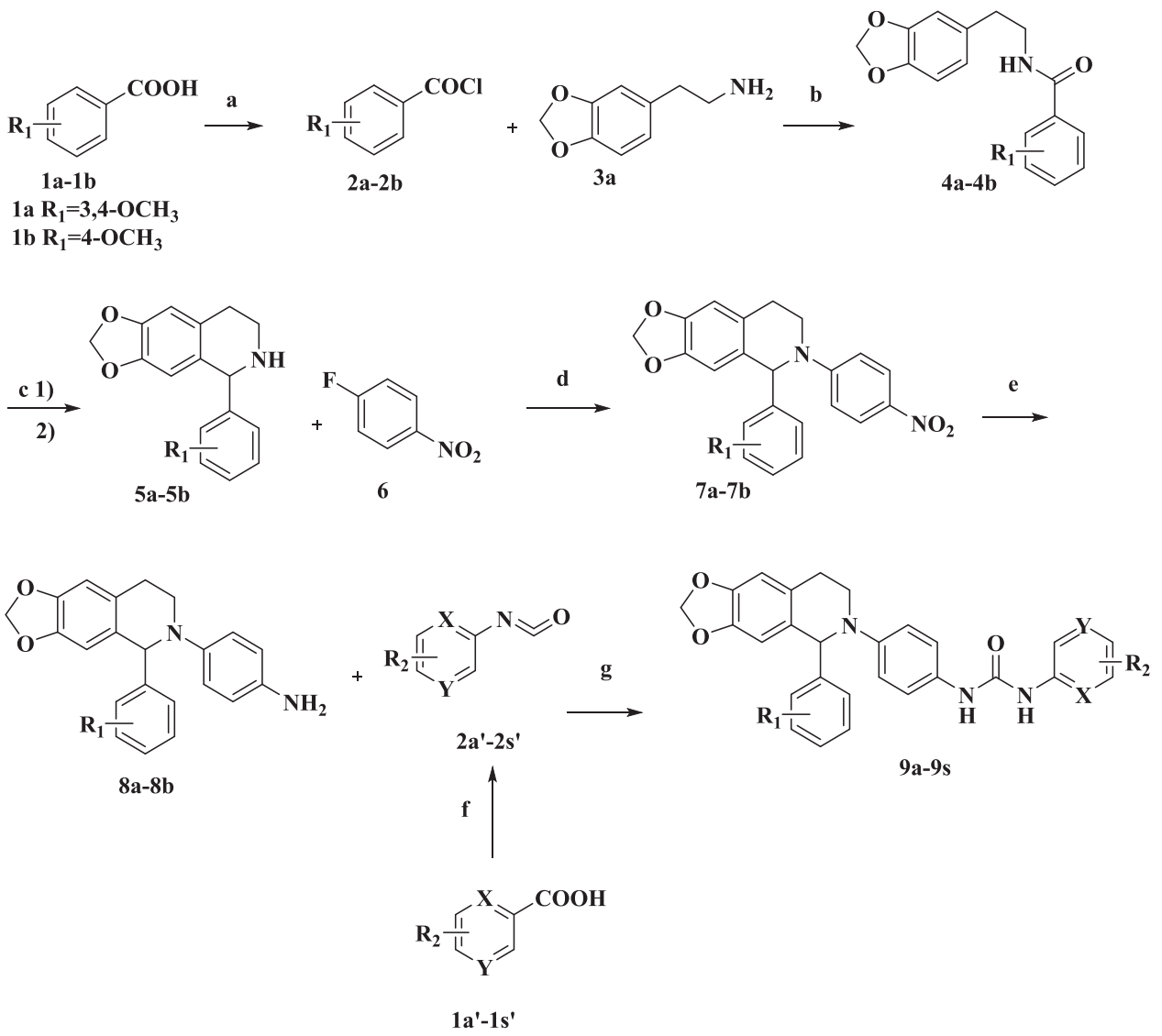
Results

Chemistry

The synthesis of target compounds **9a–9s** was depicted in Scheme 1. The tetrahydroisoquinoline intermediates **5a–5b** were prepared in accordance with our reported procedure [18]. Nucleophilic Aromatic Substitution reaction of **5a–5b** with 1-fluoro-4-nitrobenzene furnished **7a–7b**, which were subsequently reduced to corresponding aniline derivatives **8a–8b**. Meanwhile, the aromatic acids **1a'–1s'** were converted into corresponding isocyanates upon treatment with diphenyl azidophosphate (DPPA). Finally, condensation of **8a–8b** with the newly afforded isocyanates provided the tetrahydroisoquinoline-based diaryl urea derivatives **9a–9s** as the target compounds. All of them are structurally determined by ^1H NMR, ^{13}C NMR, and MS.

