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Indirubin-3′-Monoxime, a Derivative of a Chinese Antileukemia Medicine, Inhibits Angiogenesis

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ABSTRACT

Although the antiangiogenic activity of indirubin-3-monoxime (I3M), a derivative of a Chinese anti-leukemia medicine, has been demonstrated using transgenic zebrafish, the detail molecular mechanism has not been elicited. To further establish its role in antiangiogenic activity, we tested its potential against human umbilical vein endothelial cells (HUVECs) and the *in vivo* Matrigel plug model was applied to evaluate new vessel formation. We also investigated the molecular mechanisms of I3M-induced antiangiogenic effects in HUVECs. We found that I3M significantly inhibited HUVEC proliferation (2.5–20 μM), migration (2.5–20 μM), and tube formation (10–20 μM) in HUVECs. The number of microvessels growing from the aortic rings was suppressed by I3M treatment. Moreover, I3M suppressed neovascularization in Matrigel plugs in mice. The underlying antiangiogenic mechanism of I3M was correlated with down-regulation of the vascular endothelial growth factor receptor-2 activation, at least a part. These findings emphasize the potential use of I3M in pathological situations involving stimulated angiogenesis, such as tumor development. J. Cell. Biochem. 112: 1384–1391, 2011.

KEY WORDS: ANGIOGENESIS; INDIRUBIN-3-MONOXIME; VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-2; ENDOTHELIAL CELL

Angiogenesis has been described as one of the hallmarks of cancer, playing a fundamental role in tumor growth, invasion, and metastasis [Hanahan and Weinberg, 2000]. In many pathological conditions, including chronic inflammation, diabetic retinopathy, rheumatoid arthritis, or atherosclerosis, persistent upregulated angiogenesis is a common feature [Folkman, 1995; Costa et al., 2007]. Thus, the understanding of the central importance of angiogenesis and how new blood vessels are formed have led to novel therapies designed to interrupt this process [Folkman, 1995; Fayette et al., 2005; Costa et al., 2007]. Vascular endothelial growth factor (VEGF) plays important roles in the process of angiogenesis [Karkkainen and Petrova, 2000; Ferrara et al., 2003]. Binding of VEGF to VEGF receptor (VEGFR)-1 and VEGFR-2, two receptors for VEGF with intrinsic tyrosine kinase activity, leads to activation of several key enzymes, and VEGF promotes angiogenesis through activation of VEGFR-2 [Gerber et al., 1998; Eliceiri et al., 1999; Karkkainen and Petrova, 2000; Ferrara et al., 2003]. The VEGF signaling pathway has become an important target for anticancer treatment and many approaches have been developed to inhibit this pathway [Gerber et al., 1998; Eliceiri et al., 1999; Karkkainen and Petrova, 2000; Ferrara et al., 2003]. The results of the first clinical trial in the treatment of colorectal cancer by inhibition of angiogenesis are impressive [Thomas et al., 2003]. Many clinic studies have since confirmed that use of bevacizumab, the monoclonal antibody against VEGF, leads to marked survival improvement in patients with primary or metastatic cancers [Ellis et al., 2006; Grothey et al., 2008; Cohen et al., 2009].

Throughout history, natural products have afforded a rich source of compounds that have found many applications in the fields of medicine, pharmacy, and biology. Within the sphere of cancer, a number of important new commercialized drugs have been obtained from natural sources, by structural modification of natural compounds, or by the synthesis of new compounds modelled after a natural compound [Tseng and Lee, 2006]. It is generally assumed that the use of these bioactive compounds is safe and efficacious, given that they have been used for human consumption for centuries [Espín et al., 2007]. However, understanding their

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mechanisms of action as a cancer preventive and therapeutic modality is one of the main challenges for contemporary science.

Indirubin (3,2-bisindole) is an active ingredient of Danggui Luhui Wan, a mixture of 11 herbal medicines traditionally utilized against certain types of leukemias by the Chinese Academy of Medicine [Xiao et al., 2002]. Among indirubin derivatives, indirubin-3′-monoxime (I3M, Fig. 1) is the most frequently used compound for establishing biological and physiological effects of indirubin, as it has better solubility characteristics than indirubin [Marko et al., 2001]. It has been well established that I3M is a strong inhibitor of cyclin-dependent kinases (CDKs) [Leclerc et al., 2001; Marko et al., 2001; Perabo et al., 2006]. Additional studies reported that I3M induces G2/M phase cell cycle arrest by inhibiting CDK1 and glycogen synthase kinase (GSK)-3 in HBL-100 cells, and induces G2/M phase cell cycle arrest as well as G1 phase cell cycle arrest in MCF-7 cells [Damiens et al., 2001]. In addition, a study demonstrated that I3M inhibited the activation of nuclear factor (NF)-κB through inhibition of inhibitor-κB (Iκ-B)α kinase, Iκ-Bα phosphorylation and degradation, p65 nuclear translocation, DNA binding, and NF-κB-dependent reporter gene expression [Sethi et al., 2006]. More recently, I3M has been found to inhibit autophosphorylation of fibroblast growth factor receptor (FGFR)-1 and activates long-term p38 mitogen-activated protein kinase activity, which stimulates extracellular signal-regulated kinase [Zhen et al., 2007]. Although the antiangiogenic activity of I3M has been demonstrated using transgenic zebrafish with fluorescent blood vessles [Tran et al., 2007], the detail molecular mechanism is still unknown. In this study, we observed that I3M has the capability of inferring angiogenesis in HUVECs, in part through the regulation of VEGFR2 signaling, suggesting that this may be one of the antiangiogenic mechanisms of I3M towards preventing tumor growth and metastasis.

