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ORIGINAL PAPER

N-(4-(quinazolin-2-yl)phenyl)benzamide derivatives with potent anti-angiogenesis activities: synthesis and evaluation

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Abstract Expanding our studies on the anti-angiogenesis activities of 2,4-disubstituted quinazoline derivatives [8], a series of novel *N*-(2-(quinazolin-2-yl)phenyl)benzamide (SZ) derivatives were designed and synthesized. Cytotoxicity assays indicated that most of these compounds displayed similar cytotoxicity against tumor cells in comparison with our previously reported, but showed a higher cytotoxicity against HUVECs. The SZ derivatives showed a remarkable inhibitive effect against the migration and adhesion of HUVECs, in addition to demonstrating significant in vivo anti-angiogenesis activities in the chick embryo chorioallantoic membrane (CAM) assay. The results proved that the introduction of an aryl group with a basic amide side chain on the 4′ position linked to the amide of the C-2 substituted quinazoline scaffold is an effective approach to improve the anti-angiogenic activity of quinazoline derivatives.

Keywords 2,4-Disubstituted quinazoline derivatives · *N*-(2-(Quinazolin-2-yl)phenyl)benzamide (SZ) derivatives · Synthesized · Anti-angiogenesis

M. Sun and Na Lv contributed equally to the work and should be regarded as co-first authors.

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Introduction

Angiogenesis is the normal process in the growth and development of new blood vessels from the pre-existing vasculature of a parasitifer, and participates in many physiological processes, such as wound healing, the menstrual cycle, pregnancy and organ development [1]. Normally, there is a balance between molecules that either stimulate or inhibit angiogenesis so that blood vessels form only when and where they are needed. Recently, research has found that the balance is broken in the course of many diseases, especially in various tumor types including solid and hematopoietic neoplasms. Aberrant angiogenesis would lead to the formation of abnormal vessels around the tumor, an essential prerequisite for tumor cell proliferation, invasion and metastasis [2–4]. Therefore, the search for pharmaceuticals targeting angiogenesis in tumors is a promising strategy for the development of cancer therapy [5].

ZD6474 (vandetanib, AstraZeneca Company), a heteroaromatic-substituted anilinoquinazoline (Fig. 1) was found to act as a potent and reversible inhibitor of VEGF receptor-2 (VEGFR-2), EGFR and RET tyrosine kinase, and showed broad spectrum anti-tumor activity that was dependent on the inhibition of angiogenesis [6, 7]. Recently, we reported on the synthesis and angiogenesis inhibiting activity of a new 2,4-disubstituted quinazoline derivative **11d**, which shares the quinazoline scaffold of **ZD6474** [8]. As a result of our continued efforts to screen synthetic compounds exhibiting anti-tumor properties, a novel series of quinazoline-based analogs based on **11d** were designed, synthesized and evaluated for their anti-proliferative and anti-angiogenic activities. In the current research, we modified the C-2 substituted quinazoline-based scaffold of **11d** by linking a new para-substituted

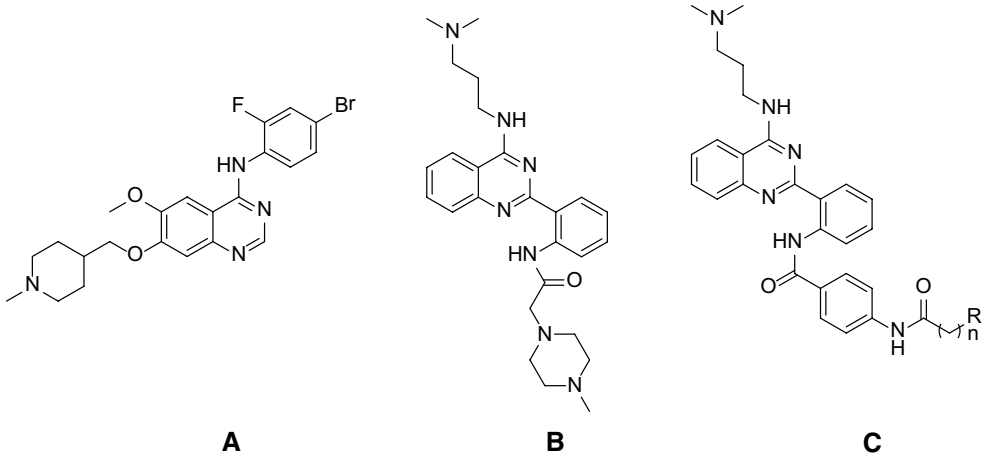


Fig. 1 Structures of **a ZD6474**, **b 11d**, and **c** *N*-(4-(quinazolin-2-yl)phenyl)benzamide (SZ) derivatives

aryl moiety to the carbonyl of the amide, while still retaining the C-4 substituted amino side chain of **11d**. Therefore, a series of *N*-(4-(quinazolin-2-yl)phenyl)benzamide (SZ) derivatives were synthesized (Fig. 1, **SZ10a-d**, **SZ11a-d**) and herein we report on the cytotoxicity and anti-angiogenic effects of these novel quinazoline analogs.

Experimental

Synthesis and characterization

^{1}H NMR spectra were recorded on a Bruker 400 NMR with tetramethylsilane (TMS) as an internal standard. NMR data are shown only for novel compounds. MS spectra were obtained using a Shimadzu LC–MS-2010A spectrometer. Elemental analysis was carried out on an Elementar Vario EL CHNS Elemental Analyzer. Melting points (m.p.) were determined using an SRS-OptiMelt automated melting point instrument without correction.

*Compound **2**: 2-(2-Nitrophenyl)quinazolin-4(3H)-one*

ZnI_2 (10 mol%), DMSO (2 mL), benzyl alcohol (1 mmol), 2-aminobenzamide (1 mmol), TBHP (70 % in H_2O, 4 eq.) and a stirring bar were added to a 50 mL pressure tube. The mixture was kept at 110°Cfor 14 h and the solvent was then removed under vacuum. The pure product was isolated by recrystallization from MeOH. Yield 57 %; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.53–7.61 (m, 1H), 7.69 (*t*, J = 7.64 Hz, 1H), 7.81–7.95 (m, 4H), 8.33 (dt, J = 8.62 Hz, 2H), 12.75 (s, 1H). ESI–MS *m*/*z*: 268 $[M + H]^+$.