Reagents and conditions used were as follows: (a) $SOCl_2$, toluene, 77°C; (b) CH_2Cl_2, Et_3N, 0°C; (c) (1) $POCl_3$, toluene, 115°C; (2) $NaBH_4$, CH_3OH, 0°C; (d) K_2CO_3, DMSO, 80°C; (e) Fe, NH_4Cl, 90% EtOH, 90°C; (f) DPPA, Et_3N, benzene, reflux; and (g) $CHCl_3$, reflux.

Scheme 1



Synthesis of compounds **9a–9s**.

Biological activity

In-vitro antiproliferative activity

All the target compounds were biologically evaluated for their antiproliferative activities against A549 (lung cancer), MCF-7 (breast cancer), and PC-3 (prostate cancer) cell lines with gefitinib as the reference using MTT assay. As a result, a majority of compounds displayed moderate antiproliferative activities (Table 1). Among them, compounds **9j**, **9o**, and **9q** are comparable to gefitinib in the antiproliferative activity against A549 cell line. Compounds **9k** ($IC_{50} = 3.24 \pm 0.71$ μmol/l) and **9s** ($IC_{50} = 2.81 \pm 0.43$ μmol/l) showed stronger inhibitory activity than gefitinib against A549 cell line. Compounds **9i**, **9j**, **9o**, **9p**, **9r**, **9k**, and **9s** were superior to gefitinib in the antiproliferative activity against MCF-7 cell line. Compounds **9j**, **9k**, **9l**, **9o**, **9q**, and **9s** were comparable or superior to gefitinib in cytotoxic activity against PC-3 cells. In general, compound **9s** exhibited the most attractive cytotoxic activity against all the tested three cell lines throughout this series with the IC_{50} at the single-digit micromolar level. Besides, compound **9k** exhibited acceptable antiproliferative activity against A549 and MCF-7 cell lines. Compounds **9k** and **9s**, as the representatives of this series, were subsequently assayed against HUVECs for evaluating their cytotoxicity toward normal cells with gefitinib as the reference. As illustrated by Table 2, they displayed comparable cytotoxic profile toward HUVECs to that of gefitinib with IC_{50} values at two-digit micromolar level.

Table 1 The inhibitory activities of compounds against antiproliferation induced by A549, MCF-7, and PC-3 ($n = 3$)

Compounds	R_1	R_2	X, Y	IC_{50} (mean ± SD) (μmol/l)		
				A549	MCF-7	PC-3
9a	3,4-OCH_3	4-OCH_3	C, C	48.33 ± 5.04	> 100	> 100
9b	3,4-OCH_3	4-CH_3	C, C	> 100	> 100	> 100
9c	3,4-OCH_3	H	C, C	38.41 ± 3.73	49.92 ± 6.54	57.6 ± 4.63
9d	3,4-OCH_3	4-Cl	C, C	57.63 ± 5.09	95.3 ± 6.67	> 100
9e	3,4-OCH_3	3-Cl	C, C	30.72 ± 2.46	38.21 ± 4.31	> 100
9f	3,4-OCH_3	2-Cl	C, C	86.40 ± 3.49	> 100	97.28 ± 5.45
9g	3,4-OCH_3	4-F	C, C	29.71 ± 2.53	58.24 ± 4.53	58.83 ± 3.57
9h	3,4-OCH_3	4-CF_3	C, C	34.56 ± 3.45	25.61 ± 2.87	44.83 ± 4.69
9i	3,4-OCH_3	4-NO_2	C, C	12.56 ± 3.52	9.62 ± 2.78	23.68 ± 3.36
9j	3,4-OCH_3	H	C, N	7.23 ± 1.08	9.76 ± 1.89	11.52 ± 2.03
9k	3,4-OCH_3	H	N, N	3.24 ± 0.71	6.42 ± 0.63	16.64 ± 1.72
9l	4-OCH_3	3,4-OCH_3	C, C	11.21 ± 1.73	16.96 ± 1.82	12.04 ± 0.77
9m	4-OCH_3	4-OCH_3	C, C	25.28 ± 2.44	33.92 ± 2.56	62.10 ± 6.66
9n	4-OCH_3	H	C, C	28.82 ± 2.52	48.64 ± 4.18	> 100
9o	4-OCH_3	4-Cl	C, C	6.29 ± 0.39	7.53 ± 0.97	14.48 ± 1.75
9p	4-OCH_3	4-CF_3	C, C	9.63 ± 1.02	4.96 ± 0.39	28.86 ± 2.08
9q	4-OCH_3	4-NO_2	C, C	7.95 ± 0.91	14.24 ± 2.08	11.17 ± 1.55
9r	4-OCH_3	H	C, N	12.32 ± 1.65	9.64 ± 1.25	22.41 ± 2.13
9s	4-OCH_3	H	N, N	2.81 ± 0.43	6.52 ± 0.75	8.09 ± 0.93
Gefitinib	–	–	–	5.62 ± 1.78	10.56 ± 0.86	14.38 ± 2.61

