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Quinazoline derivative compound **(11d)** as a novel angiogenesis inhibitor inhibiting VEGFR2 and blocking VEGFR2-mediated Akt/mTOR /p70s6k signaling pathway

Zeng Li [1], Bin Wang [3], Liang Tang [2], Shuangsheng Chen [1, 2], Jun Li [1*]

[1] Department of Pharmacy, Anhui Medical University, Hefei 230032, China
[2] Department of Chirurgery, First Affiliated Hospital of Anhui Medical University, Hefei 230032, China
[3] Department of Pharmacy, Xiangnan University, Chenzhou 423000, China

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ABSTRACT

Objective(s): We previously reported a series of quinazoline derivatives as vascular-targeting anticancer agents. In this study, we investigated the mechanism underlying the anti-angiogenic activity of the quinazoline derivative compound **11d**.

Materials and Methods: We examined the effects of quinazoline derivative **11d** on vascular endothelial growth factor receptor-2 (VEGFR2) activation via VEGFR2-specific activation assay. Reverse transcription and immunohistochemistry were used to detect vascular endothelial growth factor (VEGF), VEGFR2, and the VEGFR2-mediated Akt/mTOR/p70s6k signaling pathway in human umbilical vascular endothelial cells and hepatocellular carcinoma cells (HepG-2) after treatment with various concentrations of **11d** (0, 6.25, 12.5, and 25 μM) for 24 hr.

Results: The compound **11d** exhibited potent inhibitory activity against VEGFR2 with an IC_{50} of 5.49 μM. This compound significantly downregulated VEGF, VEGFR2, and the VEGFR2-mediated Akt/mTOR/p70s6k signaling pathway *in vitro*.

Conclusion: The mechanism underlying the anti-angiogenic activity of the quinazoline derivative **11d** possibly involves the inhibition of VEGFR2 and the downregulation of VEGF, VEGFR2, and the VEGFR2-mediated Akt/mTOR/p70s6k signaling pathway. Overall, the findings indicate that the studied class of compounds is a source of potential antiproliferative and anti-angiogenic agents, which must be further investigated.

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Introduction

Angiogenesis is the process by which the existing vascular bed expands to form new blood vessels and is a pivotal event in many physiological and pathological processes, including tumor growth and metastasis (1, 2). Recent developments in cancer therapies have focused on molecular targets, particularly tumor angiogenesis (3). Newly generated blood vessels supply oxygen and essential nutrition, support tumor growth, and later aid in the initiation of metastasis, which contributes to more than 90% of deaths in various cancers, including hepatocellular carcinoma (HCC) (4). Vascular endothelial growth factor (VEGF) is one of the central regulators in angiogenesis (5). Vascular endothelial growth factor receptor-2 (VEGFR2) is the receptor for VEGF and is the prime mediator of VEGF-induced pro-angiogenesis signaling (6). Binding of VEGF to VEGFR2 leads to dimerization of the receptors, activation of tyrosine kinase, trans-autophosphorylation, and initiation of extracellular-signal-regulated kinase, Janus kinase, signal transducer and activator of transcription, Src family kinase, focal adhesion kinase, phosphoinositide 3-kinase/AKT kinase, and mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase (p70S6K) (7–9). Considering that angiogenesis is a significant event in tumor development, blocking angiogenesis is one of the most promising strategies to treat malignancies.

Owing to the importance of VEGFR2 in angiogenesis, this receptor is the most vital target in anti-angiogenic therapy against cancer. Numerous reports on VEGFR2 inhibitors, including the commercialized vandetanib and sunitinib, have been published (10–11). At present, sorafenib is the only

*Corresponding author: Jun Li. Department of Pharmacy, Anhui Medical University, Hefei 230032, China Tel: +86-551-6512-4322; Fax: +86-551-5516-1136; email: zongshengyin1314@163.com; lj@ahmu.edu.cn;

approved angiogenesis inhibitor for treating advanced HCC, and this drug significantly improves the survival of advanced HCC patients. Moreover, sorafenib is the new standard treatment for advanced HCC patients (12). However, patient survival, which considerably varies depending on the sensitivity of the individuals, was improved only for a few months (13, 14). Therefore, the discovery of more VEGFR2 inhibitors as anti-HCC drugs remains necessary.

