SNS-032 Prevents Tumor Cell–Induced Angiogenesis By Inhibiting Vascular Endothelial Growth Factor[1]

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Abstract

Cell proliferation, migration, and capillary network formation of endothelial cells are the fundamental steps for angiogenesis, which involves the formation of new blood vessels. The purpose of this study is to investigate the effect of a novel aminothiazole SNS-032 on these critical steps for *in vitro* angiogenesis using a coculture system consisting of human umbilical vein endothelial cells (HUVECs) and human glioblastoma cells (U87MG). SNS-032 is a potent selective inhibitor of cyclin-dependent kinases 2, 7, and 9, and inhibits both transcription and cell cycle. In this study, we examined the proliferation and viability of HUVECs and U87MG cells in the presence of SNS-032 and observed a dose-dependent inhibition of cellular proliferation in both cell lines. SNS-032 inhibited three-dimensional capillary network formations of endothelial cells. In a coculture study, SNS-032 completely prevented U87MG cell–mediated capillary formation of HUVECs. This inhibitor also prevented the migration of HUVECs when cultured alone or cocultured with U87MG cells. In addition, SNS-032 significantly prevented the production of vascular endothelial growth factor (VEGF) in both cell lines, whereas SNS-032 was less effective in preventing capillary network formation and migration of endothelial cells when an active recombinant VEGF was added to the medium. In conclusion, SNS-032 prevents *in vitro* angiogenesis, and this action is attributable to blocking of VEGF.

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Introduction

Angiogenesis is a physiological process intrinsic to the formation of new blood vessels from the preexisting vasculature. This complex process is also integral to the development and progression of tumors, which involve endothelial cell migration, proliferation, and differentiation, as well as tube formation [1,2]. Angiogenesis is stimulated by several growth factors, including basic fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor-α, and vascular endothelial growth factor (VEGF) family [1,3–6]. Among these, VEGF is the most potent tumor angiogenic factor, which acts on endothelial cells and plays a central role in their proliferation, migration, and survival. VEGF is expressed abundantly by most human and animal tumors [7,8]. One of the most highly angiogenic primary solid tumors is human glioblastoma, which secretes a large quantity of VEGF [9,10]. Human glioblastoma U87MG cells express three major VEGF isoforms: $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{185}$, all of which are essential for the formation of new blood vessels and for aggressive growth of tumors [11,12].

Tumor growth and metastasis are angiogenesis-dependent. Blocking angiogenesis, specifically targeting VEGF and its receptors, is, therefore, a feasible strategy for preventing tumor growth and metastasis [13–17]. Several strategies have been used to target VEGF and its receptors, such as: 1) monoclonal antibodies that compete with activating ligands for binding with extracellular domain; and 2) small molecules that inhibit intracellular tyrosine kinase activity. An anti-VEGF monoclonal antibody, bevacizumab (Avastin; Genentech, Oceanside, CA), has now been approved as first-line therapy in the treatment of metastatic colon carcinoma, in combination with standard chemotherapy [18]. However, the role of anti-VEGF therapy in the treatment of other solid tumors is currently under intense investigation.

Perturbation of cell cycle regulation is a key factor in most human neoplasias [19]. The regulatory proteins that play key roles in controlling cell cycle progression are cyclins, cyclin-dependent kinases (CDKs), their substrate proteins, CDK inhibitors, and the tumor-suppressor gene products p53 and

Abbreviations: HUVEC, human umbilical vein endothelial cell; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription–polymerase chain reaction; HIF, hypoxia-inducible factor; COX-2, cyclooxygenase-2

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pRb. Several CDK inhibitors, such as flavopiridol, UCN-01, CYC202, and SNS-032 (formerly known as BMS-387032), are undergoing clinical evaluation [20,21]. Flavopiridol, a pan-CDK inhibitor, is effective as an anticancer agent that is associated with arresting cell growth, inducing apoptosis, inhibiting cell migration, and preventing angiogenesis [22]. In earlier reports, Brusselbach et al. [23] demonstrated the induction of apoptosis in human umbilical vein endothelial cells (HUVECs) by flavopitridol and possibly through the downregulation of survivin. Hypoxic conditions in the tumor microenvironment have been shown to induce elevated VEGF levels. It has been also reported that flavopiridol prevents hypoxia-induced VEGF in several cellular systems, including human gliomas and monocytes [24,25].

The major purposes of this research are to use a more selective CDK inhibitor and to demonstrate its efficacy in the prevention of tumor angiogenesis. A newer generation of CDK antagonists (such as SNS-032) that are more selective and less cytotoxic is now entering into clinical trials [26]. However, it is not known whether this improved selectivity retains antitumor and antiangiogenic property or compromises it. Therefore, it is necessary to obtain a clear understanding of whether selective inhibition of a small cohort of CDKs impacts angiogenesis. Preclinical studies have shown that SNS-032 induces cell cycle arrest and apoptosis across multiple cell lines. In our recent report, we demonstrated that SNS-032 strongly inhibits cyclooxygenase-2 (COX-2) expression induced by the proinflammatory cytokine IL-1β [27]. COX-2 has been widely proven to play a crucial role in tumor angiogenic development and cancer metastasis [28–30]. COX-2 inhibitors may also inhibit tumor angiogenesis, reducing prostaglandin (PG) production by acting on several potential cell sources such as tumor cells, endothelial cells, and stromal reactive cells [31–33]. The possible induction of VEGF expression by PGE_1 and PGE_2 in a nonneoplastic cell system suggests the probable role of COX-2 in the expression of VEGF and the regulation of tumor angiogenesis [34,35].

To the best of our knowledge, this is the first report to suggest that SNS-032 prevents tumor cell–induced VEGF secretion and *in vitro* angiogenesis in a tumor coculture model. As an initial step, we determined the effect of this inhibitor on endothelial cell proliferation, migration, and capillary formation. We then investigated these critical steps of angiogenesis in endothelial cells when cocultured with glioma cells in the presence of SNS-032. In addition, we demonstrated that SNS-032 significantly prevented the production of VEGF in both cell lines.

Materials and Methods

Cell Culture

The human glioblastoma U87MG cell line was purchased from the American Type Culture Collection (Manassas, VA). The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Buffalo/Grand Island, NY) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 25 mM HEPES. For all experiments, cells were grown to 75% confluency and then treated as indicated. HUVECs were purchased from Cell Applications, Inc. (San Diego, CA), and maintained in an endothelial cell growth medium (Cell Applications, Inc.). Both U87MG and HUVECs were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Materials

CDK inhibitor SNS-032 (M_W = 416.99) was obtained from Sunesis Pharmaceuticals, Inc. (San Francisco, CA), dissolved in distilled water as a 10-mM stock solution, and stored at −20°C in 100-μl aliquots. Penicillin, streptomycin, fetal bovine serum, and L-glutamine were obtained from Invitrogen (Grand Island, NY). Recombinant human VEGF was purchased from R&D Systems (Minneapolis, MN). VEGF ELISA Development Kit was purchased from Peprotech, Inc. (Rocky Hill, NJ). Antiactin antibody was purchased from Sigma Aldrich Co. (St. Louis, MO). Both standard Matrigel matrix membrane and growth factor–reduced Matrigel matrix membrane were purchased from BD Biosciences Discovery Labware (Bedford, MA).