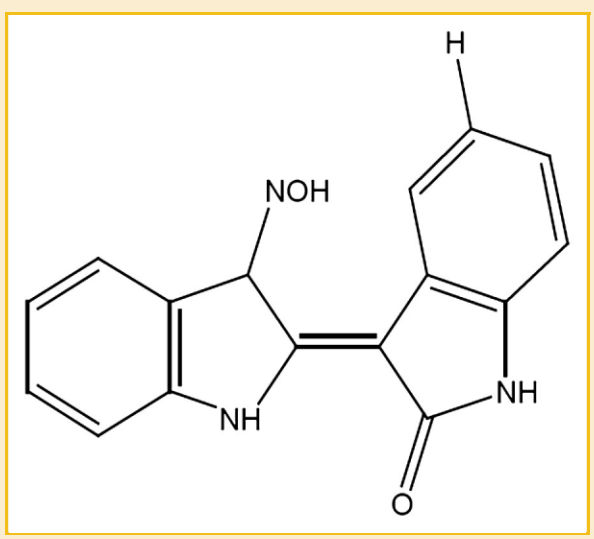

Fig. 1. Structure of I3M.

MATERIALS AND METHODS

CELL LINE, CELL CULTURING, AND REAGENTS

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD) and cultured in EGM (Lonza) at 37°C in an atmosphere with 5% CO_2. I3M was purchased from Sigma–Aldrich (St. Louis, MO). A 40 mM solution of I3M was prepared in dimethyl sulfoxide (Sigma–Aldrich), stored at −20°C, and then diluted as needed with cell culture medium for *in vitro* experiments or with PBS for animal experiments. Recombinant human and mouse VEGF-A was obtained from eBioscience (San Diego, CA) and RayBiotech (Norcross, GA), respectively. Matrigel was from BD Biosciences (San Jose, CA).

PROLIFERATION AND CYTOTOXICITY ASSAY

The effects of I3M on cell proliferation and cytotoxicity were tested using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay and CytoTox 96® Non-Radioactive Cytotoxicity Assay, respectively (Promega, Madison, WI).

MIGRATION ASSAY

HUVECs were allowed to grow into full confluence in 24-well plates precoated with 0.1% gelatin and then incubated with 10 μg/ml mitomycin C (Sigma–Aldrich) at 37°C in a 5% CO_2 atmosphere for 2 h to inactivate HUVECs. Monolayer inactivated HUVECs were wounded by scratching with 0.1 ml pipette tip. Fresh medium containing various concentrations of I3M was added. Images were taken under the AxioImager M1 microscope (Carl Zeiss, Gottingen, Germany) after 8 h incubation at 37°C.

TUBE FORMATION ASSAY

Matrigel was thawed at 4°C overnight, and each well of prechilled 24-well plates was coated with 150 μl Matrigel and incubated at 37°C for 45 min. HUVECs (4×10^4 cells) were added in 1 ml EGM and incubated with the indicated amount of I3M at 37°C in a humidified 5% CO_2 atmosphere. After 16 h incubation, the medium was removed and rhodamine-labeled phalloidin (Thermo Scientific, Rockford, IL) was added to stain F-actin. Images of fluorescently labeled cells were collected with a ThermoScientific Cellomics ArrayScan High Contents Screening Reader (Cellomics, Pittsburgh, PA) and analyzed by an automated algorithm that identified the tubes formed by the association and clustering of the endothelial cells [Basu et al., 2008].

AORTIC RING ASSAY

Aortic ring assay was performed as previously described with some modifications [Di Santo et al., 2009]. Forty-eight-well plates were covered with 100 μl of Matrigel at 4°C and incubated at 37°C, 5% CO_2 for 30 min. Aortas isolated from SD rats (KOATECH, Pyeongtek, Korea) were cleaned of periadventitial fat and connective tissues, and cut into 1- to 1.5-mm-long rings. After being rinsed with PBS, the aortas were placed on the Matrigel-covered wells and covered with another 100 μl of Matrigel. Artery rings were cultured in 1.5 ml of EGM without serum for 24 h, and then the medium was replaced with 1.5 ml of EGM with vehicle or I3M (2.5, 5, 10, and 20 μM). The medium was changed every 2 days

with the exact composition as described above. After 7 days, the microvessel growth was measured by taking photographs with the AxioImager ZI inverted microscope (Carl Zeiss) with a 4× objective lens.

