Isoindolone derivative QSN-10c induces leukemic cell apoptosis and suppresses angiogenesis via PI3K/AKT signaling pathway inhibition

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Aim: 2-(4,6-Dimethoxy-1,3-dioxoisooindolin-2-yl) ethyl 2-chloroacetate (QSN-10c) is one of isoindolone derivatives with antiproliferative activity against human umbilical vein endothelial cells (HUVECs). The aim of this study was to investigate its antitumor activity in vitro and anti-angiogenic effects in vitro and in vivo.

Methods: K562 leukemic cells and HUVECs were used for in vitro studies. Cell viability was examined using MTT assay. Cell apoptosis and mitochondrial transmembrane potential (Δψm) were detected with flow cytometry. Tube formation and migration of HUVECs were studied using two-dimensional Matrigel assay and wound-healing migration assay, respectively. VEGF levels were analyzed with RT-PCR and Western blotting. A zebrafish embryo model was used for in vivo anti-angiogenic studies. The molecular mechanisms for apoptosis in K562 cells and antiangiogenesis were measured with Western blotting.

Results: In antitumor activity studies, QSN-10c suppressed the viability of K562 cells and induced apoptosis in dose- and time-dependent manners. Furthermore, QSN-10c dose-dependently decreased the Δψm in K562 cells, increased the release of cytochrome c and the level of Bax, and decreased the level of Bcl-2, suggesting that QSN-10c-induced apoptosis of K562 cells was mediated via the mitochondrial apoptotic pathway. In anti-angiogenic activity studies, QSN-10c suppressed the viability of HUVECs and induced apoptosis in dose dependent manners. QSN-10c treatment did not alter the Δψm in HUVECs, but dose-dependently inhibited the expression of VEGF, inhibited the tube formation and cell migration in vitro, and significantly suppressed the number of ISVs in zebrafish embryos in vivo. Furthermore, QSN-10c dose-dependently suppressed the phosphorylation of AKT and GSK3β in both HUVECs and K562 cells.

Conclusion: QSN-10c is a novel antitumor compound that exerts both antitumor and anti-angiogenic effects via inhibiting the PI3K/AKT/GSK3β signaling pathway.

Keywords: isoindolone; anticancer drug; leukemia; zebrafish; angiogenesis; apoptosis; AKT; GSK3β

Introduction
Angiogenesis, the formation of new blood vessels from pre-existing vasculature, occurs under a variety of physiological and pathological conditions. Accumulating evidence indicates that angiogenesis is especially critical for the growth and progression of solid tumors. The growth of a tumor mass beyond 2 to 3 mm³ is often preceded by increased formation of new blood vessels essential for the delivery of nutrients and oxygen as well as the removal of metabolic waste from the tumor microenvironment. Moreover, many recent studies have demonstrated the important role of angiogenesis in hematologic neoplasms. Therefore, anti-angiogenic and angio-prevention therapies represent promising approaches to control tumor growth and metastasis. Neoangiogenesis is a highly ordered, multistep molecular process tightly regulated in endothelial cells that involves the proliferation of endothelial cells, motility, cell realignment, vessel formation, and the production of a new basement membrane. Of the numerous growth factors and cytokines that have been shown to possess angiogenic effects, vascular endothelial growth factor (VEGF) is an attractive therapeutic target for the development of novel anticancer agents. VEGF is produced in tumor and vascular cells, thus stimulating angiogenesis through an autocrine fashion. The inhibition of VEGF expression in endothelial cells contributes to reduced...
angiogenesis in HUVECs. In endothelial cells, VEGF has been shown to exert mitogenic activities and vasopermeability responses primarily through VEGFR-2, thereby promoting endothelial cells proliferation and migration via increased phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) (proliferation), phosphorylated-serine/threonine protein kinase family protein kinase B (AKT) (migration and survival), and c-Jun N-terminal kinase (JNK) (vascular permeability)\(^{15-18}\).