Cell Viability Assay and Growth Analysis

Cell Titer-Glo (CTG; Promega, Madison, WI) luminescent assay was performed to measure the growth curves of both HUVECs and U87MG cells. This luminescent assay determines the presence of live cells in a growing culture based on the quantitation of adenosine triphosphate released by metabolically active cells. Briefly, U87MG cells and HUVECs (2000 cells/well) were seeded in a 96-well microplate (Falcon, Franklin Lakes, NJ) in a final volume of 100 μl. After 24 hours, cells were treated with various doses of SNS-032 (0–0.5 μM) for 24, 48, or 72 hours. After completion of the treatment, 100 μl of CTG solution was added to each well and incubated for 20 minutes at room temperature in the dark. Lysate (50 μl) was transferred to a 96-well white plate (GREINER 216002, Frickenhausen, Germany), and luminescence was measured by POLARstar OPTIMA, a microplate-based multidetection reader from BMG LABTECH (Offenburg, Germany). Each point represents the mean ± SD (bars) of four values from two independent experiments. Percent cell growth was calculated by considering 100% growth at the time of SNS-032 addition.

Trypan blue exclusion (TBE) assay was performed to measure cell viability. Briefly, HUVECs and U87MG cells (3×10^5) were seeded in a 60-mm dish. Experimental conditions were the same as mentioned in the CTG assay. After treatment for indicated times, cells were trypsinized, and monolayer cell suspensions were prepared by the collection of both attached and floating cells. Resuspended cells and 0.4% trypan blue (Sigma-Aldrich Co.) solutions were mixed in equal volume, incubated at room temperature for 10 minutes, and then counted using a standard hemocytometer (Fisher Scientific, Itasca, IL). Viable cells (unstained) and nonviable cells (stained blue) were counted, and percent cell viability was calculated as: total viable cells (unstained)/total cells (stained + unstained) ×100.

Tube Formation Assay

We used standard Matrigel assay to evaluate *in vitro* angiogenesis activity by quantifying the tube formation of HUVECs, as previously described [36–38]. Briefly, 48-well culture plates were coated with 200 μl of Matrigel per well then allowed to polymerize for 30 minutes at 37°C. SNS-032 at indicated concentrations was added to HUVEC suspension 30 minutes before seeding. Cell suspensions of 150 μl (1.5×10^4 cells) were seeded on polymerized Matrigel. After incubation at 37°C for 8 hours, each culture was photographed at ×100 magnification using a Nikon (Melville, NY) camera connected to an inverted microscope. There are three parameters by which capillary structure formation can be measured: capillary length, number of capillaries or branched tubes, and relative capillary area per field. Tube formation was quantified from four randomly selected fields per experiment by measuring the total additive length of all cellular structures, including all branches, using a scale ruler.

To examine the effect of SNS-032 on tumor cell–induced tube formation of HUVECs, a conditioned medium was collected from U87MG cells as indicated and used as the growth medium for HUVECs. Briefly, cells were seeded at 70% confluency; after overnight incubation, cells were treated in the presence or in the absence of SNS-032, as indicated, for 8 hours. After 8 hours, cells were washed thoroughly with 1× phosphate-buffered saline (PBS) and further incubated in reduced serum containing DMEM for another 24 hours and collected as a conditioned medium. The conditioned medium was then used to study the *in vitro* tube formation assay in HUVECs, as described above.

To examine the effect of SNS-032 on VEGF-induced tube formation, HUVECs suspended in endothelial cell basal medium containing 0.5% fetal bovine serum (FBS) were seeded on a culture plate coated with growth factor–reduced Matrigel. SNS-032 (0.5 μM) was added to the cell suspension 30 minutes before plating the cells, and recombinant human $VEGF_{165}$ (100 ng/ml) was added at the time of seeding as indicated.

Cell Migration Assay

To investigate the cell migration of HUVECs, 8-μm-pore-size polyethylene tetraphthalate (PET) membrane inserts for 24-well plates were used (Becton Dickinson, Bedford, MA). HUVECs (2×10^4) in 500 μl of medium with or without SNS-032 were seeded onto the upper compartment (insert) of each chamber and placed into wells containing 750 μl of complete medium. Migration chambers were then incubated for 8 hours in 5% CO_2 and 95% humidified air atmosphere at 37°C to allow cells to migrate through the membrane. Following incubation, the inserts were fixed in absolute methanol for 2 minutes at room temperature and stained with 1% toluidine blue in 1% borax for 3 minutes. The cells on the upper surface of the insert were removed with a cotton-tipped swab. Images were taken using a Nikon camera connected to an inverted microscope (final magnification, ×200). Quantitation of migrating cells on the lower surface of each filter was done by manually counting 10 random fields. Each assay was performed in duplicate and repeated thrice. The data collected from independent experiments were pooled for statistical analysis.

To assay for glioblastoma cell–induced migration of endothelial cells, we performed a coculture assay using migration chambers as described by Tsujii et al. [29]. Briefly, U87MG cells were plated (70% confluency) in the outer chamber of a 24-well plate. After overnight incubation, the cells were treated with or without SNS-032, as indicated, for 8 hours. After this incubation period, the cells were thoroughly washed using 1× PBS and further incubated in reduced serum (2%) containing DMEM for another 24 hours. An insert containing 20,000 endothelial cells was then placed onto the outer chamber. After 12 hours of incubation at 37°C, migrated cells were analyzed using the same procedure as described above.

To measure VEGF-induced migration, HUVECs were suspended in growth factor–reduced endothelial cell medium and seeded in the insert with or without SNS-032. Recombinant VEGF (100 ng/ml) was then added to both inner and outer chambers containing growth factor–reduced medium. After 12 hours of incubation at 37°C, migrated cells were analyzed.

VEGF Quantification By Enzyme-Linked Immunosorbent Assay (ELISA)

The VEGF protein that was released into the conditioned medium of U87MG cells was measured using a commercially available human VEGF ELISA Development Kit (Peprotech, Inc.). Cells (5×10^5) were seeded in six-well plates in 2 ml of complete growth medium. Twenty-four hours later, cells were serum-starved for 24 hours and then exposed to SNS-032 (0–0.5 μM) with 1 ml of DMEM containing 2% FBS. After 24 hours of incubation in 5% CO_2 at 37°C and 95% humidified air to allow VEGF protein secretion, the conditioned medium was collected, and 1 mM phenyl methyl sulphonyl fluoride (PMSF) was added. The supernatant was clarified by centrifugation for 5 minutes at 14,000 rpm, aliquoted, and stored at −80°C until analysis.

Isolation and Analysis of RNA

U87MG cells were treated as indicated. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, quantified by measuring absorbance at 260 nm, and stored at −70°C. Single-tube one-step reverse transcription–polymerase chain reaction (RT-PCR) was performed using a one-step RT-PCR kit (Invitrogen). The assay was carried out in a 50-μl reaction mixture containing 2 μl (100 ng) of total RNA, 200 nM of VEGF primers 5′-CGAAGTGGTGAAGTTCATGGATG-3′ (sense) and 5′-TTCTGTATCAGTCTTTCCTGGT-3′ (antisense), 100 nM of β-actin primers 5′-GTACCACTGGCATCGTGATGGACT-3′ (sense) and 5′-ATCCACACGGAGTACTTGCGCTCA-3′ (antisense), 25 μl of a 2× reaction mixture (0.4 mM of each dNTP, 3.2 mM $MgSO_4$), and 2 μl of a SuperScript III/RT Platinum *Taq* Mix (Invitrogen, Carlsbad, CA). Cycling conditions were as follows: reverse transcription at 51°C for 25 minutes, inactivation of reverse transcriptase and activation of Platinum *Taq* DNA polymerase (Invitrogen) at 94°C for 2 minutes, 35 cycles of 94°C for 15 seconds, 55°C for

30 seconds, and 68°C for 1 minute, with a final extension at 68°C for 5 minutes. The resulting PCR product (10 μl) was analyzed on 2% agarose gels stained with ethidium bromide. Quantitation of bands was performed with the National Institutes of Health Image J software (version 1.62).