IN VIVO MATRIGEL PLUG ASSAY

All animal studies were approved by the Institutional Animal Care and Use Committee of Hallym University. I3M (0 and 10 μM) in PBS (100 μl) was mixed with Matrigel (400 μl) containing heparin (40 units/ml) and recombinant mouse VEGF-A (100 ng/ml). Prepared Matrigel then was injected subcutaneously into the flanks of 6-week-old C57BL/6 male mice (KOATECH). All treatment groups contained five mice. After 7 days, mice were sacrificed and Matrigel plugs were removed and fixed in 4% paraformaldehyde. To evaluate angiogenesis, plugs were sectioned (6 μm thickness) and stained with hematoxylin, eosin (H&E) and anti-CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to observe blood vessels within Matrigel. Experiments were performed twice with consistent results.

VEGFR-2 INHIBITION ASSAY

A 12.5 μl aliquot of the 4× reaction cocktail containing 100 ng VEGFR-2 [supplied from the HTScan VEGFR-2 kinase assay kit (Cell Signaling Technology, Danvers, MA)] was incubated with 12.5 μl of I3M for 5 min at room temperature. A 25 μl aliquot of 2× ATP/substrate peptide cocktail was then added to the preincubated reaction cocktail/I3M compound. After incubation at room temperature for 30 min, 50 μl of stop buffer (50 mM EDTA, pH 8) were added per tube to stop the reaction. Then, 25 μl of each reaction were transferred into a 96-well streptavidin-coated plate containing 75 μl H_2O/well and incubated at room temperature for 60 min. After washing the wells thrice with 200 μl/well PBS/T (0.05% Tween 20 in 1× PBS), 100 μl of primary antibody [phosphorylated tyrosine monoclonal antibody (pTyr-100), 1:1000 in PBS/T with 1% bovine serum albumin (BSA)] were added per well. After being incubated at room temperature for 60 min, the wells were washed thrice with 200 μl PBS/T. One hundred microliter of diluted HRP-labeled anti-mouse IgG (1:500 in PBS/T with 1% BSA) were added per well. After incubation at room temperature for 30 min, the wells were washed five times with 200 μl of PBS/T per well. Then, 100 μl of TMB substrate were added per well, and the plate was incubated at room temperature for 15 min. The stop solution (100 μl/well) was added and mixed, followed by incubation at room temperature for 15 min. The plate was then read at 405 nm with the SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

WESTERN BLOT ANALYSIS

HUVECs pretreated with 0–20 μM I3M for 60 min were treated with or without human recombinant VEGF-A (10 ng/ml) for 5 min. Ten microgram of total cellular protein from each sample were subjected to Western blotting with anti-VEGFR-2 (Cell Signaling Technology), anti-phospho-VEGFR-2 (Cell Signaling Technology), and anti-β-actin mAb (Sigma-Aldrich). Immunoreactive proteins were detected using a chemiluminescence Western blotting detection system (ECL Plus™ Western Blotting Reagents; Amersham Biosciences, Boston, MA).

TRANSFECTION OF SMALL INTERFERING RNA INTO HUVECS

HUVECs were transfected with indicated concentrations of VEGFR-2 small interfering RNA (siRNA; ON-TARGET plus SMARTpool; Dharmacon, Inc., Chicago, IL) or non-targeted siRNA (ON-TARGET plus Non-targeting Pool siRNA; Dharmacon) using DharmaFECT-4 (Dharmacon) as described by the vendor. Inhibition of VEGFR2 protein expression was verified by Western blot analysis.

STATISTICAL ANALYSIS

The data are depicted as means ± SEM. The values were evaluated by one-way analysis of variance with Bonferroni multiple comparison post-tests using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA). Differences with P values < 0.05 were considered statistically significant.

RESULTS

EFFECT OF I3M ON ENDOTHELIAL CELL PROLIFERATION, MIGRATION, AND TUBE FORMATION

First, we tested whether I3M inhibits the proliferation of HUVECs. Using the MTS assay, we measured HUVECs proliferation after treatment with various concentrations of I3M (0–20 μM). As shown in Figure 2A, I3M-reduced cell proliferation in a dose-dependent manner without cytotoxicity (Fig. 2B) in 24 h culture.

Since migration of endothelial cells is necessary in angiogenesis [Hanahan and Folkman, 1996], we performed wound healing migration assays to determine the effects of I3M on HUVEC migration. As shown in Figure 2C, I3M strongly inhibited the migration of HUVECs in a dose-dependent manner.