In our search for tumor inhibitors from natural sources, we identified 4,6-dihydroxyisoindol-1,3-dione (Figure 1) as a new compound isolated from Lasiosphaera fenzlii Reich. Our previous studies demonstrated that this compound has enhanced anti-angiogenic activity compared with thalidomide via inhibition of VEGF secretion. However, the compound exhibits only a slight inhibitory effect on tumor cell proliferation\(^{19}\). To find a compound with antitumor and anti-angiogenic activities in vitro, a series of isoindolone derivatives were subsequently synthesized, and their cytotoxicities were preliminarily evaluated in A549 and HUVEC cells. A new compound, 2-(4,6-dimethoxy-1,3-dioxoisoindolin-2-yl) ethyl 2-chloroacetate (QSN-10c, Figure 1), demonstrated moderate antiproliferative activities in HUVECs and K562 cells dispersed evenly in medium (containing 10% FBS) were seeded and incubated in a 96-well plate at a density of 5×10^4 cells/well for 24 and 1 h, respectively. The cells were then treated with various concentrations of QSN-10c, thalidomide, and 4,6-dihydroxyisoindol-1,3-dione for 24 h with three replicates for each treatment. Methylthiazolyldi-phenyl-tetrazolium bromide (MTT) was added to each well for 4 h. After discarding the supernatant, the insoluble formazane product was dissolved in 150 mL DMSO. The optical density was measured at 490 nm using an Elx800 microplate reader (Bio-Tek, USA). The percentage of viable cells was calculated using the following equation: \( A_s/A_c \times 100\% \), where \( A_c \) is the absorbance of the negative control and \( A_s \) is the sample absorbance.

K562 cells were seeded in 6-well plates at a density of 3×10^5 cells per well for 1 h and then treated with DMSO (0.1%) and 100 μmol/L QSN-10c for 6, 12, 24, and 48 h. To count the cells, the viable cells were distinguished using trypan blue dye. The number of viable cells was counted. The percentage of viable cells was calculated using the following equation: viable cells (sample)/viable cells (control)×100%. The assay was independently repeated thrice.

Materials and methods

Reagents

QSN-10c was prepared by the Key Laboratory of the Ministry of Education of Shenyang Pharmaceutical University with greater than 98% purity. Thalidomide was a gift from the Changzhou Pharmaceutical Co, Ltd (China). Antibodies for anti-phospho-AKT (Thr\(^{308}\)) and anti-AKT were from Bioworld (Changzhou Pharmaceutical Co, Ltd (China)). Antibodies for anti-phospho-GSK3\(^{\beta}\) (Ser\(^{9}\)), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), phospho-SAPK/JNK (Thr183/Tyr185), anti-GSK3\(^{\beta}\), anti-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), and anti-SAPK/JNK were from Cell Signaling Technology Inc (USA). Antibodies for cytochrome \( c \) (A-8), Bcl-2 (C21), Bax (P-19), VEGF (147), and anti-mouse β-actin (C4) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescent (ECL) plus reagent kit was obtained from the Beyotime Institute of Biotechnology (Haimen, China).

Cell proliferation assay

HUVEC and K562 cells dispersed evenly in medium (containing 10% FBS) were seeded and incubated in a 96-well plate at a density of 5×10^4 cells/well for 24 and 1 h, respectively. The cells were then treated with various concentrations of QSN-10c, thalidomide, and 4,6-dihydroxyisoindol-1,3-dione for 24 h with three replicates for each treatment. Methylthiazolyldi-phenyl-tetrazolium bromide (MTT) was added to each well for 4 h. After discarding the supernatant, the insoluble formazane product was dissolved in 150 mL DMSO. The optical density was measured at 490 nm using an Elx800 microplate reader (Bio-Tek, USA). The percentage of viable cells was calculated using the following equation: \( A_s/A_c \times 100\% \), where \( A_c \) is the absorbance of the negative control and \( A_s \) is the sample absorbance.

K562 cells were seeded in 6-well plates at a density of 3×10^5 cells per well for 1 h and then treated with DMSO (0.1%) and 100 μmol/L QSN-10c for 6, 12, 24, and 48 h. To count the cells, the viable cells were distinguished using trypan blue dye. The number of viable cells was counted. The percentage of viable cells was calculated using the following equation: viable cells (sample)/viable cells (control)×100%. The assay was independently repeated thrice.