To obtain more potent VEGFR2 inhibitors, we presented a restricted *de novo* design method for VEGFR2 inhibitors; this method was detailed in our previous study (15). By using this method, we synthesized and extensively tested the anti-angiogenic activities of a series of 2,4-disubstituted quinazoline derivatives. Among these compounds, **11d** exhibited potent cytotoxicity in different carcinoma cell lines, such as CNE-2 (human nasopharyngeal cancer), PC-3 (human prostatic carcinoma), and SMMC-7721 (human liver cancer), as well as moderate cytotoxicity against human umbilical vascular endothelial cells (HUVECs). Additionally, compound **11d** remarkably inhibited HUVEC migration and adhesion and demonstrated significant anti-angiogenic activities (15). However, the detailed mechanism underlying the use of this compound as an anticancer drug remains not comprehensively elucidated. Therefore, an in-depth study is required. To determine the direct anti-angiogenic effects of compound **11d** and its underlying molecular mechanisms, we evaluated the effectiveness of this compound *in vitro*. We report for the first time the effects of 2,4-disubstituted quinazoline derivative **11d** on HUVECs and HCC cells. Furthermore, we identified, at least, one mechanism of action by inhibiting VEGFR2 and blocking the VEGFR2-mediated Akt/mTOR /p70s6k signaling pathway.

Materials and Methods

Reagents

PBS buffer, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum, as well as anti-mTOR, anti-Akt, anti-p70S6K, anti-p-mTOR (Ser 2448), anti-p-Akt (Ser473), and anti-p-p70S6K (Thr389) antibodies were obtained from Cell Signaling Technology (MA, USA). Dimethyl sulfoxide (DMSO), protease inhibitor cocktail, and rabbit anti-human β-actin antibodies were obtained from Sigma (St. Louis, MO, USA). PCR kit, Trizol, and first-strand cDNA synthesis kit were purchased from TAKARA Company (Dalian, China). Polyvinylidene difluoride (PVDF) membranes and goat anti-rabbit and horseradish peroxidase-conjugated IgG were obtained from Millipore (MA, USA). TRIzol reagent and sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gels were acquired from Beyotime Biotechnology (Haimen, China).

In vitro studies on VEGFR2 tyrosine kinase activity

In vitro VEGFR2 kinase inhibition assay was performed using recombinant human VEGFR2 (Sino Biological Inc., USA) and VEGFR2 kinase assay Kit (GENMED SCIENTIFICS INC., USA). The 4× reaction cocktail containing VEGFR2 was incubated with compound **11d** (0, 1, 2, 4, 8, 16, 32, and 64 μM) and SU6668 (0, 0.1, 0.5, 1, 2, 4, 8, and 16 μM) or DMSO (0.1%) for 5 min at room temperature, and then 2× ATP/substrate peptide cocktail was added to the preincubated reaction cocktail/brucine or DMSO (0.1%). After 30 min incubation at room temperature, the reaction was stopped, transferred into a 96-well streptavidin-coated plate, and subsequently incubated for 1 hr at room temperature. Primary antibody, that is, phosphorylated tyrosine monoclonal antibody [(pTyr-100), 1:1000 in PBS/T with 1% bovine serum albumin (BSA)], was added into each well until the wells were washed thrice with PBS/T (1× PBS, 0.05% Tween-20). After 1 hr incubation at room temperature, substrate phosphorylation was monitored with HRP-labeled anti-mouse IgG antibody (1:500 in PBS/T with 1% BSA) followed by a chromogenic reaction. Finally, VEGFR2 kinase assay was performed at 450 nm by using a microplate reader. The reaction using only DMSO (0.1%) served as the vehicle control. The results were expressed as percent kinase activity of the vehicle control (100%), and IC_{50} was defined as the compound concentration resulting in 50% inhibition of enzyme activity. The kinase assay was independently performed thrice.