Preparation of **3**–**5**. Compounds **3**–**5** were synthesized according to the previously established procedure [8].

*Compound **6**: N-(2-(4-(3-(dimethylamino)propylamino) quinazolin-2-yl)phenyl)-4-nitrobenzamide*

4-Nitrobenzoyl chloride (50 mmol) was added dropwise to a solution of **5** (100 mmol) in chloroform (100 mL) at room temperature and was stirred for 10 h. The generated solid was filtered, recrystallized with ethanol, and dried, affording product 6 as a white solid. Yield: 71 %; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.88–1.98 (m, 2H), 2.41 (s, 6H), 2.68 (t, J = 5.8 Hz, 2H), 3.90 (dd, J = 7.9, 5.1 Hz, 2H), 7.37–7.58 (m, 3H), 7.60 (d, J = 8.1 Hz, 1H), 7.64 (d, J = 5.9 Hz, 1H), 7.82 (*t*, J = 8.0 Hz, 1H), 8.34 (d, J = 7.6 Hz, 2H), 8.44 (d, 2H), 8.79 (d, J = 7.5 Hz, 1H), 8.86 (d, J = 7.6 Hz, 1H), 9.14 (s, 1H), 14.73(s, 1H). ESI–MS *m*/*z* 471 $[M + H]^+$.

*Compound **7**: 4-amino-N-(2-(4-(3-(dimethylamino) propylamino)quinazolin-2-yl)phenyl)benzamide*

We adopted the same synthetic method as that of compound **5**, generating **7** as a white powder. Yield 83 %; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.90–1.98 (m, 2H), 2.44 (s, 6H), 2.70 (t, J = 5.8 Hz, 2H), 3.91 (dd, J = 7.8, 5.2 Hz, 2H), 4.1 (m, 2H), 6.70 (d, J = 8.1 Hz, 2H), 7.18 (d, J = 7.5 Hz, 1H), 7.41–7.54 (m, 2H), 7.63–7.75 (m, 2H), 7.82 (t, J = 8.3 Hz, 1H), 8.32 (d, J = 7.9 Hz, 2H), 8.79 (d, J = 7.5 Hz, 1H), 8.90 (s, 1H), 9.02 (d, J = 8.4 Hz, 1H), 14.32 (s, 1H). ESI–MS *m*/*z*: 441 $[M + H]^+$.

General acylation procedure A

A solution of the acid halide (9.6 mmol) in chloroform (10 mL) was added to a well-stirred mixture of the amino-substituted compound **7** (8 mmol), followed by the addition of Na_2CO_3 (1.6 g) in chloroform (50 mL) at 0 °C, and the

mixture was then allowed to stir overnight. The crude product formed as a precipitate was filtered off and was purified by flash column chromatography with EtOAc/petroleum ether (1:10) elution to give compounds **8**–**9**.

*Compound **8**: 4-(2-chloroacetamido)-N-(2-(4-(3-(dimethyl amino)propylamino)quinazolin-2-yl)phenyl)benzamide*

Compound **7** was treated with chloroacetyl chloride according to the general acylation procedure, forming compound **8** as a white solid. Yield 74 %; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.88–1.95 (m, 2H), 2.43 (s, 6H), 2.61–2.65 (m, 2H), 3.16 (s, 2H), 3.91 (dd, J = 10.3, 5.5 Hz, 2H), 7.24 (m, 1H), 7.42–7.55 (m, 2H), 7.61 (d, J = 8.0 Hz, 1H), 7.71–7.84 (m, 4H), 8.23 (d, J = 7.9 Hz, 2H), 8.75 (d, J = 7.9 Hz, 1H), 8.83 (s, 1H), 8.94 (d, J = 8.2 Hz, 1H), 9.52 (s, 1H), 14.39 (s, 1H). ESI–MS *m/z*: 518 $[M + H]^+$.

*Compound **9**: 4-(3-chloropropanamido)-N-(2-(4-(3-(dimet hylamino)propylamino)quinazolin-2-yl)phenyl)benzamide*

Compound **7** was treated with 3-chloropropanoyl chloride according to the general acylation procedure to form compound **9** as a white solid. Yield: 71 %; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.89-1.95 (m, 2H), 2.40 (s, 6H), 2.61-2.65 (m, 2H), 3.07 (t, 2H), 3.86 (dd, J = 10.1, 5.4 Hz, 2H), 3.96 (t, J = 5.6 Hz, 2H), 7.21 (m, 1H), 7.43-7.51 (m, 2H), 7.62 (d, J = 7.8 Hz, 1H), 7.71-7.86 (m, 4H), 8.21 (d, J = 7.9 Hz, 2H), 8.76 (d, J = 7.9 Hz, 1H), 8.88 (s, 1H), 8.96 (d, J = 8.4 Hz,1H), 10.34 (s, 1H), 14.41(s, 1H). ESI–MS *m/z*: 532 $[M + H]^+$.

General aminolysis procedure B

To a stirred suspension of compounds **8** or **9** (0.5 mmol) and KI (0.08 g) in EtOH (20 mL), the appropriate secondary amine (2.0 mL) was added dropwise; the mixture was then stirred under reflux for 6 h. Upon reaction completion, the mixture was diluted with distilled water, filtered, and washed with ether. Finally, the crude solid was purified by chromatography with petroleum ether/EtOAc elution to afford **SZ10a-10d** and **SZ11a-11d**.