Apoptosis detection by flow cytometry

The sub-diploid (sub-G\(_1\)) population was analyzed by flow cytometry using propidium iodide (PI, sigma) staining as previously described\(^{21}\). Briefly, HUVEC and K562 cells were treated with 100 μmol/L QSN-10c for 6, 12, 24, and 48 h. The cells were centrifuged at 1500 rounds per minute for 10 min at 4°C, resuspended in PBS, fixed via the drop-wise addition of ice-cold ethanol (100%) to a final concentration of 70% and incubated for 30 min on ice. The fixed cells were pelleted and treated with 500 μL propidium iodide working solution (100 μg/mL RNase A and 100 μg/mL propidium iodide in PBS) for 30 min at room temperature protected from light. DNA content was assessed using a FACSScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Annexin V staining was also used to detect apoptotic cells according to the FITC Annexin V Apoptosis Detection Kit (Becton, Dickinson
using the following equation:

\[ \text{wound area was then converted to the mean } \% \text{ recovery (} \% R \text{)} \]

VEGF mRNA levels were detected as previously reported [24].

The detection of VEGF mRNA levels using RT-PCR and each experiment was repeated at least thrice.

Endothelial cell capillary-like tube formation assay
Matrigel™ Basement Membrane Matrix (growth factor reduced; BD Biosciences, San Jose, CA, USA) was thawed at 4°C, pipetted into prechilled 96-well plates (50 µL Matrigel/well), and incubated at 37°C for 30 min. A total of 1.6×10⁴ HUVECs dispersed evenly in medium containing 1% FBS were placed onto the Matrigel layer in each well. The cells were treated with DMSO (0.1%) or various concentrations of QSN-10c (25, 50, 100, and 200 µmol/L). After 8 h of incubation, the network-like structures of endothelial cells were examined using an inverted microscope (Olympus, Tokyo) at 100× magnification. The tube-like structures were defined as endothelial cord formations connected at both ends [22]. The average number of branching points in five random fields per well was quantified using Image Pro-Plus 6.0 software. The inhibition percentage was expressed as the percentage of the vehicle control (100%). The assay was independently repeated thrice.

HUVEC migration in a wound-healing assay
An in vitro wound-healing assay was performed to measure the unidirectional migration of HUVECs. HUVECs were seeded at 5×10⁴ cells per well into a 6-well plate and allowed to grow to full confluence. After starvation with 1640 medium containing 1% FBS for 12 h to inactivate cell proliferation, the cells were wounded with a pipette tip, and washed with PBS. Fresh medium was added to the wells with or without DMSO (0.1%) or various concentrations of QSN-10c (50, 100, and 200 µmol/L) or thalidomide (100, 200 µmol/L), and the cells were incubated for 12 h. Images were obtained at the time of the wounding and at 12-h intervals thereafter using a phase-contrast microscope (Olympus, Tokyo; 100× magnification). Cell migration was quantified according to methods reported in the literature [23]. The lesion area in each field of view was measured using the data from time 0 (T₀ wound area), and the wound area was then converted to the mean % recovery (%R) using the following equation:

\[ %R = \left[ 1 - \frac{\text{wound area at } T_i}{\text{wound area at } T_0} \right] \times 100 \]

where \( T_0 \) is the wounded area 0 h and \( T_i \) is the wounded area 12 h post-injury. All experiments were performed in triplicate, and each experiment was repeated at least thrice.

The detection of VEGF mRNA levels using RT-PCR
VEGF mRNA levels were detected as previously reported [24]. HUVECs in logarithmic growth phase were seeded into 25-mL culture bottles at a density of 2×10⁵/mL. The cells were divided into control and experimental groups containing 50, 100, and 200 µmol/L QSN-10c. Total cellular RNA was isolated by TRIzol reagent extraction (TaKaRa Biotech, China) at the indicated times. Total RNA (5 µg) was reverse transcribed into cDNA using the TIANScript RT reagent kit (Tiangen Biotech Co, Ltd, Beijing). The following primers were designed and synthesized by Takara Bio: VEGF-A sense, 5’-TTG CCT TGC TCT TCC TC-3’ and VEGF-A antisense, 5’-TGCTATGATGTGTTGGACTC-3’ (280 bp product); GAPDH sense, 5’-GCA CCG TCA AGG CTG AGA AC-3’ and GAPDH antisense, 5’-ATG GTG GTG AAG ACG CCA GT-3’ (142 bp product). The following reaction conditions were used: 95°C for 5 min; 30 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s; extension at 72°C for 7 min. For the PCR product analysis, VEGF and GAPDH PCR products from the same sample were observed using 2% agarose gel electrophoresis. The images were obtained using a gel imaging system, and the target gene expression levels were measured via semi-quantitative analysis. The relative mRNA content = accumulative photon value of the target gene band/accumulative photon value of the internal standard GAPDH band.

