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An indirubin derivative, E804, exhibits potent angiosuppressive activity

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ABSTRACT

Angiogenesis, the development of neovessels from pre-existing vessels, is obligatory for solid tumors survival, growth, invasion, and metastasis. Many anti-angiogenic agents are small molecules originated from natural sources. Recently, angiosuppressive effects of indirubin and its derivatives, the active components in indigo-producing herbs, have been shown to possess anti-viral and anti-inflammatory potentials. In this study, we identified another indirubin derivative, indirubin-3′-(2,3 dihydroxypropyl)-oximether (E804), could exhibit potent angiosuppressive effects. *In vitro* study showed that E804 could significantly inhibit human umbilical vein endothelial cells proliferation, migration, and tube formation in a concentration-dependent manner (0.4–40 μM); at the concentration of 1 μmol or above, angiosuppressive potency of E804 was found to be more significant than indirubin-3′-oxime. Using *in vivo* Matrigel plug model and directed-*in vivo*-angiogenesis-assay (DIVAA), E804 was shown more effective to attenuate the VEGF/bFGF-induced neovessel formation. The hemoglobin content and the invaded endothelial cells in the implants were also greatly reduced. Results from the aortic ring assay indicated E804 (4 μM) could completely suppress *ex vivo* sprouting of endothelial cells from the rat aorta fragments; with concomitant reduction of gelatinolytic activities of matrix metalloproteinase-2 and -9. E804 also concentration-dependently (0.04–10 μM) inhibited the subintestinal vessels formation in zebrafish embryos. This study provides the first evidence that E804, a novel indirubin derivative, could more effectively inhibit angiogenesis. With the improved anti-angiogenic potency when compared with indirubin-3′oxime, E804 would be a new potential candidate in the treatment of angiogenesis-dependent diseases.

1. Introduction

Angiogenesis is a complex and multistep process that involves orchestrated interactions among angiogenic factors, extracellular matrix (ECM), and endothelial cells. The whole process is under tight regulation by a series of angiogenic stimulators (*e.g.* vascular endothelial growth factor, VEGF) and inhibitors (*e.g.* pigment epithelium-derived factor, PEDF) [1–3]. It is of key importance in a broad array of normal physiologic and pathologic processes. For the former case, it would be rarely found except for embryogenesis, wound healing, and menstruation. In several diseases, such as atherosclerosis, rheumatoid arthritis, diabetic retinopathy, and solid tumors, uncontrolled and excessive angiogenesis has been proven as part of the pathogenesis [4–6]. Therefore, anti-angiogenic strategy, proposed by Folkman in early 1970s, might constitute a new therapeutic approach for the treatment of such "angiogenic diseases". In fact, angiogenic therapy is nowadays considered as a promising approach for treatment of various cancers. In the period of 2004–2006, a variety of anti-angiogenic drugs such as Macugen (pegaptanib), Lucentis (ranibizumab) and Avastin (bevacizumab) have been approved by FDA for clinical applications [7–9].

Indirubin, a natural purple pigment, occurs as 3,2′-bisindole, has been shown to be the active compound of a traditional Chinese medicine, Danggui Longhui Wan, which comprises 11 plant ingredients, and was reported to be active for the treatment of chronic myelocytic leukemia [10]. It is also one of the major components in a series of indigo-producing Chinese medicinal herbs including *Isatis indigotica* (Brassicaceae), *Strobilanthes cusia* (Acanthaceae) and *Polygonum tinctorium* (Polygonaceae) that are commonly used for treating respiratory viral infection and other inflammatory-based diseases [11]. While parent indirubin molecule is derived from the non-enzymatic and spontaneous dimerization of colorless precursors, isatin and indoxyl, in the indigo-producing plants, a series of novel derivatives including