*Compound **SZ10a**: 4-(2-(diethylamino)acetamido)-N-(2-(4-(3-(dimethylamino)propylamino)quinazolin-2-yl)phenyl) benzamide*

Compound **8** was treated with excess diethylamine according to the general aminolysis procedure to afford **SZ10a**; purification was completed by column chromatography with petroleum ether/EtOAc (15:1) elution yielded **8** as a white solid. Yield 69 %; mp 164–166 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.12 (t, 6H), 1.81–1.89 (m, 2H), 2.41 (s, 6H), 2.59 (m, 6H), 3.14 (s, 2H), 3.76 (dd, J = 10.3, 5.2 Hz, 2H), 7.07 (t, J = 7.9 Hz, 1H), 7.38 (t, J = 7.4 Hz, 1H), 7.41 (t, 1H), 7.59 (d, J = 8.1 Hz, 1H), 7.65 (d, J = 7.6 Hz, 1H), 7.69–7.74 (m, 3H), 8.10 (d, J = 8.5 Hz, 2H), 8.72 (d, J = 8.0 Hz, 2H), 8.90 (d, J = 8.3 Hz, 1H), 9.64 (s, 1H), 14.41 (s, 1H). ESI–MS *m/z*: 554 $[M + H]^+$. Anal. Cacld for $C_{32}H_{39}N_7O_2.H_2O$: C, 69.41; H, 7.10; N, 17.71; Found: C, 69.72; H, 7.06; N, 17.69.

*Compound **SZ10b**: N-(2-(4-(3-(dimethylamino) propylamino)quinazolin-2-yl)phenyl)-4-(2-(piperidin-1-yl) acetamido)benzamide*

Compound **8** was treated with excess piperidine according to the general aminolysis procedure to afford **SZ10b**; purification by column chromatography with petroleum ether/EtOAc (15:1) elution yielded **8** as a white solid. Yield 59 %; mp 177–1789 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.53–1.62 (m, 4H), 1.76–1.83 (m, 2H), 1.90–1.96 (m, 2H), 2.43 (s, 6H), 2.66 (t, J = 5.8 Hz, 2H), 2.78 (t, J = 6.3 Hz, 4H), 3.36 (s, 2H), 3.89 (dd, J = 10.1, 5.3 Hz, 2H), 7.20 (t, J = 7.9 Hz, 1H), 7.44 (t, J = 7.2 Hz, 1H), 7.50 (t, J = 7.6 Hz, 1H), 7.68 (d, J = 8.3 Hz, 1H), 7.74–7.85 (m, 4H), 8.18 (d, J = 8.5 Hz, 2H), 8.70 (d, J = 8.0 Hz, 1H), 8.85 (s, 1H), 8.87 (d, J = 8.3 Hz, 1H), 9.14 (s, 1H), 14.43 (s, 1H). ESI–MS *m/z*: 566 $[M + H]^+$. Anal. Cacld for $C_{33}H_{39}N_7O_2.2H_2O$: C, 70.06; H, 6.95; N, 17.33; Found: C, 69.96 H, 7.01; N, 17.25.

*Compound **SZ10c**: N-(2-(4-(3-(dimethylamino) propylamino)quinazolin-2-yl) phenyl)-4-(2-morpholinoacetamido)benzamide*

Compound **8** was treated with excess piperidine according to the general aminolysis procedure to afford **SZ10c**; purification by column chromatography with petroleum ether/EtOAc (15:1) elution yielded **8** as a white solid. Yield 68 %; mp 188–189 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.81–1.86 (m, 2H), 2.39 (s, 6H), 2.54–2.63 (m, 6H), 3.21 (s, 2H), 3.78 (t, 4H), 3.93 (dd, J = 10.4, 5.9 Hz, 2H), 7.18 (t, J = 7.9 Hz, 1H), 7.36 (t, J = 8 Hz, 1H), 7.42 (t, J = 7.5 Hz, 1H), 7.63 (d, J = 8.0 Hz, 1H), 7.71–7.82 (m, 4H), 8.20 (d, J = 8.3 Hz, 2H), 8.70 (d, J = 8.0 Hz, 1H), 8.83 (s, 1H), 8.87 (d, J = 8.3 Hz, 1H), 9.19 (s, 1H), 14.41 (s, 1H). ESI–MS *m/z*: 568 $[M + H]^+$. Anal. Cacld for $C_{32}H_{37}N_7O_3.H_2O$: C, 67.70; H, 6.57; N, 17.27; Found: C, 67.76 H, 6.63; N, 17.04.

*Compound **SZ10d**: N-(2-(4-(3-(dimethylamino) propylamino)quinazolin-2-yl) phenyl)-4-(2-(4-methylpiperazin-1-yl)acetamido) benzamide*

Compound **8** was treated with excess piperidine according to the general aminolysis procedure to afford **SZ10d** purification by column chromatography with petroleum ether/EtOAc (15:1) elution yielded **8** as a white solid. Yield 44 %; mp 183–184 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.76–1.88 (m, 2H), 2.11 (s, 3H), 2.37 (s, 6H), 2.43–2.51 (m, 4H), 2.55–2.65 (m, 6H), 3.17 (s, 2H), 3.89 (dd, J = 9.4, 5.3 Hz, 2H), 7.16 (t, J = 7.9 Hz, 1H), 7.38 (t, J = 7.4 Hz, 1H), 7.42 (t, J = 7.8 Hz, 1H), 7.64 (d, J = 8.2 Hz, 1H), 7.73–7.79 (m, 4H), 8.10 (d, J = 8.4 Hz, 2H), 8.68 (d, J = 7.9 Hz, 1H), 8.74 (s, 1H), 8.91 (d, J = 8.3 Hz, 1H), 9.25 (s, 1H), 14.41 (s, 1H). ESI–MS *m/z*: 581 $[M + H]^+$. Anal. Cacld for $C_{33}H_{40}N_8O_2.H_2O$: C, 68.25; H, 6.94; N, 19.30; Found: C, 68.36 H, 6.91; N, 19.15.

*Compound **SZ11a**: 4-(3-(diethylamino)propanamido)-N-(2-(4-(3-(dimethylamino)propylamino)quinazolin-2-yl) phenyl)benzamide*

Compound **9** was treated with excess diethylamine according to the general aminolysis procedure to afford **SZ11a**; purification by column chromatography with petroleum ether/EtOAc (15:1) elution yielded **9** as a white solid. Yield: 68 %; mp 156–157 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (t, 6H), 1.91e1.95 (m, 2H), 2.38 (s, 6H), 2.49 (t, J = 5.7 Hz, 2H), 2.62–2.69 (m, 6H), 2.77 (t, J = 5.5 Hz, 2H), 3.86 (dd, J = 9.3, 5.8 Hz, 2H), 7.14 (t, J = 7.7 Hz, 1H), 7.41–7.48 (m, 2H), 7.68 (d, J = 8.39 Hz, 4H), 7.74 (d, J = 8.6 Hz, 1H), 8.06 (d, J = 8.2 Hz, 2H), 8.70 (d, J = 7.2 Hz, 2H), 8.88 (d, J = 8.3 Hz, 1H), 11.37 (s, 1H), 14.39 (s, 1H). ESI–MS *m/z*: 568 $[M + H]^+$. Anal. Cacld for $C_{33}H_{41}N_7O_2.2H_2O$: C, 69.81; H, 7.28; N, 17.27; Found: C, 69.92; H, 7.34; N, 17.26.