The antiproliferative activities of compounds against all the tested cell lines were determined using the methyl thiazolyl tetrazolium assay, and gefitinib was employed as the positive control.
The results were expressed as the IC_{50}.
Bold values represent the compounds synthesized.

Table 2 The cytotoxicity of compounds on human umbilical vein endothelial cells

Compounds	IC_{50} (μmol/l)
9k	28.09 ± 2.01
9s	14.89 ± 1.93
Gefitinib	19.38 ± 1.95

The growth inhibitory activity of compounds against HUVECs was determined using the MTT assay, and gefitinib was employed as the positive control.
The results were shown as the IC_{50} values (mean ± SD, four biological replicates).
HUVEC, human umbilical vein endothelial cell; MTT, methyl thiazolyl tetrazolium.
Bold values represent the compounds synthesized.

Effect of 9k and 9s on VEGFR-2 expression and phosphorylation

Given the importance of VEGFR-2 in VEGF signaling pathway, which mediates angiogenesis by its phosphorylation, compounds **9k** and **9s** were investigated for their effect on the expression and phosphorylation of VEGFR-2 in A549 and HUVECs by immunofluorescence assay with sorafenib as the positive control.

As shown in Fig. 3, both compounds **9k** and **9s** down-regulated the expression of VEFGR-2 in A549 cells, and effectively blocked the phosphorylation of VEFGR-2 at the concentration of 0.5 or 1.0 μmol/l. At the concentration of 1.0 μmol/l, the capability of compound **9s** to down-regulate VEFGR-2 expression was stronger than that of sorafenib, and **9k** was as effective as sorafenib. Furthermore, when investigated at the same dosage, both compounds led to more dramatic down-regulation of VEGFR-2 phosphorylation in comparison with sorafenib.

As demonstrated in Fig. 4, phosphorylation of VEGFR-2 can be induced by hVEGF in HUVECs. Compared with hVEGF (+)/drug (−) group (Fig. 5b), compounds **9k** and **9s** could down-regulate the expression of VEFGR-2 and effectively inhibit the phosphorylation of VEFGR-2 at the concentrations of both 0.25 and 0.5 μmol/l. At the concentration of 0.5 μmol/l, compound **9k** exhibited stronger suppressive efficacy against VEFGR-2 phosphorylation than that of sorafenib, whereas compound **9s** was comparable to that of sorafenib.