Statistical Analysis

All experiments were performed in triplicate. Results are expressed as mean ± SD. Statistical significance was determined by two-tailed Student's *t* test and equal variances using Microsoft Excel (Microsoft, Redmond, WA) and expressed as $P < .05$.

Results

Growth-Inhibitory Effect of SNS-032

In this study, we investigated the effects of the cell cycle inhibitor SNS-032 on several physiologic events, including migration, capillary formation, and VEGF regulation, that are critical in tumor growth and metastasis. First, we measured the growth characteristics of both endothelial and glioma cells in response to SNS-032 using the CTG assay (Promega). As shown in Figure 1, *A* and *D*, HUVECs and U87MG cells were treated with increasing concentrations of SNS-032 (0–0.5 μM) from 0 to 72 hours. In this assay, percent cell growth was calculated relative to 100% growth at the time of SNS-032 addition. We observed that SNS-032 dose-dependently inhibited the growth of both cell lines. A four-fold increase (400%) in the growth of HUVECs was noticed at 72 hours in the absence of SNS-032. Treatment with 0.1 μM SNS-032 decreased cell growth by almost two-fold (150% and 200%) at 48 and 72 hours, respectively (Figure 1*A*). Complete growth inhibition was obtained at 48 hours when HUVECs were treated with higher doses of SNS-032 (0.3 and 0.5 μM). We also noticed that the treatment of SNS-032 at these two concentrations had an even lower cell growth below 100% at 72 hours, indicating possible cell death. We then performed TBE assay (Figure 1*B*) to determine the viability of HUVECs using identical doses of SNS-032 and, at the same time, points used in CTG experiments (Figure 1*A*). Our results showed significant death of HUVECs

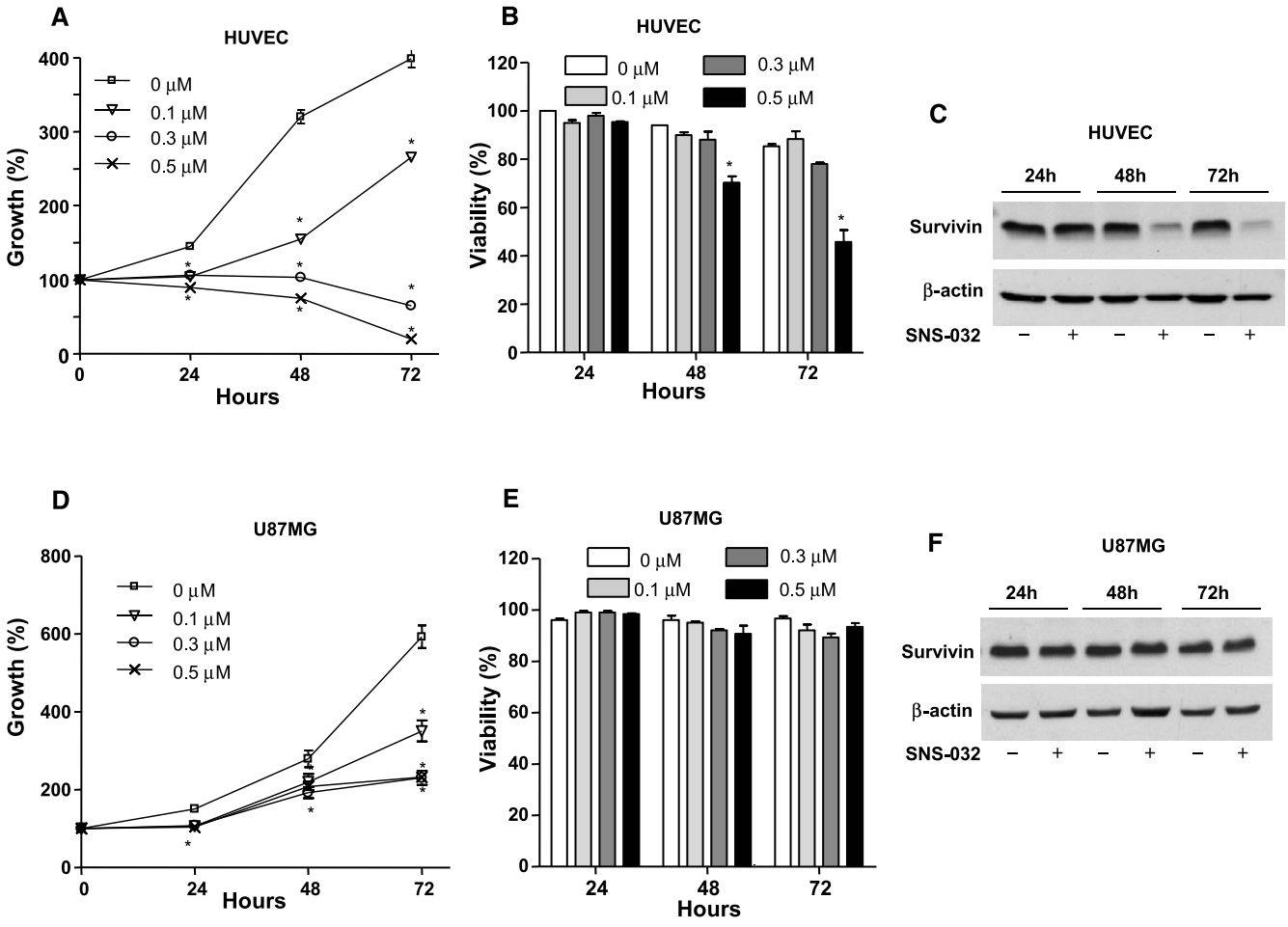

Figure 1. *Effect of SNS-032 on HUVEC and U87MG cellular growth and viability. (A and D) Cell growth was measured by CTG luminescent assay in 96-well plates in the presence of increasing doses of SNS-032, as indicated, for 24, 48, and 72 hours, and the results are expressed as percent growth inhibition in response to SNS-032 (A, HUVEC; D, U87MG). *P < .001 compared with untreated control (0 μM). (B and E) Cell viability was measured by TBE assay. Treatment conditions were identical as indicated in the CTG assay, and results were expressed as percent viability (B, HUVEC; E, U87MG). *P < .01 compared with untreated control (0 μM). (C and F) Immunoblot analysis of survivin. Cells were treated as indicated, and lysates were collected at the indicated time points and immunoblotted with survivin antibodies (C, HUVEC; F, U87MG). Bars = SD of six replicates from two independent experiments.*

(> 60%) at 72 hours when treated with 0.5 μM SNS-032. In the case of U87MG cells, a similar growth-inhibitory effect of SNS-032 was obtained. An exponential growth curve was attained in untreated cells where a six-fold growth induction (600%) was observed after 72 hours of incubation. However, a two-fold inhibition (300%) of U87MG cell growth was found at 72 hours in the presence of 0.1 μM SNS-032, and treatment with higher doses of SNS-032 (0.3 and 0.5 μM) prevented cell proliferation by almost three-fold (200%; Figure 1*D*). In contrast to HUVECs, growth inhibition of U87MG cells was not associated with cell death, as confirmed in TBE assay (Figure 1*E*). The expression level of survivin, which is an antiapoptotic protein, was also measured in response to SNS-032 in both cell lines. A temporal decrease in survivin expression was noticed in HUVECs (Figure 1*C*) in response to SNS-032, whereas no significant change in survivin level was observed in U87MG cells (Figure 1*F*) in the presence of 0.5 μM SNS-032. We observed no cytotoxic effect of SNS-032 in these two cell lines for 24 hours; therefore, we selected a treatment time frame of ≤ 24 hours for SNS-032 for the remaining experiments.