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5-methyl-indirubin (E224), 5-sulfonate-indirubin (E226), indirubin-3′-oxime (E231) (Fig. 1A), indirubin-5-sulfonic acid dimethylamide (E233), indirubin-3′-(2,3 dihydroxypropyl)-oximether (E804) (Fig. 1B), 6-bromo-indirubin-3′-oxime (6BIO), 1-methyl-6-bromo-indirubin-3′-oxime (Me6BIO), and 7-bromo-indirubin-3′-oxime (7BIO) have been synthesized by various molecular substitution of the parental indirubin backbone with improved solubility, selectivity and bioavailability [12–15]. Among these indigoids, indirubin-3′-oxime is the most widely studied indirubin derivatives. It has been shown to inhibit cyclin-dependent kinases (CDKs), glycogen synthase kinase (GSK)-3 and activate aryl hydrocarbon receptor (AhR) that finally result in growth inhibition in various human cancer cells, such as MCF-7, HBL-100 breast cancer cells, HT-29-18-C1 colon adenocarcinoma, haematopoietic cell lines Jurkat, and A498, CAKI-1, CAKI-2 renal cancer cells [15–19]. It could also suppress autophosphorylation of fibroblast growth factor receptor (FGFR)-1 but stimulate extracellular signal-regulated kinase (ERK1/2) activity through p38 mitogen-activated protein kinase [20]. On the other hand, E804, the focus of this study, was shown to inhibit signal transducer and activator of transcription (STAT) 3 in MDA-MB-468 and -435 human breast cancer cells and DU145 prostate cancer cells through direct inhibition of Src kinase activity and tyrosyl phosphorylation [13]. Besides, it could also reduce the growth of human HCT-116 colon cancer cells, MCF-7 breast cancer cells and LXFL529L lung carcinoma cells through CDK/cyclin inhibition [12]. Recently, both indirubin and indirubin-3′-oxime have been shown to exert suppression on endothelial cells proliferation, migration and tube formation [21,22]. Moreover, studies showed that indirubin's effect was due to blocking VEGFR2-mediated JAK/STAT3 signaling in endothelial cells, while indirubin-3′-oxime was suggested to act through inhibiting CDKs, AKT, p38, ERK1/2 activities [23]. For the *in vivo* studies, indirubin has been shown to inhibit intersegmental vessel formation in zebrafish embryos and neovascularization in chick chorioallantoic membrane (CAM) assay and mouse corneal model; and indirubin-3′-oxime was shown to inhibit the angiogenesis in aortic rings and Matrigel plugs, respectively [23,24].

Nowadays, many researches have been focused towards discovering anti-tumor/anti-angiogenic compounds from natural sources, or structurally modified form of their parent phytochemicals. This study provides the first evidence that E804, another novel indirubin derivative, is able to inhibit angiogenesis *in vitro* and *in vivo*. We showed that it could suppress endothelial cells proliferation, migration, tube formation; and attenuate *in vivo* neovascularization in Matrigel plug and directed-*in vivo*-angiogenesis-assay (DIVAA) assays, and subintestinal vessel formation in zebrafish embryos in a concentration-dependent manner. Moreover, this study emphasized the improved potency of E804 on angiosuppression compared with indirubin-3′-oxime. With this advancement, E804 would be a new potential candidate in the treatment of angiogenesis-dependent diseases.

2. Materials and methods

2.1. Materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA). Fetal bovine serum (FBS), trypsin-EDTA, phosphate buffered saline (PBS), penicillin–streptomycin (PS), and serum-free endothelial growth medium were obtained by Invitrogen (Carlsbad, CA, USA). Dimethyl sulphoxide (DMSO), M199 medium and heparin were obtained from Sigma (Saint Louis, MO, USA). Endothelial cell growth supplement (ECGS) was purchased from Upstate (Waltham, MA, USA). Growth factor reduced (GFR)-Matrigel was supplied by BD Bioscience (Palo Alto, CA, USA). Basic fibroblast growth factor and vascular endothelial growth factor were obtained from Promega (Madison, WI, USA) and R&D system (Minneapolis, MN, USA), respectively. Other chemicals not specified were obtained from USB (Cleeveland, OH, USA).

2.2. Chemicals

Indirubin-3′-oxime was purchased from Sigma and E804 was provided by Merck KGaA (Darmstadt, Germany). All chemicals are HPLC purified with purities of $\geq$98% and $\geq$95% for indirubin-3′-oxime and E804, respectively. Stock solutions of indirubin-3′-oxime and E804 (50 mM) were prepared in cell culture grade DMSO. Aliquoted drug were stored in −20 °C and used in dark condition.

2.3. Cell culture

HUVECs were cultured in M199 medium supplemented with ECGS (20 μg/ml), 20% heat-inactivated FBS, heparin (90 μg/ml) and 1% PS on 0.1% gelatin-coated culture flasks and maintained at 37 °C in a humidified 5% CO_2 incubator. All experiments were conducted with HUVECs from passage 2 to 8.

2.4. Maintenance of zebrafish

Wild-type strain zebrafish were obtained from local supplier, they were bred and maintained as described [25]. Briefly, as described in the zebrafish handbook, fishes were maintained on a 14 h:10 h light/dark cycle at 28 °C. The fish were fed twice daily with tropical fish food in the morning and afternoon.