Western blotting
Adherent cells were washed thrice with ice-cold phosphate buffered saline (PBS) before lysed in RIPA buffer containing the following protease and phosphatase inhibitors: aprotinin (10 mg/mL), leupeptin (10 mg/mL), phenylmethylsulfonyl fluoride (1.72 mmol/L), NaF (100 mmol/L), and NaVO₃ (500 mmol/L). K562 cells were collected and then lysed as described above. Equal amounts of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The blots were incubated with primary antibodies followed by secondary antibody conjugated to horseradish peroxidase; antibody binding was detected with BeyoECL Plus.

Anti-angiogenic activity in the zebrafish embryo model
The in vivo anti-angiogenic activity of QSN-10c was further investigated in the zebrafish embryo according to standard operation practices [25]. Briefly, zebrafish embryos were generated by natural pair-wise mating and raised at 28.5°C in embryo water (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.4 mmol/L CaCl₂, 0.16 mmol/L MgSO₄). QSN-10c was diluted in DMSO as needed and then transferred to the embryo water. Healthy, hatched zebrafish embryos were selected at 24 hpf (hours post fertilization), and treated with DMSO (0.1%) or varying concentrations of QSN-10c (5, 10, and 20 µg/mL). The embryos were then incubated in 24-well plates (10–15 embryos/well) at 28.5°C from 24 to 48 hpf. At 48 hpf, the zebrafish were removed from the 24-well plates, and viability and vessel morphological changes were assessed. The zebrafish section just below the yolk sac was selected for the assessment of complete intersegmental vessels (ISVs) by manual
counting. ISVs with blood flow were counted as effective; therefore, images could not be obtained. Embryos treated with DMSO (0.1%) served as the vehicle control. The assay was independently repeated thrice. The percentage of inhibition ratio = \[1 - \left(\frac{\text{ISVs (sample)}}{\text{ISVs (control)}}\right)\] × 100%

**Statistical analysis**

The results were expressed as the mean±SD. Statistical significance was compared between the QSN-10c-treated and control groups using Student’s t-test. Results with \( P < 0.05 \) were considered significantly different from the control. The significance of the difference between two groups was assessed using Student’s t-test.

**Results**

**QSN-10c decreases the viability of HUVEC and K562 cells**

To elucidate the anti-angiogenic effects of QSN-10c *in vitro*, the viability of human umbilical vein endothelial cell (HUVEC) treated with QSN-10c for 24 h was evaluated. In addition, the effect of QSN-10c on K562 cell viability was also examined. Similar to our previous studies, QSN-10c induced minimal reduction in the growth rate of HUVEC cells with an IC\(_{50}\) value greater than 150 μmol/L (Figure 2A). QSN-10c inhibited K562 cell viability with an IC\(_{50}\) value of 114.0±2.3 μmol/L; this effect is greater than that observed for thalidomide and 4,6-dihydroxyisoidol-1,3-dione (Figure 2B). The decrease in cell viability upon treatment with 100 μmol/L QSN-10c was time-dependent in K562 cells (Figure 2C).

**QSN-10c induces apoptosis in a time- and concentration-dependent manner in K562 cells**

The sub-diploid (sub-G1) population after PI staining was quantified to determine the extent of apoptosis induced by QSN-10c. A time-dependent increase in sub-G1 cells was visible after treatment with 100 μmol/L QSN-10c (Figure 3A, 3B, 6, 12, 24, and 48 h after treatment resulted in 3.47%±0.11%, 4.31%±0.46%, 5.38%±0.51%, 11.27%±0.73% sub-G1 cells, respectively, vs 1.75%±0.17%, 2.10%±0.28%, 2.16%±0.29%, 2.25%±0.21% in the respective control groups). However, no significant apoptotic induction was observed in HUVEC cells treated with the same QSN-10c concentration (data not shown).