When HUVECs are plated on a basement membrane matrix (Matrigel) in short-term culture, they align into networks of tubules (Fig. 2D), a process that is dependent upon proteolytic degradation of the matrix, cell realignment, and apoptosis; however, directed cell migration and proliferation are not involved in this process [Vailhé et al., 2001]. I3M-reduced HUVEC tubule formation in a concentration-dependent manner (Fig. 2D), with a significant reduction observed at 10 and 20 μM.

EFFECT OF I3M ON MICROVESSEL OUTGROWTH FROM RAT AORTINC RING

We next evaluated the antiangiogenic effects of I3M in an ex vivo aorta sprout outgrowth assay. The 1- to 1.5-mm-long aortic rings were placed on Matrigel and covered by another Matrigel layer and EGM with or without I3M. After 7 days of incubation, the numbers of microvessel outgrowths from the aortic rings in the presence or absence of I3M were compared. As shown in Figure 3, the presence of 10 or 20 μM I3M inhibited the microvessel sprouting from rat thoracic aorta, suggesting that I3M inhibited angiogenesis.

EFFECT OF I3M ON ANGIOGENESIS *IN VIVO*

To further verify the inhibitory effect of I3M on angiogenesis, we used the Matrigel plug assay *in vivo*. We subcutaneously injected Matrigel containing recombinant mouse VEGF and heparin with or without I3M (10 μM) into the midventral abdominal region of