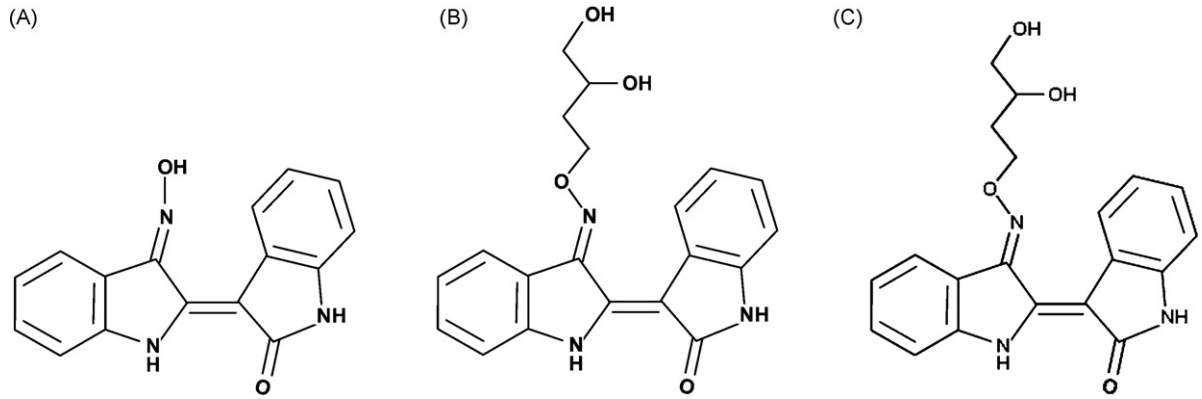

Fig. 1. Chemical structures of (a) indirubin-3′-oxime (E231) and (b) indirubin-3′-(2,3 dihydroxypropyl)-oximether (E804).

2.5. Collection of zebrafish embryos

Embryos were generated by natural pair-wise mating when fishes were matured (6-month-old or above). The breeding tank was placed into the fish tank just before switching on the light in the morning (10:00 a.m.). After 2 h, the breeding tank was removed from the fish tank and the embryos were collected and transferred to Petri dishes containing fresh water. Embryos were maintained in embryo water at 28 °C for about 22 h (~24 h post fertilization (pf)). Healthy, limpid and regular embryos were selected, dechorionated and transferred to 24-well plate (8 embryos per well).

2.6. In vitro proliferation assay

The cell viability was measured as described previously [26]. Briefly, HUVECs were seeded at a density of 1×10^4 cells/well into 96-well plate and incubated in growth medium supplemented with 20% FBS and ECGS (20 μg/ml) and allowed to attach for 24 h. Then cells were incubated with various concentrations of indirubin-3′-oxime and E804 (0.05–90 μM) for 48 h. Cell viability was determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The culture medium was then removed and formazan product was solubilized by DMSO; absorbances at wavelength 540 nm and 690 nm (reference) were measured by the microplate reader (Infinite F200, Tecan, Switzerland). Each sample was tested in quadruplicate and the experiment was repeated in triplicate.

2.7. In vitro cell migration assay

Cell motility ability was determined using cell migration assay as described previously [27]. Briefly, HUVECs (3×10^4 cells/well) were seeded onto gelatin (0.1%) precoated 96-well plates and incubated for 24 h. An artificial wound was created by mechanical scratching of the cell monolayer using home-made mechanical wounder at time zero ($T = 0$ h). Then the image of denuded area (wound) in each well was captured using Motic AE31 inverted microscope with attached digital camera, Moticam 2300 (Motic Instruments Inc., Richmond, Canada). Then, cell culture medium was replaced with fresh serum-free medium containing various concentrations of indirubin-3′-oxime and E804 (0.5–50 μM) at 37 °C. Besides, medium and solvent controls were set in parallel by using fresh serum-free medium and medium containing corresponding percentage of DMSO. After incubation for 16 h ($T = 16$ h), denuded area in each well was captured again, images at 0 and 16 h were analyzed using Java's *Image J* software (http://rsb.onfo.nih.gov). The migration of cells towards the wounds was expressed as percentage of recovery.

$$\%\ \text{of recovery} = \left[\frac{(A_{t=0} - A_{t=16})}{A_{t=0}}\right] \times 100\%$$

where, $A_{t=0}$ is the area of wound measured immediately after scratching, $A_{t=16}$ is the area of wound measured 16 h after scratching. Each sample was tested in quadruplicate and the experiment was repeated in triplicate.