*Compound **SZ11b**: N-(2-(4-(3-(dimethylamino) propylamino)quinazolin-2-yl)phenyl)-4-(3-(piperidin-1-yl) propanamido)benzamide*

Compound **9** was treated with excess piperidine according to the general aminolysis procedure to afford **SZ11b**; purification by column chromatography with petroleum ether/EtOAc (15:1) elution yielded **9** as a white solid. Yield 63 %; mp 159–160 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.55–1.65 (m, 4H), 1.77–1.83 (m, 2H), 1.88–1.94 (m, 2H), 2.45 (s, 6H), 2.56 (t, J = 5.8 Hz, 2H), 2.67 (t, J = 5.5 Hz, 2H), 2.77 (t, J = 6.4 Hz, 4H), 2.90 (t, J = 5.8 Hz, 2H), 3.89 (dd, J = 10.0, 5.9 Hz, 2H), 7.19–7.24 (m, 1H), 7.42–7.48 (m, 1H), 7.50–7.53 (m, 1H), 7.64 (d, J = 7.9 Hz, 1H), 7.68 (d, J = 8.6 Hz, 2H), 7.74–7.79 (m, 1H), 7.84 (d, J = 8.2 Hz, 1H), 8.17 (d, J = 8.6 Hz, 2H), 8.79 (dd, J = 8.0, 1.5 Hz, 1H), 8.89 (t, J = 4.4 Hz, 1H), 8.96 (dd, J = 8.3, 0.7 Hz, 1H), 11.45 (s, 1H), d 14.42 (s, 1H). ESI–MS *m/z*: 580 $[M + H]^+$. Anal. Cacld for $C_{34}H_{41}N_7O_2.H_2O$: C, 70.44; H, 7.13; N, 16.91; Found: C, 70.66; H, 7.25; N, 17.04.

*Compound **SZ11c**: N-(2-(4-(3-(dimethylamino) propylamino)quinazolin-2-yl) phenyl)-4-(3-morpholinopropanamido)benzamide*

Compound **9** was treated with excess piperidine according to the general aminolysis procedure to afford **SZ11c**; purification by column chromatography with petroleum ether/EtOAc (15:1) elution yielded **9** as a white solid. Yield 60 %; mp 174–176 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.78–1.84 (m, 2H), 2.37 (s, 6H), 2.56 (t, J = 5.8 Hz, 2H), 2.59–2.67 (m, 6H), 2.77 (t, J = 5.6 Hz, 2H), 3.81–3.88 (m, 6H), 7.17 (t, J = 7.3 Hz, 1H), 7.39 (t, J = 7.8 Hz, 1H), 7.46 (t, J = 7.6 Hz, 1H), 7.56 (d, J = 8.3 Hz, 1H), 7.64–7.69 (m, 3H), 7.77 (d, J = 8.2 Hz, 1H), 8.12 (d, J = 8.5 Hz, 2H), 8.74 (d, J = 8.0 Hz, 1H), 8.82 (s, 1H), 8.93 (d, J = 8.3 Hz, 1H), 11.13 (s, 1H), 14.39 (s, 1H). ESI–MS *m/z*: 582 $[M + H]^+$. Anal. Cacld for $C_{33}H_{39}N_7O_3.H_2O$: C, 68.14; H, 6.76; N, 16.86; Found: C, 68.09; H, 6.71; N, 16.91.

*Compound **SZ11d**: N-(2-(4-(3-(dimethylamino) propylamino)quinazolin-2-yl) phenyl)-4-(3-(4-methylpiperazin-1-yl)propanamido) benzamide*

Compound **9** was treated with excess piperidine according to the general aminolysis procedure to afford **SZ11d**; purification by column chromatography with petroleum ether/EtOAc (15:1) elution yielded **9** as a white solid. Yield: 47 %; mp 165–166 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.85–1.96 (m, 4H), 2.18 (s, 3H), 2.22 (s, 6H), 2.35–2.44 (m, 6H), 2.49–0.57 (m, 4H), 2.67 (t, J = 6.6 Hz, 2H), 3.67 (dd, J = 11.4, 6.6 Hz, 2H), 7.26 (t, J = 7.2 Hz, 1H), 7.54 (t, J = 7.8 Hz, 1H), 7.61 (t, J = 7.6 Hz, 1H), 7.76(d, J = 8.3 Hz, 1H), 7.81–7.87 (m, 3H), 8.04 (d, J = 8.0 Hz, 2H), 8.33 (d, J = 8.0 Hz, 1H), 8.68–8.77 (m, 2H), 8.84 (d, J = 8.1 Hz, 1H), 11.07 (s, 1H), 14.35 (s, 1H). ESI–MS *m/z*: 595 $[M + H]^+$. Anal. Cacld for $C_{34}H_{42}N_8O_2.2H_2O$: C, 68.66; H, 7.12; N, 18.84; Found: C, 68.60; H, 6.99; N, 18.91.

Materials and biological assays

Stock solutions of all compounds (10 mM) were made with DMSO and stored at 4 °C. HUVEC and tumor cell lines were obtained from the Animal Laboratory Center of Sun

Yat-Sen University. The cells were maintained in a DMEM medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL of streptomycin in 25 cm^2 culture flasks at 37 °C humidified atmosphere with 5 % CO_2. Chick embryos were purchased from Anhui Agricultural University.