To further characterize QSN-10c-induced apoptosis in K562 cells, annexin V staining was performed. The results demonstrated an approximate 3- to 11-fold increase in the apoptotic fraction of the groups treated with 50–150 μmol/L QSN-10c compared with the control (Figure 3C, 3D). Whereas, 100-200 μmol/L QSN-10c displayed minimal induction of apoptosis in HUVEC cells (Figure 3E).

**QSN-10c-induced apoptosis is accompanied by mitochondrial changes and mitochondria-related protein alterations**

Many anti-neoplastic drugs induce apoptosis in cancer cells via the mitochondrial apoptotic pathway\(^{[26–29]}\). A hallmark of death induction via this pathway is a rapid and early loss of the mitochondrial membrane potential (Δψm). Thus, we analyzed the integrity of mitochondrial function after treatment with QSN-10c. QSN-10c treatment resulted in a significant concentration-dependent breakdown of the Δψm (Figure 4A, 4B). HUVEC cells treated with QSN-10c showed no change in Δψm (Figure 4B).

Given that changes in the mitochondrial membrane potential are typically associated with increased permeability of the outer mitochondrial membrane that promotes efflux of apoptogenic proteins to the cytosol\(^{[30]}\), we measured the release of cytochrome c from the mitochondria to the cytosol by Western blot. As expected, the expression of cytoplasmic cytochrome c increased in a dose-dependent manner (Figure 4C). Changes in mitochondrial membrane potential can result from the
action of pro- and/or anti-apoptotic members of the Bcl-2 family. Thus, we examined the effects of QSN-10c on Bcl-2 and Bax protein expression. The Western blot revealed that QSN-10c reduces the expression of the anti-apoptotic protein Bcl-2 and increases the levels of the pro-apoptotic protein Bax in a dose-dependent manner (Figure 4C).

The effects of QSN-10c on tube formation in HUVECs
Although angiogenesis is a complex procedure involving various cell types, endothelial cell tube formation is a key step of angiogenesis. To further assess the effects of QSN-10c on endothelial cell tube formation, we used a two-dimensional Matrigel assay to examine the effect of QSN-10c on HUVEC
tube formation. When HUVECs were seeded on the Matrigel, robust tubular-like structures were formed. Capillary tube formation on Matrigel was inhibited by approximately 7.4% after treatment with 25 μmol/L QSN-10c for 8 h (P>0.05). Tube formation was inhibited by approximately 50% with 50 μmol/L QSN-10c, and tube formation was almost completely inhibited upon treatment with 200 μmol/L. The QSN-10c-mediated effects were more robust than the results of treatment with 4,6-dihydroxyisoindol-1,3-dione and thalidomide (Figure 5A–5G, P<0.05, P<0.01).

The effects of QSN-10c on HUVEC migration

As cell migration is also essential for angiogenesis in endothelial cells[33], we investigated the inhibitory effects of QSN-10c on the chemotactic motility of endothelial cells using a wound-healing migration assay. We discovered that QSN-10c inhibits HUVEC migration in a dose-dependent manner, with significant inhibition at 100–200 μmol/L (Figure 6A–6G, P<0.01, P<0.001). These findings indicate that QSN-10c may inhibit angiogenesis in vitro by preventing endothelial cell migration and tubular structure formation.

VEGF levels are decreased in HUVEC cells

To investigate whether QSN-10c modulates the VEGF-mediated autocrine effects on endothelial cells, RT-PCR and Western blot were performed to examine VEGF expression in HUVECs. The results indicated that 24 h of treatment with 50–100 μmol/L QSN-10c clearly decreased VEGF mRNA levels (Figure 7A, 7B, P<0.05). We further determined the effect of QSN-10c on VEGF expression by Western blot. QSN-10c decreased VEGF protein in a dose-dependent manner compared with the control (Figure 7C). These results suggest that QSN-10c-mediated VEGF inhibition potentially contributes to reduced angiogenesis by HUVECs.