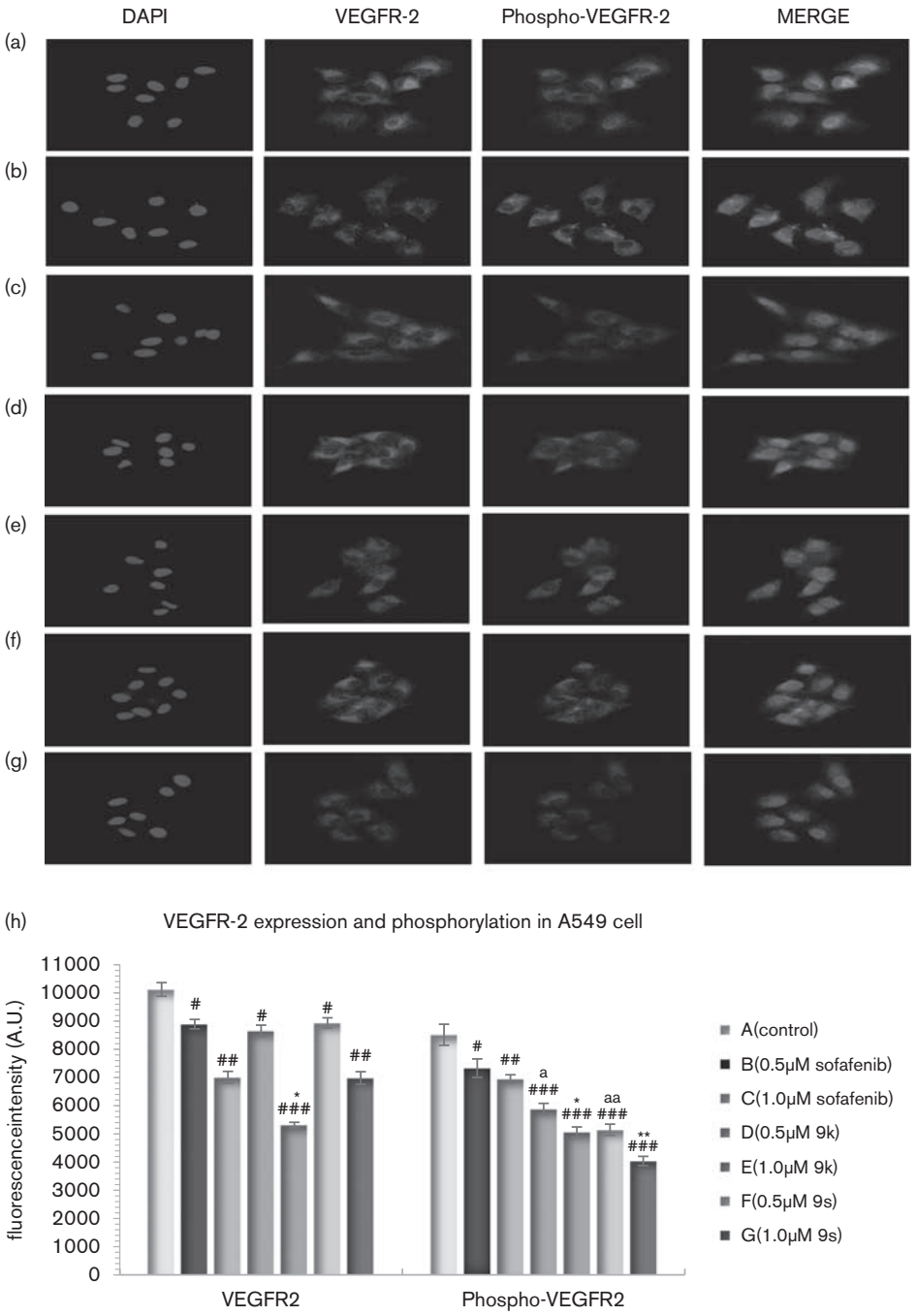
Tube formation assay

Compounds **9k** and **9s** were further evaluated for their capability to suppress the tube formation. According to the efficacy of them at the concentrations of 0.25, 1.0 and 4.0 μmol/l (Fig. 5), compounds **9k** and **9s** inhibited tube formation in a dose-dependent manner in HUVECs. They led to a dramatic inhibition of tube formation at the concentration of 4.0 μmol/l; however, both compounds were ineffective at the dosage of 0.25 μmol/l. Besides, at the concentration of 4.0 μmol/l, their potency was slightly inferior to sorafenib.