Cell line, cell culture, and cell viability assays

HUVECs and HepG2 cells were purchased from the American Type Culture Collection and were cultured in DMEM (GIBCO, USA) supplemented with 10% (v/v) fetal bovine serum (GIBCO, USA), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were cultured at 37°C humidified atmosphere with 5% CO_2.

HepG2 cells (5 × 10^4 cells/well) were seeded on 96-well plates treated with various concentrations of compound **11d** (0, 1.25, 2.5, 5, 10, 20, and 40 μM) for 24 hr. After 4 hr of incubation, 20 μl of MTT (5 mg/ml) was added. DMSO (100 μl) was then added into each well, and the optical density (OD) was recorded at 490 nm. All measurements were obtained in triplicate.

Reverse transcription PCR (RT-PCR)

To evaluate the mRNA expression of human VEGFA and VEGFR2, we allowed the cells to grow in their media and then treated them with various concentrations of compound **11d** (0, 6.25, and 12.5 μM) or with vehicle alone for 24 hr. Total RNAs of the

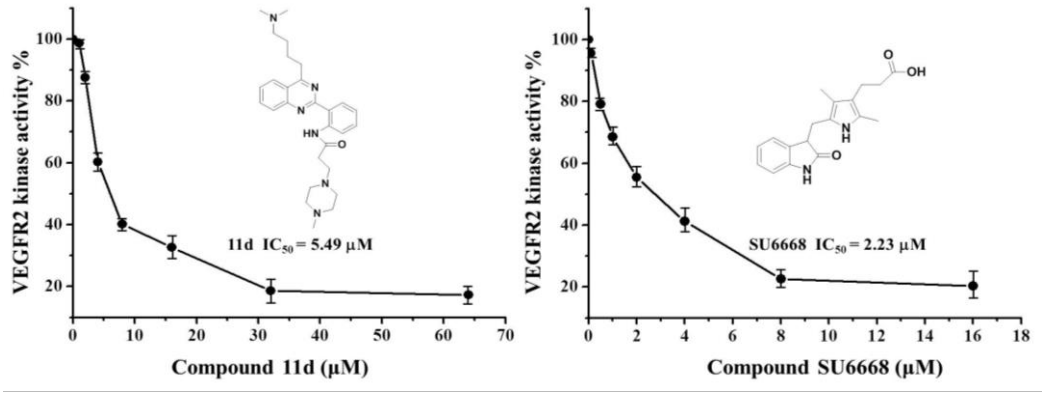

Figure 1. Inhibition of VEGFR2 kinase activity by quinazoline derivative **11d** and **SU6668** was analyzed using an *in vitro* HTScan® VEGF receptor 2 kinase kit (Cell Signaling Technology, Danvers, MA, USA) combined with colorimetric ELISA detection according to the manufacturer's instructions. Values are mean ± SEM (n = 6) of three independent experiments

cells were extracted with TRIZOL reagents in accordance with the manufacturer's protocol. Any potential DNA contamination was removed by RNase-free DNase treatment. cDNA was synthesized from the total RNA by using AMV reverse transcriptase. The primers for human VEGFA are Sense: 5'GAAGA-GAGAGACGGGGTCAGAGAG3', Antisense: 5' AGCCC-AGAAGTTGGACGAAAAGT3'; and those for human VEGFR2 are Sense: 5'CCATGTTCTTCTGGCTACTTC-TTGT3', Antisense: 5' CTCACTGTGTGTTGCTCCTTCTTT 3' (16).