The PI3K/AKT/GSK3β pathway is involved in QSN-10c-mediated antiangiogenesis and K562 cell apoptosis

To investigate the molecular mechanism of QSN-10c-mediated antiangiogenesis, we examined the effect of QSN-10c on the activity of AKT and GSK3β, important components of the PI3K/AKT signaling pathway that plays a crucial role in HUVEC migration[34]. Our data indicated that AKT and GSK3β phosphorylation were significantly reduced by QSN-10c in a dose-dependent manner in HUVEC cells. However, QSN-10c did not affect the phosphorylation of ERK1/2 and JNK, signal proteins involved in the MAPK pathway (Figure 8A). To investigate the role of the PI3K/AKT signaling pathway in QSN-10c-mediated apoptosis in K562 cells, we also examined the expression of AKT and GSK3β in K562 cells treated with various concentrations of QSN-10c. The result suggest that QSN-10c also inhibits the expression of p-AKT and p-GSK3β in a dose-dependent manner (Figure 8B), indicating that QSN-10c induces K562 cell apoptosis at least in part via inhibition of the PI3K/AKT pathway.

Anti-angiogenic activity in zebrafish embryo model

Based on QSN-10c activities in vitro, we further evaluated the anti-angiogenic property of QSN-10c in a zebrafish embryo model. Zebrafish embryos were treated with DMSO (0.1%) or various concentrations of QSN-10c (5, 10, and 20 μg/mL) from the shield stage (24 hpf) to 48 hpf. Significant reductions
in the number of complete ISVs were observed in the 10–20 μg/mL QSN-10c-treated groups compared with the vehicle control group (Figure 9, *P*<0.05).

**Discussion**

Angiogenesis is now recognized as playing a crucial role in the initiation and progression of many diseases, such as cancer and RA, and anti-angiogenic interventions are becoming a promising approach for the treatment of these diseases. Many reported agents possess anti-angiogenic activity via the inhibition of HUVEC proliferation, migration, and tube formation *in vitro*. Moreover, bevacizumab (Avastin, anti-VEGF monoclonal antibody) and vatalanib (VEGFR inhibitor) are approved for the treatment of various malignant tumors by targeting angiogenic pathways. Thalidomide [α-(N-phthalimido)-glutarimide] is an immunomodulatory and anti-angiogenic drug. Recent research has shown that the drug has antitumoral properties, and promising results from thalidomide treatment in patients with myeloma, myelodysplastic syndrome, and variety of solid tumors has been reported[35]. However, thalidomide has been shown to require bio-activation to exert its anti-angiogenic effect in isolated blood vessels and endothelial cells[36, 37]. Moreover, thalidomide displayed varying anti-angiogenic activities in endothelial cells (ECs) in the literature[38, 39]. Thus, the specific mechanism of antitumoral action for thalidomide remains unclear. In this study, thalidomide was used as positive control and displayed minimal anti-angiogenic activities, similar to previous findings[39].

The preliminary comparison between 4,6-dihydroxyisoindol-1,3-dione and thalidomide has been previously performed. Because 4,6-dihydroxyisoindol-1,3-dione exhibits specific anti-angiogenic activities, a series of derivatives were subsequently synthesized, and then their cytotoxicity and anti-angiogenic activity was evaluated in tumor cells to identify candidates with enhanced properties. Among the derivatives, QSN-10c displayed moderate cytotoxicity in A549 and HUVEC

![Figure 5. QSN-10c inhibits capillary structure formation of endothelial cells on Matrigel.](image-url)