SNS-032 Inhibited Endothelial Tube Formation

An important function of HUVEC is that it can form capillary-like structures spontaneously on Matrigel; this function is crucial in blood vessel formation. Because SNS-032 strongly inhibits HUVEC cell survival and growth, we examine the effect of this drug on HUVEC function. In this study, a HUVEC suspension containing increasing doses of SNS-032 (0.0, 0.1, 0.3, and 0.5 μM) was plated on Matrigel-coated plates. In a normal growth medium, HUVECs align and form cords that ultimately become the pattern for new capillary structure (Figure 2*A*). In this study, we counted the total number of branched tubes per field formed after 8 hours of treatment with SNS-032. The data showed that treatment of SNS-032 resulted in a dose-dependent inhibition of capillary tube formation. Capillary formation was inhibited by 27% at 0.1 μM and by 65% at 0.3 μM, whereas at 0.5 μM SNS-032, > 90% of capillary formation was inhibited (Figure 2*B*). A bar graph (pooled data from three independent experiments) shows that SNS-032 significantly reduced the average number of branched tubes compared to untreated controls, and reduction was in an SNS-032 dose-dependent fashion.

SNS-032 Inhibited Endothelial Cell Migration

The migration and invasion of endothelial cells through basement membranes are a crucial step in the development of new blood vessels [39,40]. In this experiment, we measure the ability of SNS-032 in these critical events in endothelial cells using a migration chamber. HUVECs (2×10^4) were seeded onto chamber inserts and allowed to migrate for 8 hours. Representative images are shown in Figure 3*A*, and pooled results from three independent experiments are plotted in Figure 3*B*. The data demonstrated that SNS-032 treatment efficiently suppressed HUVEC migration for 8 hours in a dose-dependent manner. SNS-032 treatment inhibited 36%, 50%, and 60% of endothelial cell migration at 0.1, 0.3, and 0.5 μM, respectively. We also monitored HUVEC migration in the presence of SNS-032 at an earlier time point (3 hours) and found similar results (data not shown).

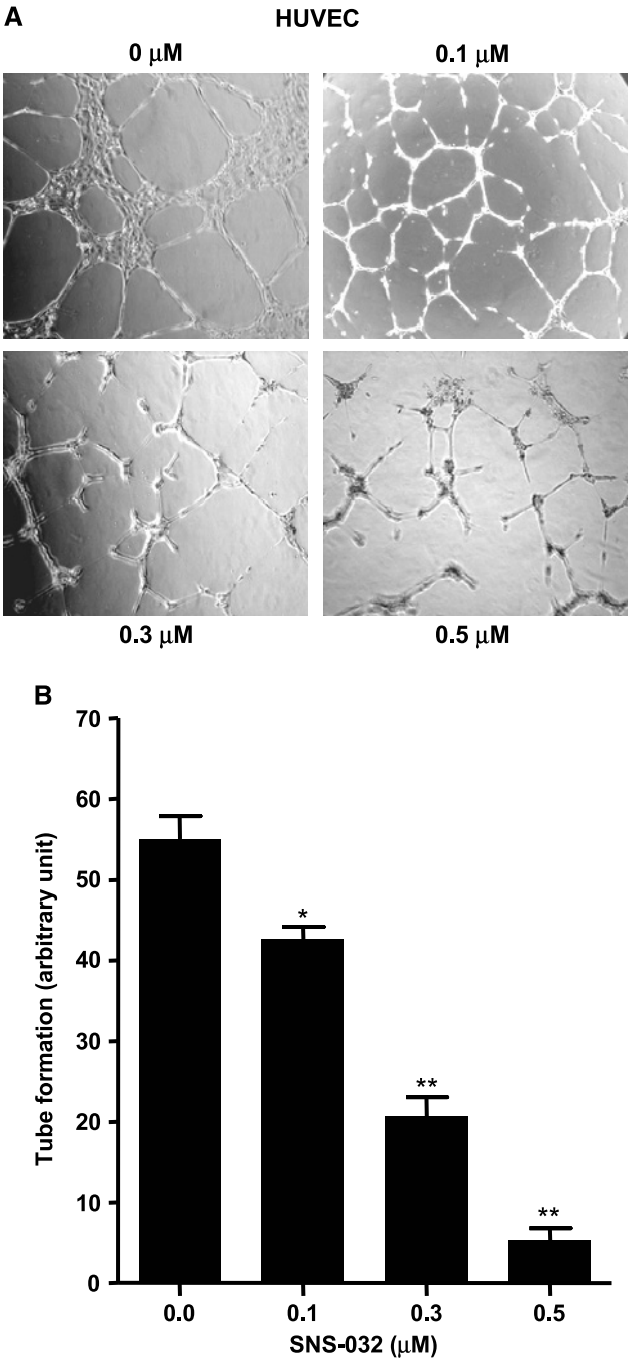

Figure 2. *Effect of SNS-032 on tube formation by HUVECs. Cells were seeded on Matrigel-coated wells in the presence of different doses of SNS-032, as indicated, and incubated for 8 hours to form a capillary network, and the total number of branched tubes was then counted. The assay was run in duplicate and repeated thrice. (A) Representative images of capillary network formation. (B) Statistical analysis of three independent experiments. Bars, SD. *P < .05 and **P < .001 compared with untreated control.*

SNS-032 Inhibited VEGF Expression

VEGF is a critical factor in new blood vessel formation [4,41,42]. In a tumor microenvironment, cancer cells secrete a high level of VEGF that binds to receptors on surrounding