Western blot analysis

Western blot analysis was performed using previously reported methods (16). After treatment with various concentrations of compound **11d** (0, 6.25, 12.5, and 25 μM) for 24 hr, the cells were harvested and lysed on ice for 30 min in lysis buffer containing protease inhibitors and phosphatase inhibitors to prevent proteolysis and/or dephosphorylation. After centrifugation at 12000 rpm for 5 min, the supernatant was harvested as cellular protein extract. Protein content was determined using a BCA protein assay kit (Beyotime Biotechnology, Haimen, China). The total cellular protein extracts were separated by SDS-PAGE and then transferred onto PVDF membranes (Millipore, USA); the transferred proteins were assessed by staining with Ponceau S solution. The membranes were blocked with 5% dehydrated skim milk in TBST for 1 hr at room temperature and then incubated overnight at 4°C with primary antibody. The blots were washed thrice in TBST buffer and subsequently incubated with corresponding HRP-linked secondary antibodies for 1 hr at room temperature. The specific proteins in the blots were visualized using an enhanced chemiluminescence reagent (Thermo Scientific, USA).

Statistical analysis

Results are expressed as mean ± standard deviation. Statistical comparisons were performed using Student's *t*-test and one-way ANOVA. The minimum level of significance was $P < 0.05$.

Results

Compound 11d attenuates VEGFR2 tyrosine kinase activity

VEGFR2 plays a crucial role in VEGF-dependent angiogenesis. To investigate the molecular mechanism of quinazoline-derivative-induced angiogenic inhibition, we examined the effects of quinazoline derivative **11d** on VEGFR2 activation by using a VEGFR2-specific activation assay; the previously reported VEGFR2 receptor SU6668 was used as a positive control. Accordingly, ELISA-based tyrosine kinase assay was conducted to examine the effects of these compounds on VEGF-stimulated P-VEGFR2. It was found that compound **11d** could dose-dependently suppress the kinase activity of VEGFR2 with an IC_{50} of 5.49 μM, which is in the same order of magnitude as the reference compound SU6668 (IC_{50}= 2.23 μM) (Figure 1).

Compound 11d modulates VEGF and VEGFR2 expression

Considering that VEGF plays a crucial role in tumor angiogenesis, we first examined the VEGF transcription in HUVECs in response to compound **11d**. HUVECs were treated with increasing concentrations of compound **11d** (0, 6.25, 12.5, and 25 μM) for 24 hr. Afterward, the mRNA level of VEGFA was determined using RT-PCR. As shown in Figure 2, compound **11d** changed the expression levels of VEGF in a dose-dependent manner. In addition, VEGF transmitted angiogenic signals through the VEGF receptors. We subsequently examined the VEGFR2 expression in HUVECs in response to compound **11d**. Consistent with the VEGF induction results, compound **11d** significantly downregulated the VEGFR2 mRNA expression (Figure 1).

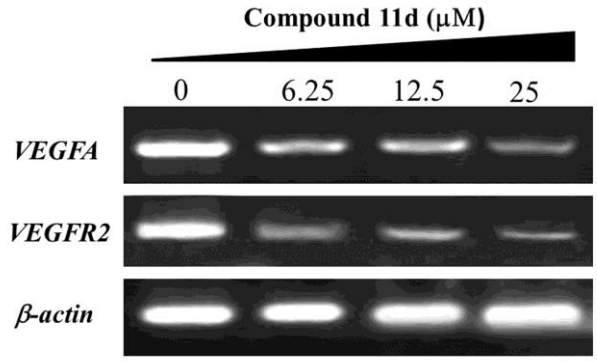

Figure 2. mRNA expression of VEGF and VEGFR2. Compound **11d** reduced the mRNA expression of VEGFA and VEGFR2 in a dose-dependent manner. HUVECs were treated with increasing concentrations of compound **11d** for 24 hr

Compound 11d inhibits activation of VEGFR2-mediated Akt/mTOR/P70S6K signaling in HUVECs