Conclusion

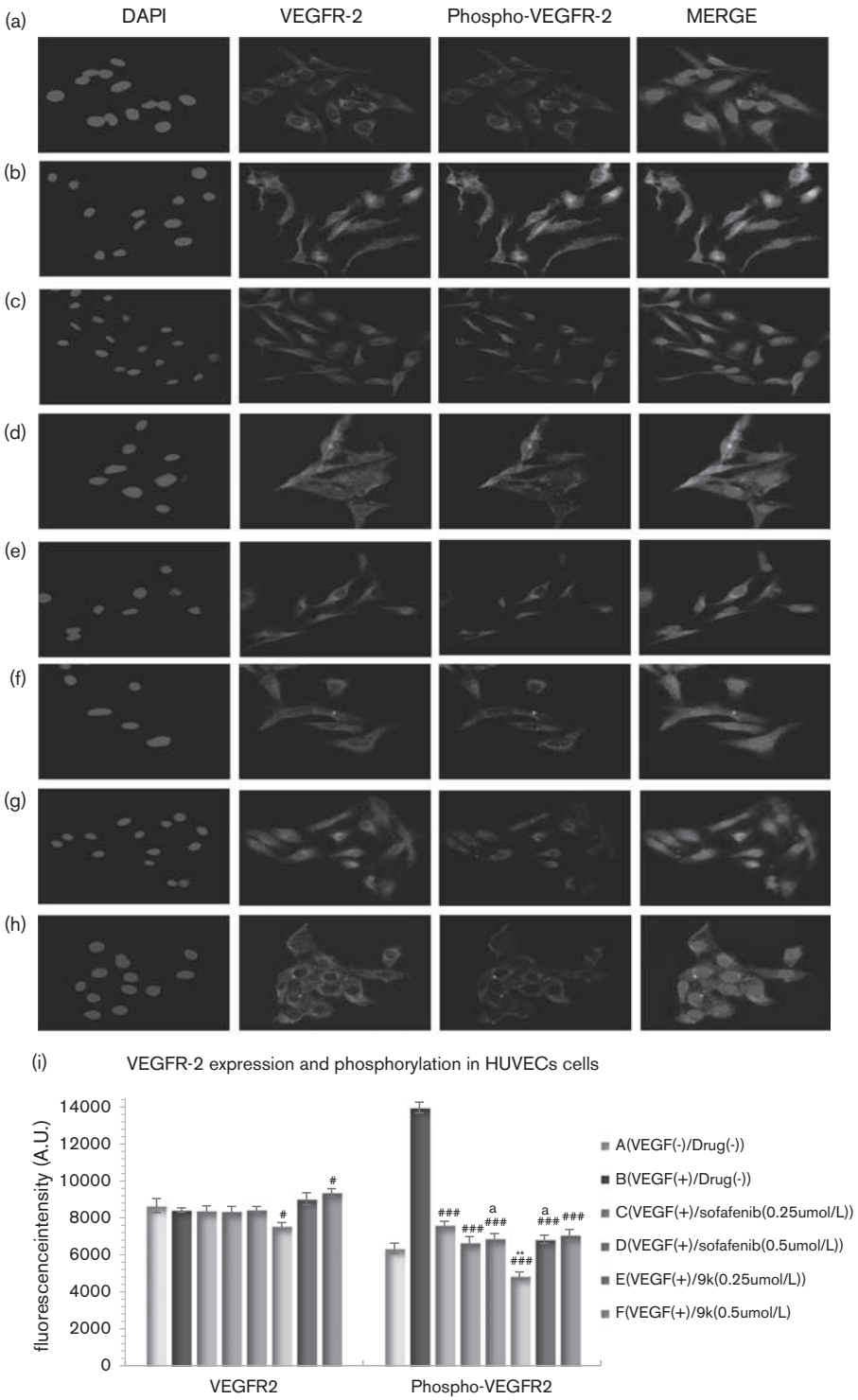
Tetrahydroisoquinoline serves as a well-established template for developing anticancer agents, whereas the diaryl urea moiety is a vital structural element of compounds that suppress VEGFR-2 signaling. Thus, during our efforts to explore novel modulators of VEGFR-2 signaling, the pharmacorphore-combination strategy was introduc ed by incorporating diaryl urea moiety to the tetrahydroisoquinoline scaffold. Among the obtained