Methyl thiazolyl tetrazolium (MTT) assay

The HUVEC, CNE-2, PC-3, and SMMC-7721 cells were seeded in 96-well plates at a concentration of 5000 cells/well. After overnight incubation, the cells were exposed to different concentrations of the derivatives for 48 h. Subsequently, a 10 mL 3-(4,5-dimethylthiazol-2-yl)-2,5diphenylte-trazolium bromide (MTT, 5 mg/mL) solution was added to each well and further incubated for 4 h at 37 °C. Next, to each well 100 mL of DMSO was added, and the optical density (OD) was read on the microplate reader at 490 nm. The IC_{50} values were determined from the mean OD values of the triplicate tests versus drug concentration curves [9, 10].

Cell adhesion assay

The cell adhesion assay has been reported previously [11, 12]. Collagen type I (0.02 mg/mL) from the rat trail tendon was seeded in 96-well plates at room temperature overnight. Then, the wells were washed with PBS in triplicate and blocked with 0.2 % bovine serum albumin (BSA) for 2 h at 37 °C. The wells were washed three times with PBS (100 mL) again. Subsequently, the HUVECs (50 μL, 10^4 cells/ml) and related compounds (50 μL, at various concentrations) were added to each well. After a 1 or 3 h incubation period, the plates were washed three times with 100 mL of PBS. Then, the attached cells were fixed and stained with 0.2 % crystal violet in 20 % methanol for 10 min, and washed three times with 100 mL of PBS. Finally, the cell cultures were solubilized by 2 % SDS and the OD was determined at 570 nm; each experiment was repeated three times.

Endothelial cell migration assay

The cell migration assay was used to confirm the effect of the derivatives on cell invasion activity [13, 14]. HUVEC cell lines were plated in triplicate in pre-treated 48-well culture plates (2×10^4 cells/well) and incubated for 24 h. The cells were then scraped away with a sterile disposable rubber policeman. The debris was removed by washing with PBS (phosphate-buffer saline) and the compounds were added to a DMEM medium. After 24 h incubation, the HUVEC cells that migrated into the wounded area or that protruded from the border of the wound were visualized, photographed under an inverted microscope and the area of the migrations was measured using Photoshop CS3. All measurements were done in triplicate.

Chicken chorioallantoic membrane (CAM) assay

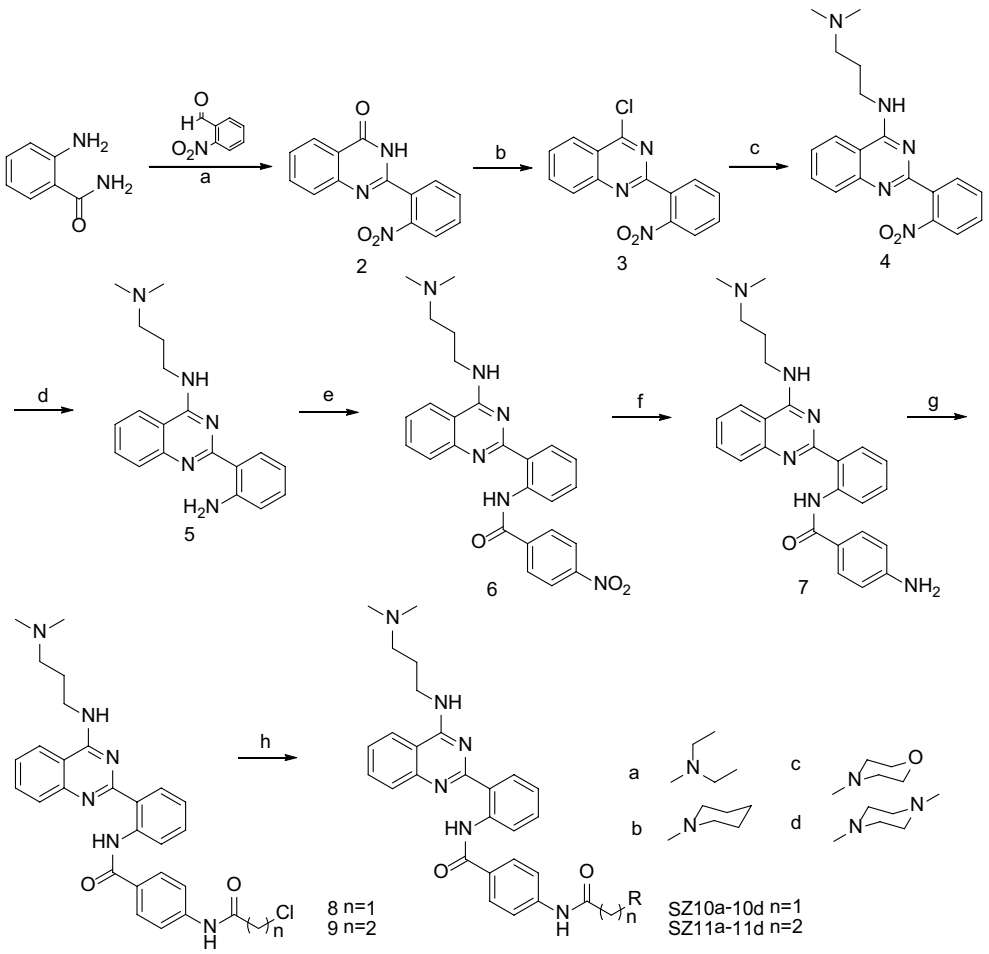
The anti-angiogenic activity of compounds on Chorioallantoic Membrane (CAM) was assayed as described previously [15–17]. First, fertilized chicken eggs were incubated for 8 days. Then, a 1 cm × 1 cm window was punched on the broad side of the egg to expose the CAM. Sterilized filter paper disks saturated with 0.9 % NaCl solution or 30 mg/10 mL of compounds were placed upon the CAM. The eggs were then incubated at 37 °C for a further 48 h. Finally, an appropriate volume of 4 % paraformaldehyde was fixed upon the CAMs to observe the density and length of the vessels toward the CAM face, and the vessel number from three random fields around the sterilized filter paper disk was pictured using a microscope.