VEGFR2 binds with VEGF to activate numerous downstream signaling molecules responsible for cell migration, proliferation, and survival. We previously found that compound **11d** exhibits significant anti-angiogenic activities in the chick embryo via chorioallantoic membrane assay. To understand the molecular mechanism underlying the compound **11d**-mediated anti-angiogenic properties, we examined the signaling molecules and pathways through Western blot assays. Activation of the Akt/mTOR/P70S6K pathway is required for the proliferative and migratory effects of VEGF on endothelial cells. Thus, we investigated the potential inhibitory effect of compound **11d** being mediated by its ability to interfere with the VEGF-triggered activation of the Akt/mTOR/P70S6K signaling pathway. To determine whether compound **11d** can regulate the active signaling pathways involved in cell functions, we incubated the HUVECs with various doses of compound **11d** *in vitro*. By examining the key components regulating the endothelial cell function during angiogenesis, we found that compound **11d** effectively suppressed the VEGF-induced AKT activation (Figure 3). As a result of AKT inhibition, compound **11d** blocked the activation of mTOR and p70S6K, suggesting that compound **11d** inhibits tumor angiogenesis by blocking the PI3K/AKT/mTOR signaling pathway.

Compound 11d induces HCC cell death by inhibiting Akt/mTOR/P70S6K signaling

Considering that PI3K/AKT/mTOR activation plays a crucial role in carcinogenesis by maintaining cancer cell proliferation and preventing apoptosis, recent studies have focused on developing novel anticancer agents targeting this pathway. Therefore, we investigated the effects of compound **11d** on the PI3K/Akt/mTOR pathway in HCC cells (Figure 4).

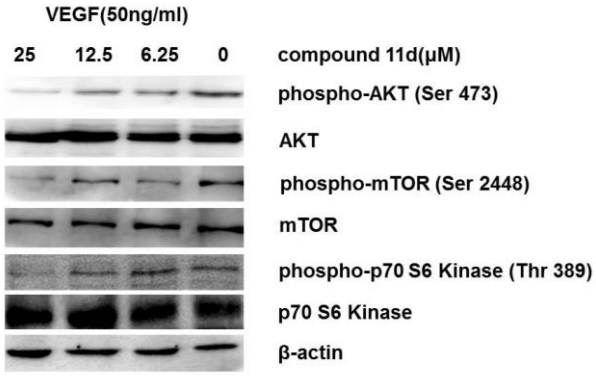

Figure 3. Compound **11d** inhibited the activation of AKT/mTOR/P70S6K signaling in HUVECs

The HCC cell viability was determined by MTT assay. At 5–40 μM concentration, compound **11d** significantly inhibited HepG-2 cell proliferation at an IC_{50} value of 10.3 μM (Figure 4A). By treating the HCC cells with various concentrations of compound **11d** (0, 6.25, 12.5, and 25 μM), we found that the phosphorylation level of AKT and its downstream factor mTOR were effectively suppressed in a dose-dependent manner. In addition, mTOR activation resulted in phosphorylation of effectors, such as p70S6K, leading to mTOR-dependent gene transcription, which regulates cell proliferation, protein synthesis, and metabolism. Therefore, we further determined the effect of compound **11d** on p70S6K and found that p70S6K phosphorylation was downregulated by compound **11d** compared with the control.

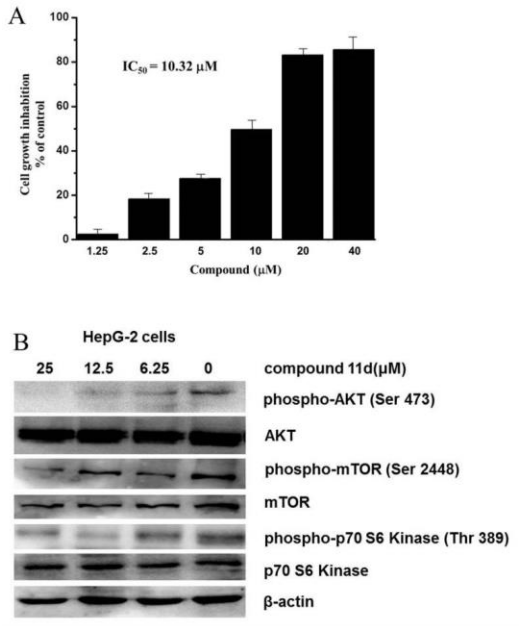

Figure 4. (A) Compound **11d** inhibited HepG-2 cell growth. Values are expressed as mean ± SEM ($n = 6$) of three independent experiments; $P < 0.05$ versus vehicle control. (B) Compound **11d** inhibited the VEGFR2-mediated AKT/mTOR/P70S6K pathway in HCC cells