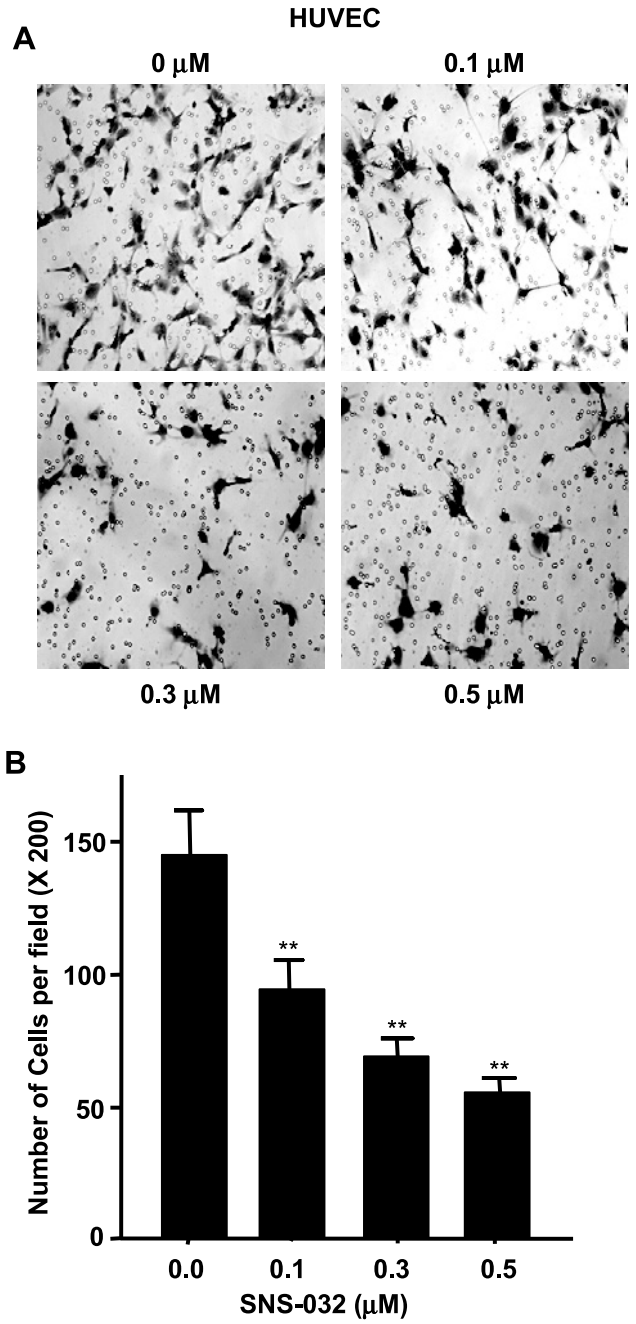

Figure 3. *Effect of SNS-032 on the migration of HUVECs. Cells were seeded onto PET membrane insert with or without SNS-032, as indicated. The insert was then placed into the well; after 8 hours, migrated cells were stained and photographed. The assay was run in duplicate and repeated thrice. (A) Representative images of endothelial cell migration were shown. (B) The total number of migrated cells per field (×200) was measured. Bars = SD calculated from three independent experiments. **P < .001, significantly different from untreated control.*

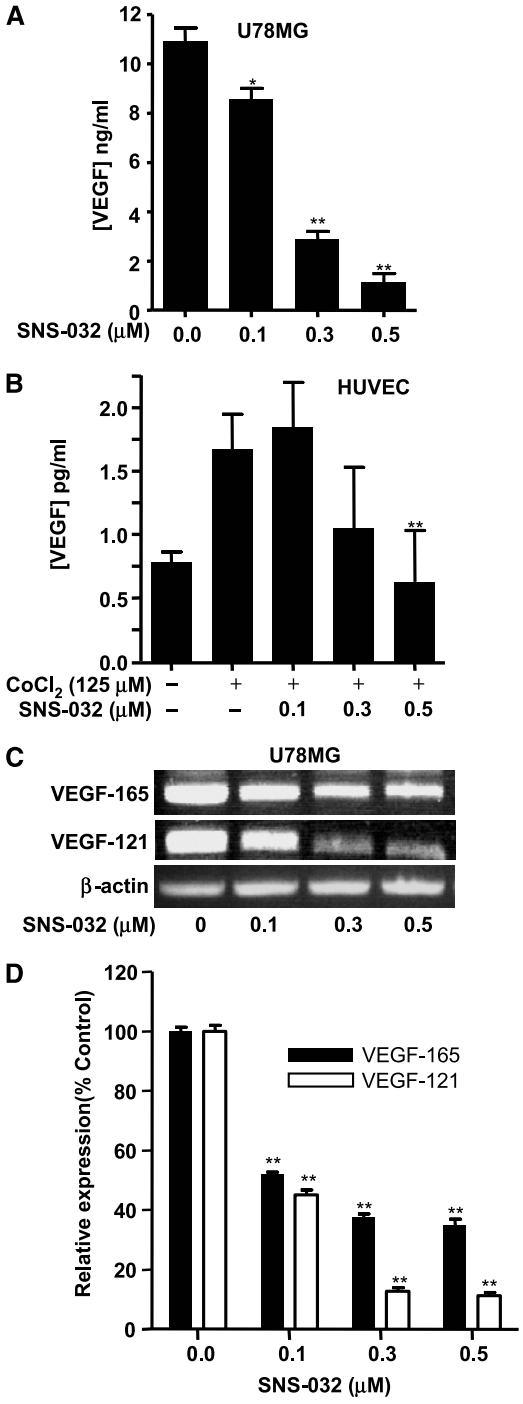

Figure 4. *SNS-032 prevents VEGF expression in both glioblastoma and endothelial cells. Expression of VEGF in U87MG and HUVECs was determined by ELISA (A and B), RT-PCR (C), and quantitation of VEGF mRNA bands (D). Cells were exposed to different doses of SNS-032, as indicated, for 24 hours. (A) VEGF protein released by U87MG cells into the conditioned medium was measured by ELISA kit. Bars = SD (n = 3). *P < .05 and **P < .001 compared with untreated control. (B) HUVECs were treated as indicated. Conditioned media were collected and then concentrated up to 10-fold using a 10-kDa molecular weight cutoff centricon concentrator (Amicon; Millipore, Billerica, MA). VEGF protein was measured by ELISA kit. Bars = SD (n = 3). **P < .001 compared with $CoCl_2$ treatment. (C) Effect of SNS-032 on VEGF mRNA in U87MG cells. The expression of VEGF mRNA was analyzed by RT-PCR. β-Actin expression was included as an internal control. (D) VEGF mRNA expression in U87MG cells was quantitated by densitometry and then normalized with β-actin, as shown in the bar graph. The ratios of VEGF/β-actin at 0 μM SNS-032 were assigned to be 100%. Bars = SD (n = 3). **P < .001 compared with untreated control.*

endothelial cells, promoting endothelial cell migration, proliferation, and differentiation, as well as tube formation [43,44]. In this experiment, we measure the effect of SNS-032 on the VEGF level in these two cell lines (U87MG and HUVECs) at different concentrations by ELISA. Data presented in Figure 4*A* show that the treatment of SNS-032 at 0.3 μM for 24 hours markedly reduced (72%) the secretion of VEGF by U87MG cells. An almost-complete inhibition was

observed at 0.5 μM SNS-032 at the same time point, whereas a 21% inhibition of VEGF level was obtained at 0.1 μM SNS-032. Pooled results from three independent experiments are shown in Figure 4*A*. We also demonstrated that this inhibition of VEGF by SNS-032 in U87MG cells was not due to cell death, as shown in Figure 1. In contrast, untreated HUVECs released a very low level VEGF (0.75 pg/ml), as shown in Figure 4*B*; therefore, we use $CoCl_2$ to induce VEGF expression in this cell line. $CoCl_2$ is a hypoxia mimetic agent and is known to induce VEGF expression by upregulating hypoxia-inducible factor (HIF) 1 [25]. A 2.5-fold induction of VEGF was achieved on treatment with $CoCl_2$, and a dose-dependent decrease of VEGF was noticed when increasing concentrations of SNS-032 were added in the presence of $CoCl_2$ (Figure 4*B*). To investigate whether SNS-032 inhibited VEGF secretion through its ability to inhibit gene transcription, we next determined the effect of SNS-032 treatment on the levels of VEGF mRNA expression in U87MG cells. Thus, after collecting conditioned medium for VEGF estimation, total RNA was extracted from remaining cells, and mRNA levels of VEGF were measured by RT-PCR (Figure 4*C*). VEGF exists in at least four different isoforms of 121, 165, 189, and 206 amino acids that are produced by the alternative splicing of VEGF mRNA from a single gene [45,46]. In the majority of cells and tissues, isoforms of $VEGF_{121}$ and $VEGF_{165}$ are detected. Our results show that treatment with SNS-032 reduces the level of these two isoforms in U87MG cells in a dose-dependent fashion (Figure 4*C*). Data presented in Figure 4*D* are a densitometric analysis of the relative expression level of these two isoforms that were performed in three independent experiments. Around 85% and 60% reduction in $VEGF_{121}$ and $VEGF_{165}$ isoforms, respectively, were obtained in response to 0.3 μM SNS-032 in U87MG cells ($P < .001$).