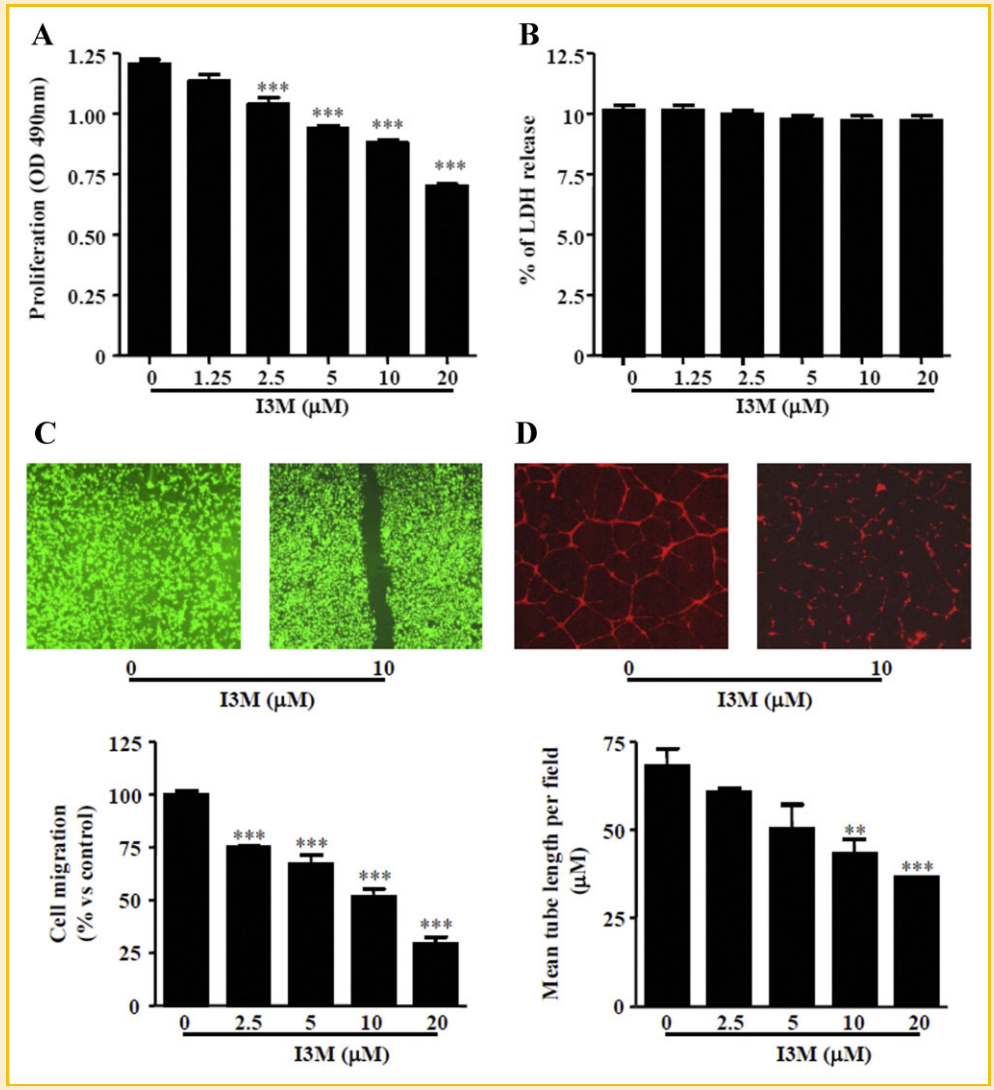

Fig. 2. Inhibition of endothelial cell proliferation, migration, and capillary-like tubule formation by I3M. A: For cell proliferation, HUVECs cells were plated in a 96-well plate and allowed to attach overnight; vehicle or the indicated concentrations of I3M was then added. The proliferation was measured as described in the Materials and Methods section. B: HUVECs were treated the same as in Figure 1A, and LDH release was determined. C: For cell migration, monolayer inactivated HUVECs were wounded by scratching with a 0.1 ml pipette tip, and fresh medium containing vehicle or the indicated concentration of I3M was added. After 24 h, migration of HUVECs was quantified as described in the Materials and Methods section. D: For capillary-like tubule formation, HUVECs (4×10^4/well) were seeded onto Matrigel-coated 24-well plates and incubated with vehicle or the indicated concentration of I3M at 37°C for 16 h. Endothelial tubules were photographed and quantified as described in the Materials and Methods section. The results are reported as mean ± SEM of four independent experiments in triplicate. Statistical significance is based on the difference versus 0 μM I3M-treated cells ($^{**}P < 0.01$, $^{***}P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

C57BL/6 mice. After 7 days, the mice were sacrificed and the Matrigel plugs were removed, sectioned, and stained with H&E. Plugs containing VEGF and heparin were red, indicating that occurrence of angiogenesis (Fig. 4A). In the presence of I3M, plugs were clear and pale yellow in appearance, indicating the absence of angiogenesis (Fig. 4A). H&E staining, as well as CD31 immunostaining of sections, revealed significantly suppressed angiogenesis by I3M treatment (Fig. 4B).

EFFECT OF I3M ON VEGFR-2 PHOSPHORYLATION AND ACTIVITY

Since VEGFR-2 is the primary receptor for VEGF that mediates angiogenic activity, we tested whether I3M interacted with the VEGF/VEGFR-2 signaling pathway. VEGFR-2 was phosphorylated by exogenous VEGF in HUVECs (Fig. 5A), and I3M blocked this phosphorylation. The total steady state levels of VEGFR-2 proteins remained unchanged, indicating that I3M specifically interferes with VEGFR-2 phosphorylation.

To verify the inhibitory effect of I3M on VEGFR-2, we examined the effects of various concentrations of I3M on the specific activation of VEGFR-2 using the HTScan® VEGFR-2 kinase assay kit according to the suggested protocol (Cell Signaling Technology). We found that I3M inhibited VEGFR-2 kinase activity with an IC_{50} of 6.58 μM (Fig. 5B), indicating that I3M is a potent VEGFR-2 inhibitor.

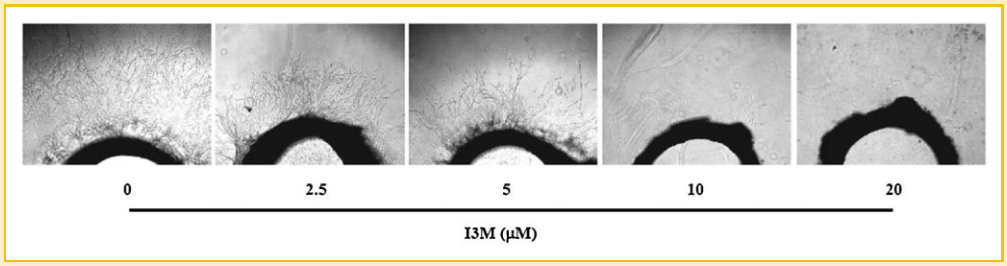

Fig. 3. Effect of I3M on microvessel outgrowth arising from rat aortic ring. Aortic rings isolated from SD rats were embedded in Matrigel in 48-well plates, then fed medium containing various concentrations of I3M for 7 days. Representative photographs of three independent experiments are shown.

VEGFR-2 SIGNALING IS NECESSARY FOR THE INHIBITION OF ANGIOGENESIS BY I3M

To directly assess the functional role of VEGFR-2 in I3M-induced inhibition of angiogenesis, VEGFR-2 expression was inhibited by introducing short-interfering RNA (siRNA) into HUVECs. As a control, a non-targeted siRNA that targets a non-human mRNA sequence was introduced into HUVECs as well. As shown in Figure 6A, almost complete inhibition of VEGFR-2 protein expression was