Results and discussion

Chemistry

The new quinazoline derivatives (**SZ** derivatives) were prepared as depicted in Scheme 1, a route that closely follows the previously reported procedure [8]. To obtain the quinazolinedione intermediate 2, we employed 2-nitrobenzaldehyde as the reagent instead of 2-Nitrobenzoyl chloride [18]. Intermediate **6** was prepared by mixing the C-2 substituted quinazoline analog **5** with 2-nitrobenzoyl chloride in chloroform at room temperature. The nitro-group on intermediate **6** was then reduced to the amino group by $Pd/C/H_2$ in isopropanol, affording intermediate **7**, which was then treated with various acyl chlorides in dichloromethane to generate compounds **8-9**. Finally, intermediates **8** and **9** were reacted with the appropriate secondary amines through a nucleophilic substitution reaction to form the target compounds **SZ10a–d** and **SZ11a–d**. The correct structure of the desired compounds was confirmed through analytical and spectral data (MS, NMR, etc.).

Reagent: (a) ZnI_2 (10 mol %), TBHP (70 % in H_2O, 4 eq.), DMSO, 110 °C 14 h. (b) *N*,*N*-diethylaniline, $POCl_3$, toluene, reflux, 6 h; (c) 3-aminopropyldimethyl-amine, THF, reflux, 5 h; (d) 10 % Pd/C, 80 % $N_2H_4H_2O$, isopropanol, reflux, 2 h; (e) 4-nitrobenzoyl chloride, K_2CO_3, CH_3Cl_3, rt, 24 h; (f) 10 % Pd/C, 80 % $N_2H_4.H_2O$, Isopropanol, reflux, 2 h; (g) $Cl(CH_2)nCOCl$, K_2CO_3, CH_3Cl_3, rt, 24 h; (h) R_2NH, KI, ethanol, reflux.



Scheme 1 Synthesis route for the formation of derivatives SZ10a-d and SZ11a-d

Cell growth inhibition

We previously reported on the cytotoxic properties of **11d**, but there are fewer studies on the in vitro cytotoxicity of the newly-synthesized **SZ** derivatives [8]. As was previously observed with **11d,** inhibition of cell proliferation was observed following treatment of both normal cell lines (endothelial cell) and tumor cell lines with the SZ derivatives.

The MTT cytotoxicity assay was carried out to determine the cytotoxic activities of the **SZ** derivatives against Human Umbilical Vein Endothelial Cells (HUVEC) and human tumor cell lines, including PC-3 (human prostatic carcinoma), CNE-2 (human nasopharyngeal cancer) and SMMC-7721 (human liver cancer). Table 1 shows the IC_{50} values (cytotoxicity potency indexes) of all the derivatives against four types of cells lines. Comparing quinazoline derivative **11d** with the novel derivatives, **SZ10a-d** and **SZ11a-d** which bear an additional aromatic moiety, we find that the latter (some compounds) have a similar cytotoxicity against the tumor cells and a higher cytotoxicity against the HUVEC cell line. (Table 1). Like in the previous study with **11d**, the IC_{50} values of all the **SZ** derivatives tested on the tumor cell lines also fall in the range of 10–28.1 μM; however, the IC_{50} value on the HUVEC line was reduced

Table 1 IC_{50} cytotoxicity values (μM) of quinazoline derivatives SZ10a-d and SZ11a-d against tumor cells

Compound	IC_{50} (μM)			
	CNE-2	PC-3	SMMC-7721	HUVEC
SZ10a	15.7 ± 0.2	14.2 ± 0.1	16.6 ± 0.1	40.2 ± 0.5
SZ10b	16.8 ± 0.2	12.4 ± 0.1	22.5 ± 0.3	33.6 ± 0.3
SZ10c	21.2 ± 0.4	28.1 ± 0.3	19.7 ± 0.5	41.5 ± 0.2
SZ10d	14.7 ± 0.3	17.2 ± 02	13.6 ± 0.1	28.4 ± 0.5
SZ11a	13.9 ± 0.2	12.7 ± 0.2	16.4 ± 0.3	35.3 ± 0.2
SZ11b	17.7 ± 0.2	16.5 ± 0.2	15.4 ± 0.3	29.7 ± 0.4
SZ11c	24.5 ± 0.5	20.8 ± 0.3	22.4 ± 0.1	46.2 ± 0.3
SZ11d	15.1 ± 0.2	14.6 ± 0.1	12.9 ± 0.5	21.8 ± 0.3
11d	11.3 ± 0.2	10.6 ± 0.5	12.4 ± 0.2	45.6 ± 0.2

Data derived from the mean of three independent assays

to 21.8 μM (**SZ11d**). Among compounds having the same R-group, those bearing the less basic morpholine group (**SZ10c** and **SZ11c**) had the weakest cytotoxicity to various cancer cells lines and to the normal cell line. In contrast, compounds with a more basic amino terminus (*N*-methyl-piperazino analogs, **SZ10d** and **SZ11d**) showed a higher activity on all the cell lines. On the other hand, comparing **SZ10d** and **SZ11d**, which have the same R group but vary in the length of the amide side chain ($n = 1$ and $n = 2$, respectively), we observed no significant effect on the IC_{50} values. The results demonstrate the importance of the basicity of the amide side chain to achieve strong cytotoxicities on the cell lines.

Cell adhesion assays

Cell adhesion plays an important role in maintaining cell shape, regulation of cell division and movement, as well as being an important step in the process of angiogenesis. Therefore, we performed HUVEC cell adhesion assays to investigate the inhibitory effects of these **SZ** derivatives on the attachment of endothelial cells to type I collagen and thalidomide was selected as positive control.

As shown in Table 2, the inhibition rate of the SZ derivatives for HUVEC adhesion to collagen showed a strong inhibitory effect at both 1 and 3 h (Inhibition rate range: 31.4 ± 0.3–74.3 ± 0.1 %). **SZ11d** ($n = 2$), containing the more basic piperazino moiety, results in the strongest inhibition among all the derivatives tested (69.2 ± 0.2 % at a dose of 30 μM, 1 h; 74.3 ± 0.1 % at a dose of 30 μM, 3 h incubation at 37 °C). An analogous result is observed among the **SZ10** derivatives, with **SZ10d** displaying the greatest inhibition among the series, albeit lower than that of **SZ11d**. Derivative **SZ10c**, with a shorter amide side chain and the less basic morpholine group, showed the weakest inhibition of HUVEC adhesion (34.3 ± 0.2 % at a dose of 30 μM; 43.2 ± 0.2 % at a dose of 30 μM, 3 h incubation at 37 °C). Similar to the results of the cytotoxicity assays, the basicity of the R group was proven to be an important factor in the potency of the derivative for inhibition. Comparing the **SZ10a-d** and **SZ11a-d** derivatives, we can also detect a strengthening of the inhibitory effect in all cases as the alkyl chain is lengthened from $n = 1$ to $n = 2$. So we can find that all **SZ** compounds with a new phenyl group onto the scaffold of quinazoline derivatives gave an improvement of inhibition effect of HUVEC adhesion.