Discussion

The cancer-related high mortality rate and significant morbidity, the apparent toxicity, and the poor response rates to current chemotherapy have been significant impetuses to identify novel compounds suppressing tumor growth. The development of anti-angiogenic agents is the current trend in molecular-targeted therapy for cancer (17). Angiogenesis plays a significant role in cancer progression because newly formed tumor veins serve as feeding tubes to deliver nutrients and oxygen supply to the growing tumor and ultimately act as conduits for the dissemination of tumors that have escaped from the primary tumor (18). The present strategies for anticancer therapy become ineffective once tumor cells reached the secondary organs and generate metastatic foci. Consequently, angiogenesis suppression has become a hotspot in the fight against cancer progression.

To date, several angiogenic activators and inhibitors have been identified. Among the many pro-angiogenic mechanisms, VEGF signaling has been implicated as the key regulator of tumor neovascularization. VEGF is associated with the initiation of angiogenesis by regulating proliferation, migration, invasion, survival, and differentiation of endothelial cells (7). VEGF exerts its biological effects by binding to transmembrane receptors, such as VEGFR1 and VEGFR2, both of which contain a cytoplasmic tyrosine kinase domain. The binding of VEGF to VEGFRs leads to conformational changes in the receptors followed by dimerization and autophosphorylation (3). Interestingly, we found that the 2,4-disubstituted quinazoline derivative **11d** significantly downregulated the phosphorylation of VEGFR2 via VEGFR2 kinase inhibition assay. Compound **11d** exhibited strong inhibitory activity, with an IC_{50} value of 5.49 μM, which is in the same order of magnitude as that of some VEGFR2 kinase inhibitors, such as semaxanib (IC_{50} = 0.29 μM) (19) and SU6668 (IC_{50} = 2.23 μM). These results suggest that the anti-angiogenic effects of the 2,4-disubstituted quinazoline derivatives are partially mediated by inhibition of VEGR2 activation. We also further validated that the antiangiogenic properties of compound **11d** significantly inhibited the mRNA levels of VEGF and VEGFR2 in HUVECs.

The mammalian target of rapamycin (mTOR) is a significant regulator of tumor growth, metastasis, and angiogenesis (20, 21). This pathway is overactive in many cancers, and enhanced signaling through this pathway is a crucial contributor to the formation of new blood vessels (22, 23). Moreover, AKT is a serine/threonine kinase that plays a key role in a range of cellular functions, including cell proliferation, migration, protein synthesis, and angiogenesis (24). P70S6K kinase, a downstream of AKT, also plays a crucial role in regulating tumor microenvironment and angiogenesis. P70S6K activation is found in various human cancers, such as thyroid, breast, and ovarian cancers (24–26). In addition, the AKT/mTOR/p70S6K signaling is recently identified as a novel, functional mediator in angiogenesis (23). Our studies have shown that treatment of HUVECs and HCC cells with compound **11d** significantly reduced the phosphorylation of mTOR and p70S6K and its upstream kinase AKT, suggesting that compound **11d** suppresses tumor angiogenesis both by inhibiting VEGFR2 and by blocking its multiple downstream signaling components.

Conclusion

In summary, the quinazoline derivative **11d** inhibits angiogenesis by down regulating the phosphorylation of VEGFR2 tyrosine kinase and suppressing the VEGFR2-mediated signaling pathway, which plays multiple roles in regulating neovascularization. Therefore, our results strongly suggest that the quinazoline derivative **11d** is an effective antagonist of the VEGF/VEGFR2-stimulated angiogenesis and a potential candidate for the development of a multifunctional anticancer agent owing to its inhibitory activity on several aspects of tumor growth and angiogenesis.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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