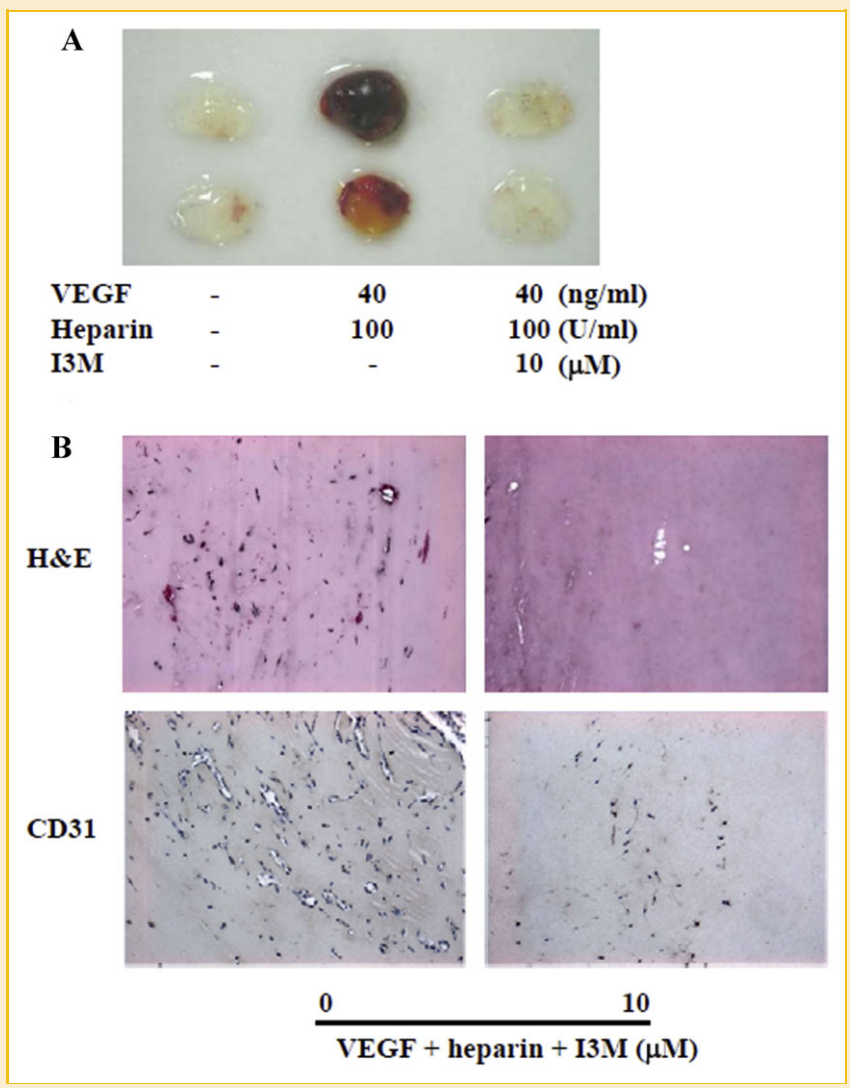

Fig. 4. Effect of I3M on Matrigel plug *in vivo* angiogenesis. Matrigel mixed with VEGF and heparin or I3M was injected into the abdominal region of 5- to 6-week-old C57BL/6 mice (five mice per group). Seven days later, the Matrigel plugs were harvested. A: The macroscopic appearance of Matrigel plugs from indicated groups. B: Matrigel plug cross-sections were stained with H&E and anti-CD31. Original magnification, ×200. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