Table 2 Inhibitory effects of **SZ** derivatives on adhesion of HUVEC to collagen at a dose of 15 and 30 μM

Compound	1 h Inhibition rate (%)		3 h Inhibition rate (%)	
	Dose 15 μM	Dose 30 μM	Dose 15 μM	Dose 30 μM
SZ10a	39.7 ± 0.2	46.6 ± 0.1	40.4 ± 0.3	47.7 ± 0.2
SZ10b	36.6 ± 0.1	41.9 ± 0.4	42.3 ± 0.2	49.2 ± 0.1
SZ10c	31.4 ± 0.3	34.3 ± 0.2	35.7 ± 0.5	43.2 ± 0.2
SZ10d	43.4 ± 0.2	51.3 ± 0.1	45.2 ± 0.5	52.6 ± 0.2
SZ11a	41.1 ± 0.4	47.9 ± 0.2	46.2 ± 0.3	55.4 ± 0.5
SZ11b	40.7 ± 0.2	53.6 ± 0.2	46.2 ± 0.2	69.3 ± 0.3
SZ11c	35.4 ± 0.1	51.3 ± 0.4	43.2 ± 0.1	60.8 ± 0.2
SZ11d	51.3 ± 0.5	69.2 ± 0.2	56.4 ± 0.3	74.3 ± 0.1
11d	46.5 ± 0.2	66.3 ± 0.3	52.6 ± 0.2	73.1 ± 0.4
Thalidomide	39.7 ± 0.4	54.6 ± 0.2	42.6 ± 0.3	64.7 ± 0.1

Data derived from the mean of three independent assays

Migration inhibition assays

Endothelial cell migration is an essential step in angiogenesis, hence inhibition of this process could hinder the formation of new blood vessels. Based on the most satisfactory results of the HUVEC adhesion assay, derivatives **SZ10d**, **SZ11d**, **thalidomide** and 2,4-disubstituted quinazoline derivative **11d** were chosen to evaluate their inhibition against HUVEC migration in a wound-healing migration assay. All compounds were tested at concentrations of both 15 and 30 μM, and were all able to inhibit HUVEC migration. Compared with **11d**, **SZ10d** and **SZ11d,** with a longer amide side chain in the 4-position of the added aryl group, showed a greater inhibitory potential on HUVEC migration at 15 μM (Fig. 2); in all cases, the inhibitory effects were more significant when the concentration was increased to 30 μM. These results demonstrated that the inhibitory effect on the migration of HUVECs is significantly enhanced for derivatives bearing an added aryl group on the C-2 substituted quinazoline scaffold, which further supports the results of the adhesion studies.

Derivatives inhibiting angiogenesis in vivo in the CAM assay

The anti-angiogenic activity of the most potent derivatives, **SZ10d** and **SZ11d,** was tested using the chick chorioallantoic membrane (CAM) assay which represents an excellent animal model for the study of angiogenesis, since the early 1970s when it was developed by Folkmann et al. [10]. Chorioallantoic membrane was treated with thalidomide (a clinical anti-tumor drug targeting tumor angiogenesis, 30 mg/10 mL), **SZ10d** (30 mg/10 mL), **SZ11d** (30 mg/10 mL) or the vehicle control for 48 h. As shown in Fig. 3, when **SZ** derivatives were added on the CAMs, there was a considerable inhibition of the angiogenic responses. In the group treated with **SZ11d**, the formation of vessels in the chick embryos was greatly inhibited, compared

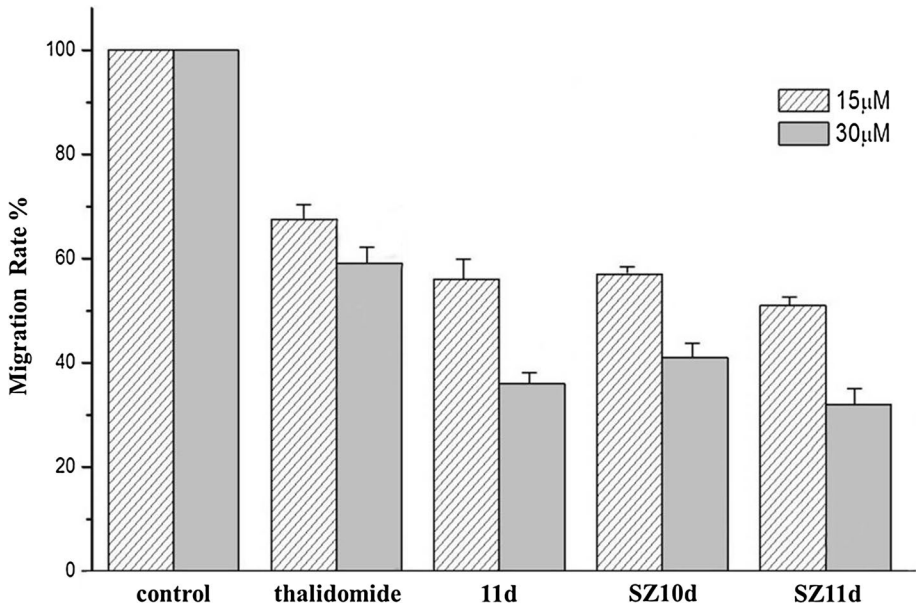


Fig. 2 The inhibitory effects of quinazoline derivatives on migration of HUVEC at a dose of 15 and 30 μM