Fig. 3



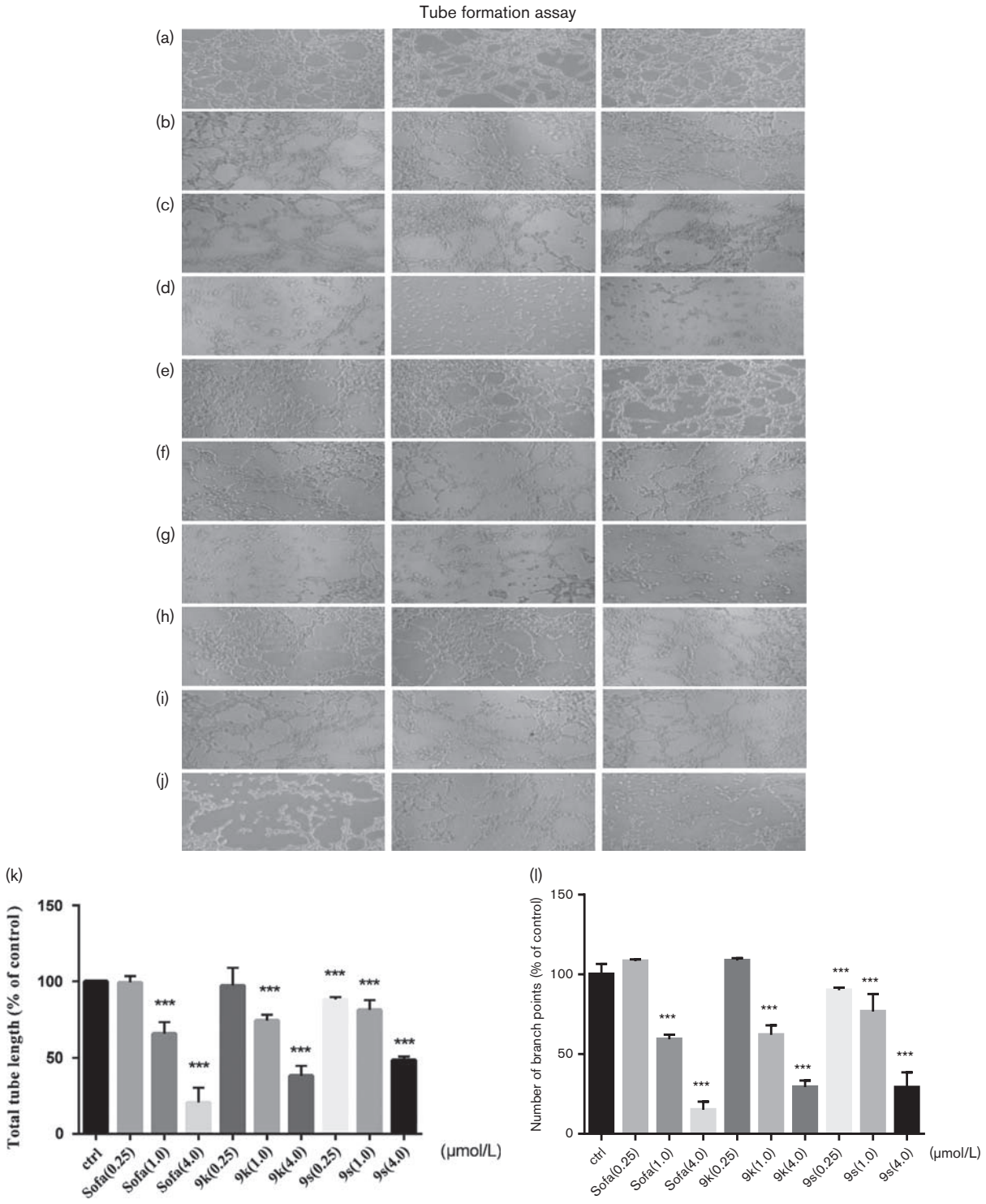
The effect of compounds on VEGFR-2 expression and phosphorylation in A549 cells. The A549 cells were treated with sorafenib, **9k** and **9s** at the concentration of 0.5 and 1.0 μmol/l, respectively. (a) The A549 cells without compound treatment; (b, c) the A549 cells treated with 0.5 or 1.0 μmol/l sorafenib; (d, e) the A549 cells treated with 0.5 or 1.0 μmol/l **9k**; (f, g) the A549 cells treated with 0.5 or 1.0 μmol/l **9s**. Images were taken at a magnification of × 200; (h) the bar chart presented the quantification of fluorescence intensity of each group (mean ± SD, three biological replicates). Compound-treated group versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; d and f versus b, $^{a}P < 0.01$, $^{aa}P < 0.001$; e and g versus c, $^{*}P < 0.01$, $^{**}P < 0.001$.