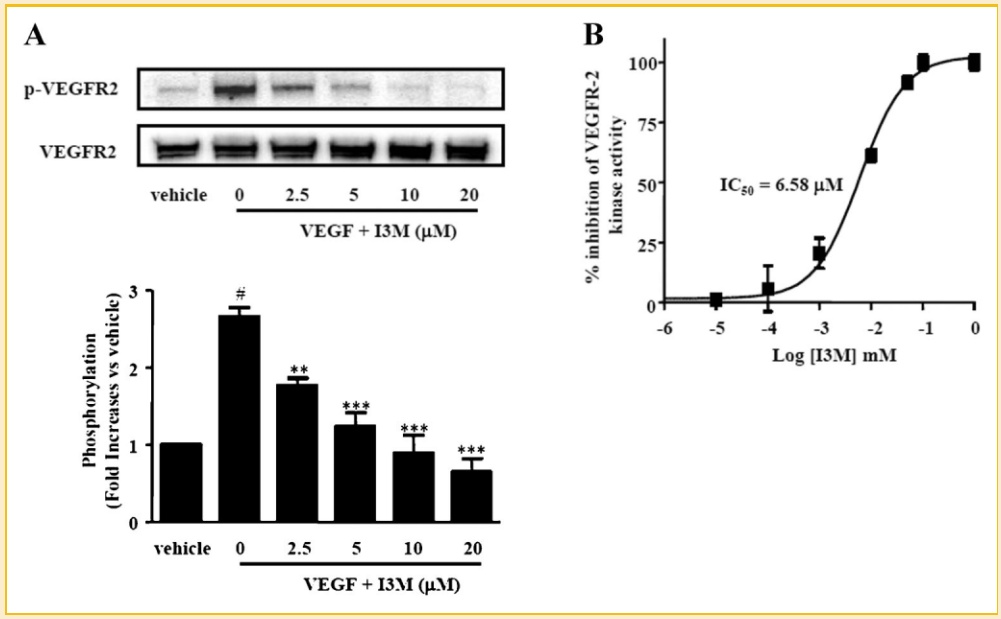

Fig. 5. Inhibition of VEGFR-2 signaling pathway by I3M. A: HUVECs were incubated with VEGF (10 ng/ml) for 5 min with or without pretreatment of the indicated I3M concentration. Total cell lysate was prepared, proteins were separated by SDS–PAGE and Western blot was performed using corresponding antibodies. The band intensities were quantified by densitometry and are represented in the bar graph. The results are reported as mean ± SEM for four independent experiments with identical conditions. Statistical significance is based on the difference versus VEGF-stimulated cells ($^{**}P < 0.01$, $^{***}P < 0.001$). #Significant difference between vehicle and VEGF treatment alone, $P < 0.001$. B: Inhibition of VEGFR-2 activation by I3M in a specific VEGFR-2 inhibition assay. The results are reported as mean ± SEM of triplicate assays.

shown at 48 h after transfection (Fig. 6A). A concentration of 25 nM siRNA was chosen for subsequent experiments because this concentration could significantly inhibit VEGFR-2 protein expression, whereas the non-targeted siRNA had no effect. I3M inhibited endothelial cell migration and tube formation in cells transfected with non-targeted siRNA (Fig. 6B,C). In contrast, I3M treatment did not suppress the migration and tube formation of HUVECs in which VEGFR-2 had been depleted by VEGFR-2 siRNA (Fig. 6B,C). Taken

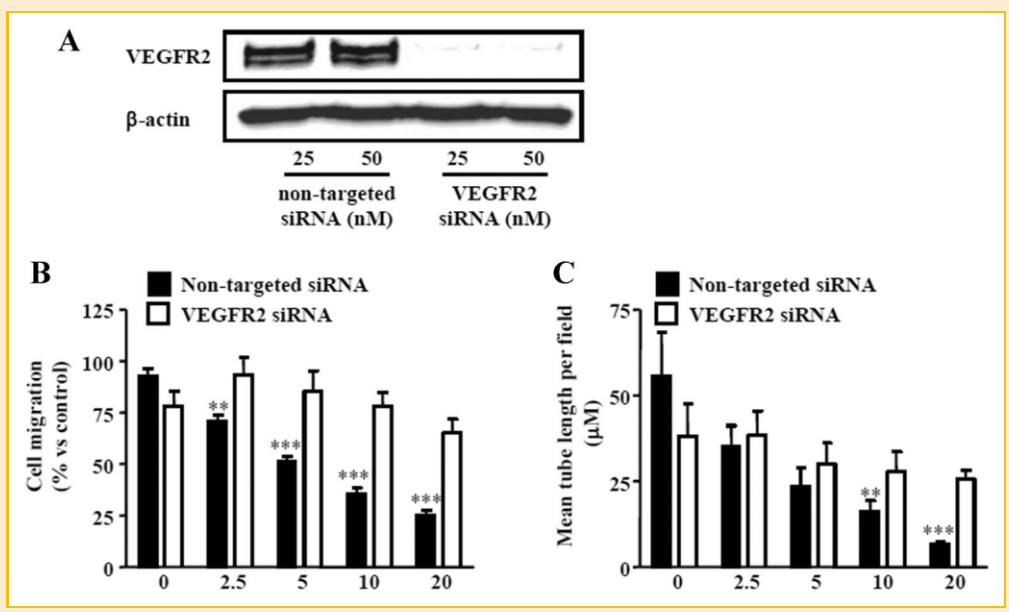

Fig. 6. Requirement of VEGFR-2 for I3M-induced inhibition of angiogenesis. HUVECs were transfected with indicated concentrations of VEGFR-2 siRNA or non-targeted siRNA. A: Inhibition of VEGFR-2 protein expression was verified by Western blot analysis. B: Inhibition of endothelial cell migration was measured as described Figure 2C. C: Inhibition of capillary-like tube formation was measured as described Figure 2D. The results are means ± SEM of three independent experiments in triplicate. Statistical significance is based on the difference versus μM I3M-treated cells ($^{**}P < 0.01$, $^{***}P < 0.001$).

together, these finding indicates that I3M has the capability of inferring angiogenesis in HUVECs, in part through the regulation of VEGFR2 signaling.