SNS-032 Inhibited U87MG Cell–Induced Capillary Formation of Endothelial Cells

In this experiment, our objective was to determine: 1) whether glioblastoma cells can induce the capillary formation of HUVECs; and 2) to monitor the effect of SNS-032 on this event. Therefore, we used conditioned media from U87MG cells treated with or without SNS-032, as described in Materials and Methods section. A HUVEC suspension was then prepared in this conditioned medium and plated onto a Matrigel-coated plate. Images were taken after 8 hours of incubation; results are presented in Figure 5*A*, and pooled results from three independent experiments are shown in Figure 5*B*. It is clearly seen from the results that the conditioned medium (without SNS-032) from U87MG cells induced tube formation by three-fold compared to controls (nonconditioned media, DMEM only). However, SNS-032–treated conditioned media from U87MG cells produced a complete prevention of tube formation (Figure 5*B*; $P < .001$). We also measured the VEGF level in the conditioned medium obtained from U87MG cells, and a 50% inhibition of VEGF production was noticed in the presence of SNS-032 (Figure 5*C*). These results demonstrate that the function of HUVEC is augmented by glioblastoma cells as measured by capillary formation and that this activity is downregulated when treated with SNS-032. Blocking VEGF synthesis in glioblastoma cells is one of the major factors causing the inhibition of HUVEC function.

SNS-032 Inhibited U87MG Cell–Induced Migration of Endothelial Cells

Using a coculture assay, we next tested the effect of SNS-032 on endothelial cell (HUVEC) migration induced by tumor cells. As mentioned earlier, the coculture study was performed based on the previous assay performed by Tsujii et al., and a schematic diagram of this assay is presented in Figure 6*A*. Representative images are shown in the middle panel (Figure 6, *B–D*), and pooled results from three independent experiments performed in triplicate are shown as a bar graph in the lower panel (Figure 6*E*). As shown in Figure 6*E*, HUVECs cocultured with U87MG cells migrated 2.6-fold faster compared to HUVECs alone (Figure 6*B*). This increased migration of HUVECs was prevented significantly when U87MG cells were treated with SNS-032. In a parallel experiment, we collected the medium from the bottom chamber and measured the VEGF level, finding it to be significantly inhibited in the presence of SNS-032 (data not shown). These results show an increased migration of endothelial cells through a matrix when cocultured with glioblastoma cells, and this migration is severely inhibited when U87MG cells are treated with SNS-032.

SNS-032 Partially Inhibited VEGF-Induced Capillary Formation and Migration of Endothelial Cells

In the previous experiment, we demonstrated that SNS-032 inhibited tumor cell–induced capillary formation and the motility of endothelial cells using a coculture assay. The purpose of this experiment is to investigate the effect of SNS-032 on endothelial cell capillary formation and migration when a functionally active VEGF was added to the culture medium. An active form of human recombinant VEGF (100 ng/ml) was added to the growth factor–reduced endothelial cell medium either in the presence or in the absence of SNS-032, and then we measured capillary formation and migration. Results presented in Figure 7, *A* and *B*, show that exogenous treatment of VEGF induced tube formation by almost three-fold when compared to untreated controls. However, the induction was partially inhibited (~23%), as opposed to near-complete inhibition observed in Figure 5*B* by SNS-032 treatment. In the next experiment, we determined the effect of SNS-032 on HUVEC migration induced by the addition of exogenous VEGF. Results indicate a two-fold induction of endothelial cell migration at 8 hours after the addition of VEGF, and partial reduction in migration (< 25%) was observed in the presence of SNS-032 (Figure 7, *C* and *D*). These results indicate that SNS-032 prevents *in vitro* angiogenesis primarily by blocking the production of VEGF.

Discussion

Angiogenesis, or the formation of new blood vessels, is important for tumor growth and progression, as the process