(A) HUVECs cultured on Matrigel were treated with only DMSO (0.1%). (B, C) HUVECs cultured on Matrigel were treated with 100 μmol/L of thalidomide and 4,6-dihydroxyisoindol-1,3-dione, respectively. (D–F) HUVECs cultured on Matrigel were treated with various concentrations of QSN-10c (50, 100, and 200 μmol/L). (G) Quantitative comparison of the numbers of branching points in different groups. Cells receiving only DMSO (0.1%) served as a vehicle control, thalidomide as a positive control. Data are expressed as percentages of the vehicle control (100%) in mean±SD from three independent experiments. *P*<0.05, *P*<0.01 compared with control.
cells in our previous study. To further elucidate the effects of QSN-10c on angiogenesis and hematologic neoplasms, we employed in vitro HUVEC models (migration, tube formation, and VEGF expression) and an in vivo zebrafish embryo model. In addition, the cytotoxicity of QSN-10c was investigated in K562 cells. Under the conditions used in this study, QSN-10c exhibited better antiproliferative activity than thalidomide and 4,6-dihydroxyisoindol-1,3-dione in K562 cells. Additional studies suggested that 100 μmol/L QSN-10c induces apoptosis via the mitochondrial apoptotic pathway. In endothelial cells, 50–200 μmol/L QSN-10c also exhibited enhanced inhibition of HUVEC migration and tube formation as well as VEGF expression. Furthermore, we showed that QSN-10c inhibits angiogenesis in an in vivo zebrafish model.

With regard to molecular targeting mechanisms, VEGF-mediated biological effects initiate the binding of VEGF to VEGFR-2, thereby resulting in the autophosphorylation of tyrosine residues in the receptors. In fact, the Tg (VEGFR2:GFP) transgenic zebrafish model that expresses fluorescent tags in endothelial cells was first employed to evaluate the anti-angiogenic activity of QSN-10c. In this transgenic line, QSN-10c showed no inhibitory activity (data not shown), indicating that QSN-10c possibly has no effect on VEGFR2 protein. Consequently, a non-transgenic zebrafish embryo model was used for further studies, and the primary downstream pathways were investigated by Western blot to elucidate in part the anti-angiogenic mechanism of QSN-10c. Our preliminary studies indicate that QSN-10c inhibits the activities of AKT (protein kinase B) and GSK3β, crucial proteins in the PI3K/AKT signaling pathway. PI3K is activated in response to diverse mitogenic signals and catalyzes the formation of the secondary lipid messenger phosphatidylinositol-3,4,5-triphosphate. AKT binds to phosphatidylinositol-3,4,5-triphosphate via pleckstrin homology domains and is recruited to the plasma membrane, where AKT is phosphorylated at Thr308 by phosphoinositide dependent kinase-1 (PDK1) and subsequently activated. Also in K562 cells, the decrease of AKT and GSK3β was found. Interestingly, EC migration is AKT-phosphatidylinositol-3-kinase (PI3K) dependent[40–42], and the PI3K/AKT pathway transduces anti-apoptotic signals.
in tumor cells. AKT inhibition has been reported to follow the induction of the downstream mitochondrial apoptotic pathway by alterations in the Bcl-2/Bax ratio. According to increasing evidence regarding the importance of angiogenesis in hematologic neoplasms, the antiproliferative and antiangiogenic activity of QSN-10c in hematologic K562 cells may support further systematic studies.

VEGF is a potent pro-angiogenic factor that stimulates endothelial cells. Kim KH et al found that galbanic acid isolated from F. assafoetida could inhibit the proliferation of HUVECs stimulated by VEGF better than HUVECs under non-proliferative conditions (ie, without additional angiogenic growth factors). Given the similarity between QSN-10c and thalidomide as well as the unclear molecular mechanisms of QSN-10c, the effect of QSN-10c on HUVEC proliferation, migration, and capillary structure formation was evaluated without VEGF stimulation. Based on the data from our study, 50–200 μmol/L QSN-10c demonstrated specific anti-angiogenic activities in endothelial cells as well as inhibition of the AKT signaling pathway. We hypothesize that QSN-10c might display enhanced activity under mitogen-activated conditions (ie, VEGF stimulation); however, this hypothesis requires further testing.

In conclusion, our findings indicate that QSN-10c inhibits angiogenesis in vitro, and this effect is mediated by inhibition of endothelial cell tube formation and migration. The inhibition of these endothelial cell activities is mediated in part through inhibition of the AKT pathway, which plays multiple roles in the regulation of neovascularization. Furthermore, QSN-10c also directly induces apoptosis in K562 leukemia cells via the mitochondrial apoptotic pathway. On the basis of these findings, QSN-10c could serve as an effective candidate for further study. In addition, more derivatives with enhanced efficacy can be designed.

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