DISCUSSION

Indirubin was originally identified as an active compound in the herbal medicine, Danggui Longhui Wan, which has been widely used as a traditional Chinese treatment for chronic myelogenous leukaemia [Xiao et al., 2002]. Indirubin displays marked antitumor properties and relatively low toxicity in animal studies [Hoessel et al., 1999]. I3M is a derivative of the *bis*-indole alkaloid indirubin and is mainly recognized as an inhibitor of CDKs and GSK-3 [Leclerc et al., 2001; Marko et al., 2001; Meijer et al., 2003; Perabo et al., 2006]. Previous studies have demonstrated that I3M is a promising anticancer agent because it is able to inhibit the proliferation and induce the apoptosis of various cancer cells with minimal toxicity to normal cells [Marko et al., 2001; Perabo et al., 2006]. Shi and Shen [2008] demonstrated that I3M induce apoptosis through extrinsic pathway with type II response mediated by the pro-apoptotic Bcl-2 family members on human cancer cell cells, such as cervical cancer HeLa, hepatoma HepG2, and colon cancer HCT116. In addition, it has been shown that I3M induces growth arrest and apoptosis in renal cell cancer cell lines [Perabo et al., 2009]. In addition, an *in vivo* study in a rat model proved its efficacy in arresting tumor growth [Selenica et al., 2007]. Recently, I3M was shown to be a potent angioinhibitory compound [Tran et al., 2007]. However, little is known about the precise mechanism of I3M on angiogenesis.

Recent gains in our knowledge of endothelial cell physiology and tumor angiogenesis are providing the necessary background to develop ever more effective antiangiogenic strategies for cancer therapy. Identification of new pharmacologically active compounds of natural origin and identification of their molecular mechanisms are opening new perspectives in preventive oncology. In this study, we identified I3M as a novel VEGFR-2 inhibitor and comprehensively showed that I3M inhibited angiogenesis *in vitro* and *in vivo*. Our work focuses on the inhibitory effects of I3M on proliferation, migration, and tube formation of HUVECs, fundamental characteristics of endothelial cells in angiogenesis.

Our *in vitro* studies with HUVECs demonstrated that I3M inhibited the proliferation, migration, and capillary-like structure formation (Fig. 2). A similar phenomenon was observed in rat aortic ring assay (Fig. 4), suggesting that I3M has antiangiogenic effects on endothelial cells. The Matrigel plug assay mimics normal, physiological conditions very well for the quantitative assessment of neo-angiogenesis, yet also reflects many of the features of tumor angiogenesis. Angiogenic growth factors are locally released from a growing tumor to stimulate endothelial cell proliferation and migration and extracellular matrix degradation, which is needed to allow invasion and vessel formation. Our study showed that I3M virtually abolished angiogenesis in this assay (Fig. 5). These results strongly suggest that I3M inhibits angiogenesis not only *in vitro* but also *in vivo*.

VEGF is a key mediator of tumor angiogenesis that functions primarily through VEGFR-2. VEGFR-2 is the primary receptor in the VEGF signaling pathway that regulates endothelial cell proliferation, migration, differentiation, tube formation, and angiogenesis [Gerber et al., 1998; Eliceiri et al., 1999; Karkkainen and Petrova, 2000; Ferrara et al., 2003]. To understand the molecular mechanism of the I3M-mediated antiangiogenic effect, we examined whether I3M inhibits the activation of VEGFR-2. As shown in Figure 5A, VEGFR-2 was phosphorylated following addition of exogenous VEGF to HUVECs. Pretreatment of the cells with I3M significantly blocked the VEGF-induced phosphorylation of VEGFR-2 without affecting the overall VEGFR expression levels, suggesting that I3M is an inhibitor of VEGFR-2. The mechanism by which I3M inhibits angiogenesis was first investigated by measuring the VEGFR-2 activation. We found that I3M directly inhibited the kinase activity of purified VEGFR-2, a novel activity of I3M that has not been characterized. As far as we are aware, this is the first study to demonstrate the inhibitory effect of I3M on angiogenesis via inhibition of VEGF/VEGFR-2 signaling.

How I3M inhibits VEGFR-2 kinase activity remains unknown. It has previously been demonstrated that indirubin and its analogues selectively inhibit CDKs by competing with ATP for binding to the catalytic site of the kinase [Hoessel et al., 1999]. Indirubins are also potent ATP competitive inhibitors of GSK-3 [Leclerc et al., 2001]. Based on these previous reports and the results that I3M inhibits the kinase activity of purified VEGFR-2, I3M might be a potent ATP competitive inhibitor of VEGFR-2 kinase.

Since previous results indicated that I3M affect the signal pathways of NF-κB and bFGF which are involved angiogenesis [Gerber et al., 1998; Eliceiri et al., 1999; Karkkainen and Petrova, 2000; Ferrara et al., 2003; Suhardja and Hoffman, 2003], we tested whether I3M involves these signal pathways in HUVECs (Supplementary Fig. 1). I3M impaired the phosphorylation of FGFR-1 but not NF-κB activation (Supplementary Fig. 1). Based on these findings, we consider that I3M could downregulate angiogenesis via the blocking VEGFR-2 and FGFR-1 signal pathways, at least a part.

In summary, our studies show that I3M functions as an inhibitor of the VEGFR-2 signaling pathway, leading to inhibition of angiogenesis. Our data suggest a new mechanism of action for I3M and its potential use as an antiangiogenic and anticancer agent.

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