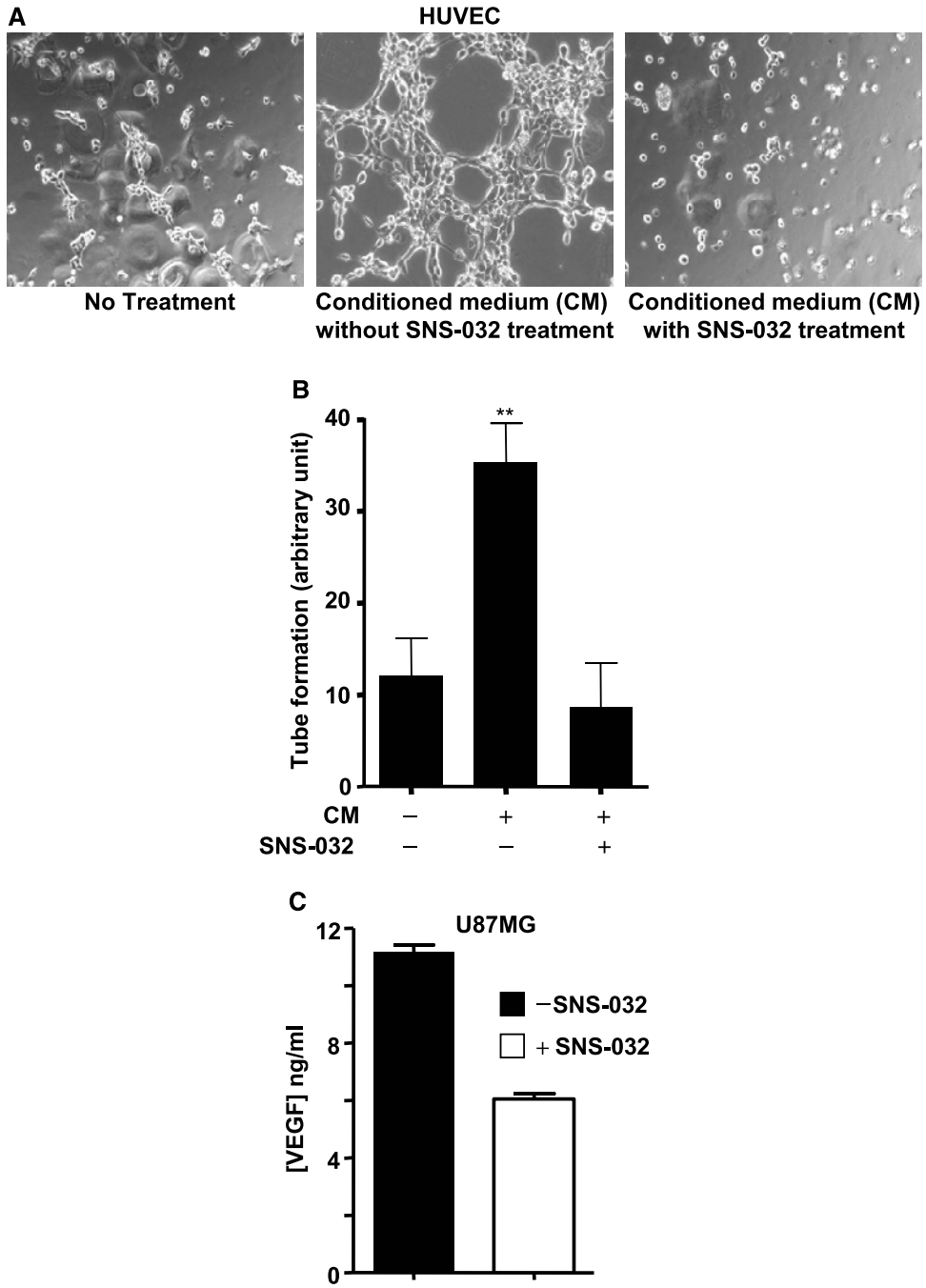

Figure 5. *SNS-032 inhibited the U87MG cell–induced tube formation of HUVECs. U87MG cells were treated with or without SNS-032 for 8 hours and then incubated with fresh media without SNS-032 for 24 hours, followed by the collection of conditioned media (CM). (A) Capillary network formation of HUVECs using CM. No-treatment panel indicates that a blank medium (serum-free DMEM) was added to HUVECs for network formation. In the other two panels, CM was added to HUVECs, as indicated. The tube formation assay of HUVECs was performed using CM prepared from U87MG cells instead of HUVEC medium. (B) The total number of branched tubes was counted, and results from three independent experiments were presented for statistical analysis. Error bars = SD. **P < .001 between untreated CM and SNS-032–treated CM or blank CM. (C) VEGF level was analyzed in the conditioned medium by ELISA.*

oxygenates and feeds the proliferation of tumor cells while quickly ridding cells of CO_2 and waste metabolites. It has been widely reported that tumor cells produce numerous angiogenic factors, including VEGF, FGF, epidermal growth factor (EGF), PDGF, and matrix metalloproteinase enzymes. These factors play a pivotal role in the development of tumor angiogenesis by stimulating endothelial cell proliferation, migration, and capillary tube formation [4,47,48]. However, among all angiogenic factors, VEGF is identified as a key mediator of angiogenesis [4,42]. Glioblastomas are one of the most highly vascularized and angiogenic solid tumors known, where aggressive blood vessel formation and glioma cell invasion result in the failure of regular conventional therapies such as surgery, radiation, and chemotherapy [12]. These tumors secrete a very large quantity of VEGF protein into the surrounding microenvironment, thereby allowing endothelial cell proliferation, migration, and tube formation [40,49,50].

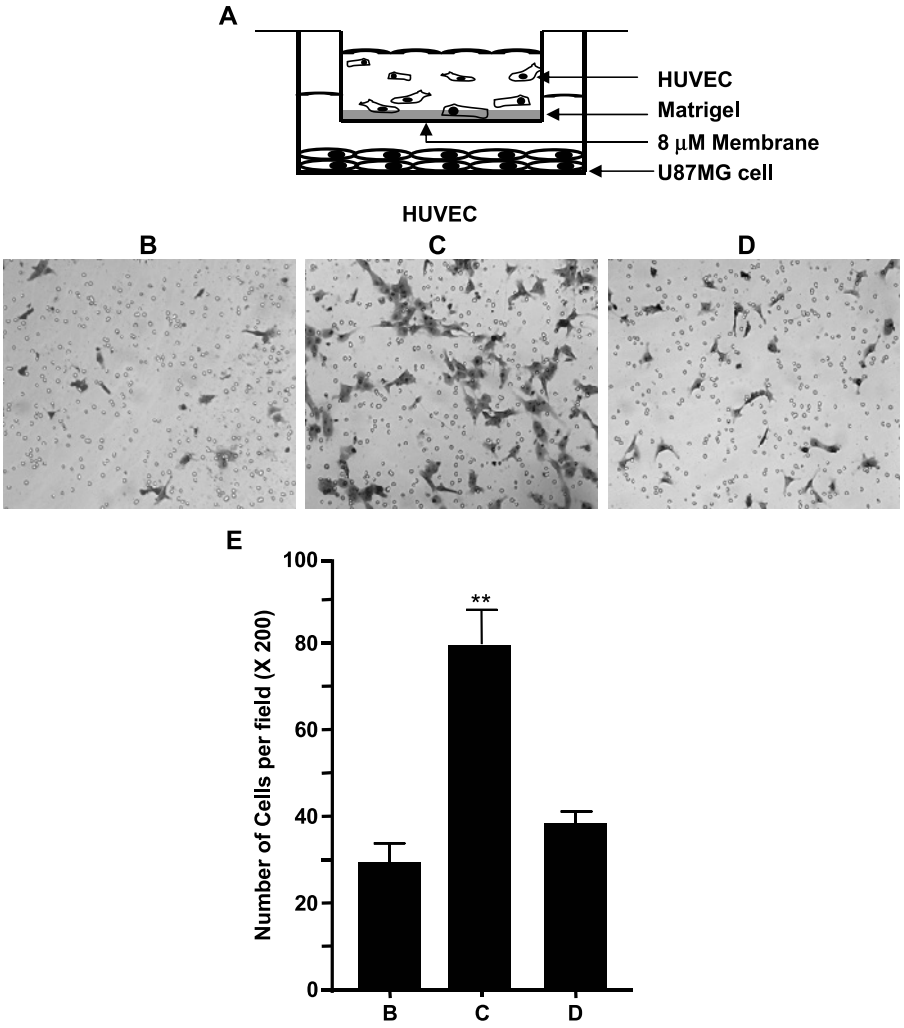

Figure 6. *Effect of SNS-032 on the migration of HUVECs cocultured with U87MG cells. U87MG cells were cultured in the well (lower chamber). Cells were then treated with or without SNS-032 for 8 hours. After treatment, the medium from the lower chamber was replaced with fresh DMEM without SNS-032, and the insert (upper chamber) containing a monolayer of HUVECs in DMEM was then placed into the well. After 8 hours, migrated cells were photographed and counted. (A) Schematic diagram of coculture assay. (B) Control. HUVECs were seeded onto the upper chamber, whereas the bottom chamber contains only DMEM without U87MG cells. (C) HUVECs cocultured with U87MG cells untreated with SNS-032. (D) HUVECs cocultured with U87MG cells pretreated with 0.5 μM SNS-032. (E) Pooled data from three experiments for statistical analysis comparing the number of migrated cells in coculture with and without SNS-032 treatment. Bars = SD. **P < .001 between treated and untreated U87MG cells with SNS-032 or blank culture medium.*

In the present study, we show that SNS-032 blocked the proliferation, capillary formation, and migration of endothelial cells, all of which are critical steps for angiogenesis. Flavopiridol, a similar class of inhibitor, showed antiproliferative activities by blocking cells at either G_1/S or G_2/M phases of the cell cycle and is associated with the downregulation of cyclins and other biologic CDK inhibitors [51,52]. Tran et al. [53] and Wall et al. [54] showed that induction of apoptosis in endothelial cells by cell cycle inhibitors may be due to the downregulation of survivin, which is an anti-apoptotic protein overexpressed in endothelial cells and is associated with resistance to apoptosis. We also observed a time-dependent decrease of survivin expression in HUVECs by SNS-032.

We found that the basal VEGF level in U87MG cells is significantly high and that SNS-032 dose-dependently downregulates both mRNA and protein levels of VEGF (Figure 4, *A* and *C*). The basal level of VEGF in HUVEC was very low, but in the presence of $CoCl_2$, an increase in VEGF release in the media was noticed, and this induction was inhibited by SNS-032 (Figure 4*B*). Melillo et al. [24] have previously shown that flavopiridol prevents VEGF in human monocytes under hypoxic conditions. In U87MG cells, induction of VEGF was also prevented by flavopiridol, possibly through the downregulation of HIF-1α [25]. In our study, we also observed the downregulation of $CoCl_2$-induced HIF-1α in HUVECs by SNS-032 (data not shown). In this study, using a coculture assay, we examined the effect of this inhibitor on glioblastoma cell–induced migration and on the tube formation of endothelial cells. Treatment with SNS-032 decreased VEGF secretion by U87MG cells and thereby reduced endothelial cell migration and tube formation.