Fig. 4



The effect of compounds on VEGFR-2 expression and phosphorylation in HUVECs. The A549 cells were treated with compounds sorafenib, **9k**, and **9s**. Each compound was divided into two concentration groups of 0.5 and 1.0 μmol/l, respectively. (a) The human umbilical vein endothelial cells (HUVECs) without treatment; (b) the HUVECs induced phosphorylation by hVEGF and without treatment; (c, d) the HUVECs induced phosphorylation by hVEGF and treated with 0.25 or 0.5 μmol/l sorafenib; (e, f) the HUVECs induced phosphorylation by hVEGF and treated with 0.25 or 0.5 μmol/l **9k**; and (g, h) the HUVECs induced phosphorylation by hVEGF and treated with 0.25 or 0.5 μmol/l **9s**. Images were taken at a magnification of × 200; (i) the bar chart presented the quantification of fluorescence intensity of each group (mean ± SD, three biological replicates). Compound-treated group versus b, $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$. e and g versus c, $^{a}P < 0.05$. f versus c, $^{**}P < 0.01$.

Fig. 5



The effect of compounds on tube formation in HUVECs. The human umbilical vein endothelial cells (HUVECs) were treated with sorafenib, **9k**, and **9s**. Each compound was divided into three concentration groups of 0.25, 1.0 and 4.0 μmol/l, respectively. (a) The HUVECs without treatment; (b–d) the HUVECs treated with 0.25, 1.0, or 4.0 μmol/l sorafenib; (e–g) the HUVECs treated with 0.25, 1.0, or 4.0 μmol/l **9k**; and (g, h, j) the HUVECs treated with 0.25, 1.0, or 4.0 μmol/l **9s**. The density of tubules and tubular junctions elaborated by HUVEC were visualized in Matrigel culture. The dose-dependent effect of compounds **9k** and **9s** on the total tube length (k) and number of branches (l) in HUVECs. Results are representative of three independent experiments. Images were taken at a magnification of ×200. The treated group versus control, $^{***}P < 0.01$, $n = 4$.

tetrahydroisoquinoline-based compounds, compounds **9k** and **9s** exhibited comparable or superior antiproliferative activity to that of gefitinib against the tested three cell lines, including A549, MCF-7, and PC-3. To investigate their cytotoxicity toward normal cells, both compounds were assayed against HUVECs. Consequently, they displayed comparable cytotoxicity against HUVECs to that of gefitinib.

Afterward, using **9k** and **9s** as tool molecules, we further evaluated their capability to interfering with VEGFR-2 signaling by monitoring their influence on VEGFR-2 expression and VEGFR-2 phosphorylation by immunofluorescence assay. In A549 cells, both compounds **9k** and **9s** down-regulated the expression of VEFGR-2 and effectively blocked the phosphorylation of VEFGR-2 at the concentration of 0.5 or 1.0 μmol/l. At the concentration of 1.0 μmol/l, the capability of compound **9s** to down-regulate VEFGR-2 expression was stronger than that of sorafenib, and **9k** was as effective as sorafenib. Besides, at this dosage, both compounds led to more dramatic down-regulation of VEGFR-2 phosphorylation in comparison with sorafenib.

Accumulating researches have indicated that VEGFR-2 signaling plays an intimate role in tube formation. Thus, **9k** and **9s**, with capability to down-regulate VEGFR-2 signaling, were then evaluated for their efficacy in suppressing tube formation with sorafenib as the reference. As a result, **9k** and **9s** inhibited tube formation in a dose-dependent manner in HUVEC cells and led to a dramatic inhibition of tube formation at the concentration of 4.0 μmol/l. At the dosage, their potency was slightly inferior to that of sorafenib. However, given tube formation is not merely regulated by VEGFR-2 signaling and sorafenib is a well-established multitarget kinase inhibitor, it is acceptable that sorafenib exhibits a stronger effect on tube formation. The decreased potency of both compounds **9k** and **9s** than sorafenib in tube formation may imply sorafenib is capable to suppress tube formation via modulating pathway (s) other than VEGFR-2 signaling.

Taken together, owing to their favorable in-vitro performance, both **9k** and **9s** are valuable for further investigation and modification for exploring novel VEGFR-2 signaling modulators.

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Conflicts of interest

There are no conflicts of interest.

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