2.8. In vitro tube formation assay

Tube formation assay was performed as described previously [28]. Briefly, HUVECs (2×10^4 cells/well) were seeded onto 96-well plate coated with GFR-Matrigel and incubated with various concentrations of indirubin-3′-oxime and E804 (0.5–50 μM) at 37 °C for 16 h. Medium and solvent control were performed in parallel. Tubular network was fixed by methanol and images of tubular network formed in each well were captured using stereomicroscope (Olympus SZX16) with attached digital camera (Olympus DP71) (Olymus America Inc). The angiosuppresive capabilities of the testing drugs were determined by counting the number of branch points of the tubular network formed in the well. Three independent experiments were performed and each experiment was run in triplicate.

2.9. Ex vivo aortic ring sprouting assay

Rat aortic rings were prepared as described previously [29]. Briefly, the aortic fragments were collected from Sprague-Darley (SD) rat (6 weeks old) and embedded into GFR-Matrigel precoated 96-well plate. Additional GFR-Matrigel (40 μl) was added to overlay the aortic fragments and the gel was allowed to solidify in the 37 °C incubator. The cultures were incubated in serum-free endothelial growth medium containing ECGS (200 μg/ml) with or without indirubin-3′-oxime or E804 (0.4, 4 and 40 μM) at 37 °C. Microvascular outgrowths were distinguished from the fibroblasts based on their unique morphology. Culture medium was replaced with fresh medium on day 4. The aortic fragments were visualized on day 8 under inverted microscope and microvascular sprouting was captured using Moticam 2300 digital camera (Motic Instruments Inc., Richmond, Canada). The microvascular sprouting area was measured and calculated by Java's *Image J* software. The experiment was repeated three times on six individual rats and each culture was performed in triplicates.

2.10. Gelatin zymography

Culture supernatants from different treatment group of rat aortic ring culture were collected on day 8. Gelatinase activity in the culture supernatant was measured by in-gel gelatin zymography. Culture supernatants were mixed with 4× zymograph sample buffer (10% SDS, 40% glycerol, 0.25 M Tris–HCl. pH 6–8, 0.02% bromophenol blue) without heat denaturation. Electrophoresis was performed on 8% (for MMP-9) and 10% (for MMP-2) polyacrylamide gels containing gelatin (1 mg/ml) at 4 °C for 2 h. After electrophoresis, the gels were washed in renaturing solution (50 mM Tris–HCl, pH 7.4, 2% Triton X-100) and incubated in substrate buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X100, 5 mM $CaCl_2$, 0.02% Tween 20) at 37 °C overnight. Gels were stained with 0.5% Coomassie Blue R-350 followed by destaining with 10% acetic acid in 40% methanol. Gelatinolytic activity was visualized by negative staining. Pre-stained SDS-PAGE protein standards were used for estimation of apparent molecular weights of the protein samples. The intensity of the bands on zymogram was quantified by Kodak 1D software.

2.11. In vivo Matrigel plug assay

Matrigel plug assay was performed as described previously [29,30]. Briefly, a total of 500 μl injection mixture containing 400 μl liquefied GFR-Matrigel, angiogenic supplements including bFGF (125 ng/ml) and heparin (32 U); and testing drugs – indirubin-3′-oxime and E804 (0.4, 4, 40 μM) were injected subcutaneously into the left and right abdomen of Balb/c mice (6 weeks old). After injection, the Matrigel rapidly formed a single, solid gel plug. Mice were sacrificed 5 days after injection. Plugs were then removed and the extent of neovascularization was assessed by measuring the hemoglobin content using the hemoglobin quantitation kit (BioSystems S.A., Barcelons, Spain) according to the manufacturer's instructions. A control was performed in parallel with another set of mice by injecting GFR-Matrigel alone. At least two mice were used for each treatment group and the experiment was done at least twice.