Overexpression of VEGF and VEGF receptors correlates with increased microvessel density, proliferation, and tumor growth rate, which lead to poor patient prognosis in a variety of malignancies [1,55]. In this study, we analyzed cellular

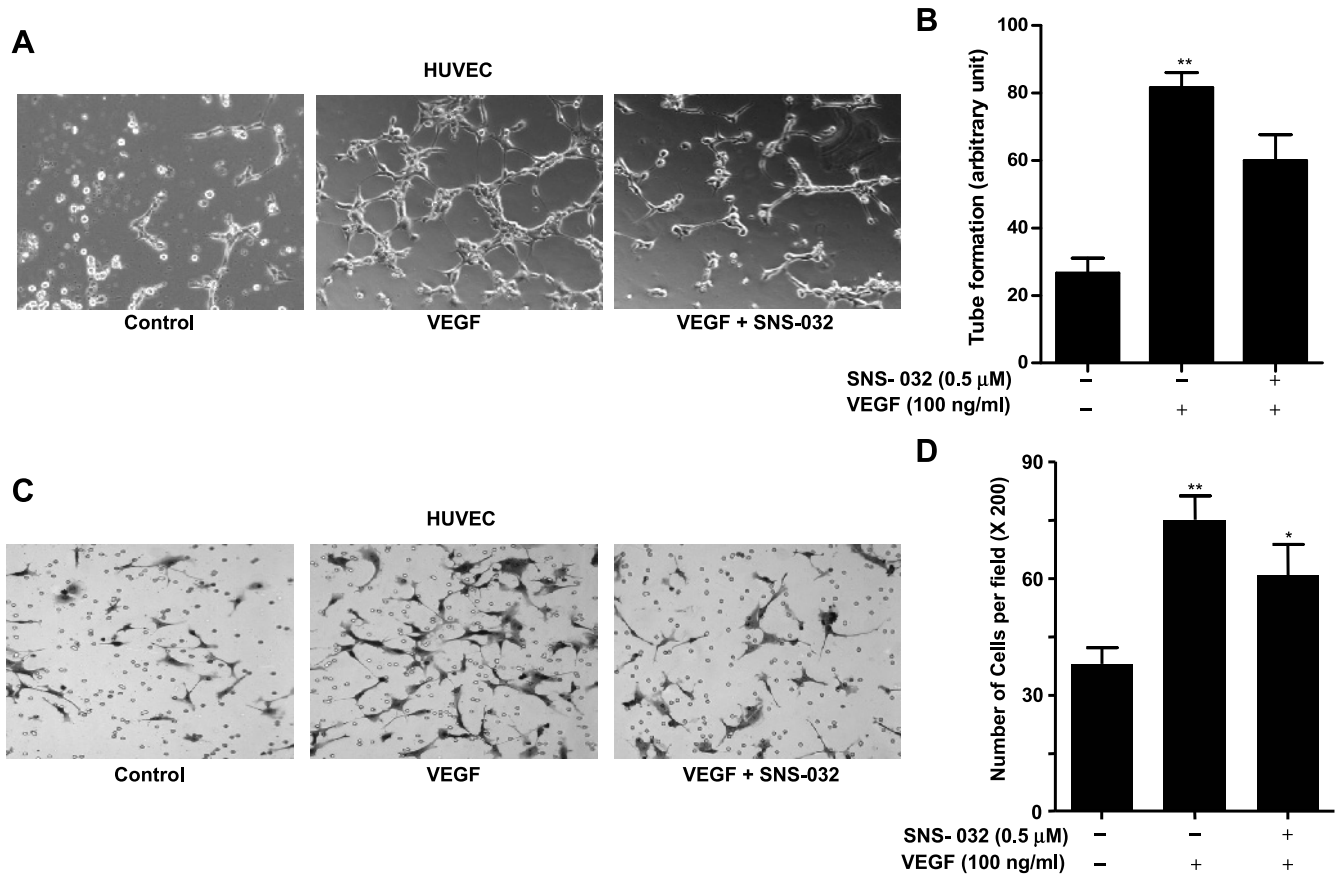

Figure 7. *Effect of SNS-032 on exogenously added VEGF-induced capillary network formation and migration of HUVECs. (A) HUVECs in endothelial cell basal medium seeded onto a growth factor–reduced Matrigel and then treated with SNS-032 (0.5 μM) for 1 hour. Recombinant $VEGF_{165}$ (100 ng/ml) was added into the endothelial cell basal medium; after 8 hours, capillary network formations by HUVECs were analyzed. (B) Branched tubes were counted, and the numbers of branched tubes with and without VEGF and VEGF + SNS-032 treatment were compared. Bars = SD (n = 3). *P < .001, significantly different from untreated control (0 μM SNS-032) or from VEGF + SNS-032 treatment. (C) Cells were seeded onto a Biocoat (Bedford, MA) (PET) membrane in growth factor–reduced endothelial cell medium, treated with SNS-032 followed by the addition of rhVEGF (100 ng/ml). (D) After 8 hours, migrated cells were counted, and statistical analysis was performed. Bars = SD. **P < .001, compared with untreated control. *P < .05, compared with the sample treated with VEGF.*

proliferation by measuring the growth of HUVECs and U87MG cells in the presence of SNS-032 at increasing concentrations. In both cell lines, it was noticed that SNS-032 prevented growth at all concentrations up to 48 hours (Figure 1, *A* and *D*). In general, antiangiogenic inhibitors exhibit cytotoxicity and induce the apoptosis of endothelial cells. When cells were continuously exposed to SNS-032 for longer periods of time (up to 72 hours), significant endothelial cell death was noticed. However, almost no cell death was observed in U87MG cells even after 72 hours of exposure of SNS-032; instead, these cells experienced complete growth arrest (Figure 1, *D* and *E*). Because the experiments were performed within 24 hours of exposure of SNS-032, reduction in VEGF, migration, and capillary network formation occurred mostly in the absence of significant cell death.

Multiple factors are involved in the upregulation of VEGF synthesis. Tsujii et al. [56] demonstrated that the overexpression of COX-2 in Caco2 cells induced VEGF levels. Furthermore, using a coculture assay, Tsujii et al. reported that a COX-2–specific inhibitor (NS-398) blocked PGE_2 synthesis in colon carcinoma cells, thus affecting tube formation and the migration of endothelial cells. As mentioned earlier, we showed that SNS-032 inhibits IL-1β–induced COX-2 expression and PGE_2 synthesis in lung tumor cells [27]. But the precise mechanism of COX-2–dependent VEGF regulation is still not clear. We demonstrated that SNS-032 mostly inhibits the transcription of VEGF. However, we noticed that SNS-032 was also effective in preventing either capillary formation or migration when an active recombinant VEGF was added exogenously into the growth factor–reduced endothelial cell medium. Currently, we are elucidating the molecular pathways of this event in response to SNS-032. In conclusion, our data suggest that SNS-032 downregulates these critical steps for tumor-induced angiogenesis and that this action is partially attributable by the blocking of VEGF.

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