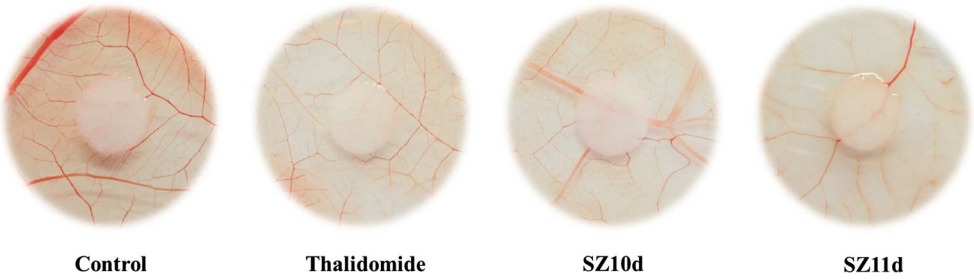


Fig. 3 The results of the CAM assay. 0.9 % NaCl solution and thalidomide were used as a negative and positive control, respectively. Representative pictures of independent experiments performed in duplicate for **Thalidomide**, derivatives **SZ10d**, **SZ11d** and *N*-(4-(quinazolin-2-yl)phenyl)benzamide dose and controls. All filter discs had the same size; the apparent visual size difference is due to the various distances from which pictures were taken

with those treated with **SZ10d** and **thalidomide**, a result in accord with the aforementioned studies on cytotoxicity, migration and adhesion.

Conclusion

In our efforts towards the discovery of novel 2,4-disubstituted quinazoline derivatives with enhanced anti-angiogenesis activities, a series of *N*-(2-(quinazolin-2-yl)phenyl) benzamide (**SZ**) derivatives were designed and synthesized. Based on previous reports of the use of amide bonds on ligand design, a new benzene ring was attached to the scaffold of quinazoline derivatives through amide bond. In comparison with the previously reported 2,4-disubstituted quinazoline derivative **11d**, cytotoxicity assays indicated that most of these novel compounds (with the exception of derivative **SZ11c**) displayed similar cytotoxicities against tumor cells but showed increased cytotoxicity towards HUVECs. Among these derivatives, compound **SZ11d** with an *N*-methyl piperazino terminus of the amide side chains ($n = 2$, with three bonds between basic N terminus and carbonyl group) exhibited the most potent inhibitory effect on the proliferation of HUVECs, CNE-2, PC-3, and SMMC-7721 cells (IC50 = 21.8 ± 0.3, 15.1 ± 0.2, 14.6 ± 0.1, and 12.9 ± 0.5 μM, respectively). Furthermore, cell adhesion and migration inhibition assays demonstrated the anti-angiogenic potential of some derivatives on HUVECs and their use as possible targets for further optimization studies. In addition, the chick embryo chorioallantoic membrane (CAM) assay showed that compounds **SZ10d** and **SZ11d** inhibited the angiogenic responses at 30 mg/10 mL. In conclusion, the results proved that introduction an aryl group linked to the amide of the quinozaline C-2 substituted scaffold with a basic amide side chain on the 4′ position is an effective approach to increase angiogenesis inhibition,

which will provide us with a framework for structural modification in the future.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. P.C. Stapor, R.S. Sweat, D.C. Dashti, A.M. Betancourt, W.L. Murfee, J. Vasc. Res. **51**, 163–246 (2014)
2. J. Folkman, Adv. Cancer Res. **43**, 175–203 (1985)
3. N. Ferrara, R.S. Kerbel, Nature **438**, 967–974 (2005)
4. J. Folkman, Nat Rev Drug Discov **6**, 273–286 (2007)
5. R.S. Herbst, Expert Opin Emerg Drugs **11**, 635–650 (2006)
6. A. Morabito, M.C. Piccirillo, F. Falasconi, G. De Feo, A. Del Giudice, J. Bryce, M. Di Maio, E. De Maio, N. Normanno, F. Perrone, Oncologist **14**, 378–390 (2009)
7. J.A. Meyerhardt, M. Ancukiewicz, T.A. Abrams, D. Schrag, P.C. Enzinger, J.A. Chan, M.H. Kulke, B.M. Wolpin, M. Goldstein, L. Blaszkowsky, A.X. Zhu, M. Elliott, E. Regan, R.K. Jain, D.G. Duda, PLoS One **7**, e38231 (2012)
8. G. Yu, Z. Li, L. Tang, Q. Xiong, Molecules **19**, 8916–8932 (2014)
9. R.J. D'Amato, M.S. Loughnan, E. Flynn, J. Folkman, Proc. Natl. Acad. Sci. USA **91**, 4082–4085 (1994)
10. F. Xu, H. Xu, X. Wang, L. Zhang, Q. Wen, Y. Zhang, W. Xu, Bioorg Med Chem. **22**, 487–495 (2014)
11. J. Vukanovic, A. Passaniti, T. Hirata, R.J. Traystman, B. Hartley-Asp, J.T. Isaacs, Cancer Res. **53**, 1833–1837 (1993)
12. K.M. Malinda, M. Nomizu, M. Chung, M. Delgado, Y. Kuratomi, Y. Yamada, H.K. Kleinman, M.L. Ponce, FASEB J. **13**, 53–62 (1999)
13. H. Zhengfu, Z. Hu, M. Huiwen, L. Zhijun, Y. Xiaoyi, C. Xiujun, Biochem Biophys Res Commun. 30180–30187 (2015) **(pii S0006-291X)**
14. I.S. Yun, M.H. Lee, D.K. Rah, D.H. Lew, J.C. Park, W.J. Lee, Plast. Reconstr. Surg. **136**, 44e–53e (2015)
15. N. Lu, Y. Yang, Q.D. You, Y. Ling, Y. Gao, H.Y. Gu, Cancer Lett. **258**, 80–89 (2007)
16. N.A. Lokman, A.S. Elder, C. Ricciardelli, M.K. Oehler, Int. J. Mol. Sci. **13**, 9959–9970 (2012)
17. S. Majidian Eydgahi, J. Baharara, S. Zafar Balanezhad, M. Asadi Samani, Avicenna J. Phytomed. **5**, 174–181 (2015)
18. M. Sharif, J. Opalach, P. Langer, M. Beller, X.F. Wu, RSC Adv. **4**, 8–17 (2014)