2.12. Directed in vivo angiogenic assay (DIVAA)

In vivo angiogenesis was further studied using DIVAA kit from Trevigen (Gaithersburg, MD, USA) as previously described [31]. Briefly, surgical-grade silicone tubes (angioreactors ~10 mm long, with only one end open) were filled with a total of 20 μl of Matrigel containing VEGF (62.5 ng/ml), FGF (187.5 ng/ml) and heparin (100 ng/ml) (as positive control), or mixed VEGF/FGF/heparin with indirubin-3′-oxime or E804 (0.4 and 40 μM). After the Matrigel was solidified at 37 °C, the angioreactors were implanted subcutaneously into the dorsal flanks of anesthetized Balb/c mice (6 weeks old). After 9 days, the mice were sacrificed and the tubes were removed from the skin pockets. Matrigel was removed from angioreactors and digested in 300 μl CellSperse™ solution at 37 °C for 1 h. Then, endothelial cells were collected by centrifugation at 250 × *g* for 10 min; subsequently, cells were incubated in 10% FBS supplemented DMEM at 37 °C for 1 h and followed by fluorescent labeling with DIVAA™ FITC-Lectin. Quantitation of angiogenesis in the angioreactors was determined by measuring the fluorescence emitted from the FITC-Lectin labeled endothelial cells. The amount of fluorescence was measured at wavelength 485 nm (excitation) and 510 nm (emission) by the microplate reader (Infinite F200, Tecan, Switzerland). At least two mice were used for each treatment group and the experiment was done in triplicates.

2.13. Zebrafish angiogenesis assay

Healthy, limpid and regular embryos were sorted for viability. Selected embryos were dechorionated and transferred to 24-well plate (8 embryos per well). Embryos were then treated with various concentrations of indirubin-3′-oxime and E804 (0.04, 0.4, 4, 10 μM) at 28 °C for 48 h (~72 h pf). Embryos nourished in milli-Q water or DMSO served as medium and vehicle control, respectively. After 48 h of incubation, embryos were visually inspected for viability and gross morphological defects.

2.14. Endogenous alkaline phosphatase–based vascular staining

On day 3 of development (3 dpf), fish embryos were fixed in 10% formalin at 4 °C for 20 h. The endogenous melanin in the pigment cells on fish embryo body was first removed by incubating in 1% KOH and 3% H_2O_2 for 20 min, followed by two rounds of PBST (0.1% Tween 20) rinsing. Then fish embryos were immersed in acetone for 30 min at −20 °C followed by washing twice for 5 min each in PBST. For staining, fish embryos were equilibrated with NTMT buffer (100 mM Tris–HCl, pH 9.7, 100 mM NaCl, 50 mM $MgCl_2$ and 0.1% Tween 20) three times for 15 min at room temperature. After that, all the blood vessels were stained with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (AMRESCO) at room temperature under dark condition. Staining reaction was stopped by adding PBST; fish embryos were fixed again in 10% formalin and followed by three rounds of PBST rinsing. Finally, stained fish embryos were examined under stereomicroscope (Olympus SZX16) with digital camera (Olympus DP71) (Olymus America Inc). The experiment was repeated five times with indicated numbers of embryos per treatment group.

2.15. Data and statistics analysis

Data are presented as mean ± standard deviation (S.D.) of control. Statistical comparisons between groups were performed using the Student's *t*-test.

3. Results

3.1. Inhibition of HUVECs proliferation

First, we determined the effect of indirubin-3′-oxime and E804 on HUVECs proliferation by MTT assay. As shown in Fig. 2, both indirubin analogs (0.1–90 μM) suppressed HUVECs proliferation

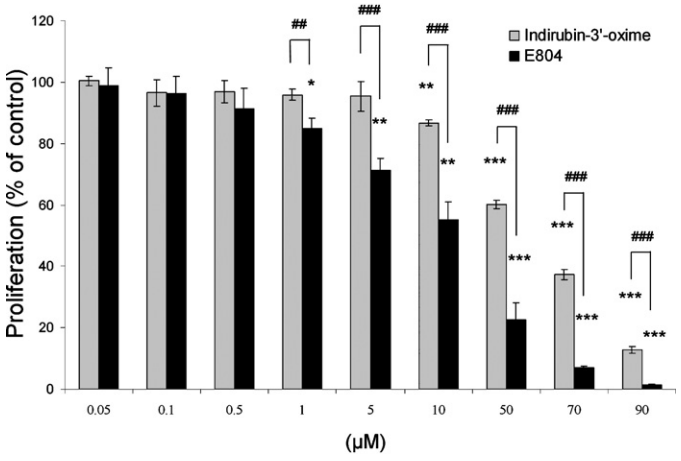

Fig. 2. Inhibition of HUVECs proliferation by indirubin-3′-oxime and E804. Cells were incubated with various concentrations of indirubin-3′-oxime and E804 (0.05–90 μM) for 48 h and cell proliferation was determined by the MTT assay. The proliferation inhibition was presented as the percentage of medium control. The data represented mean ± S.D. from triplicate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with medium alone; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared with indirubin-3′-oxime.

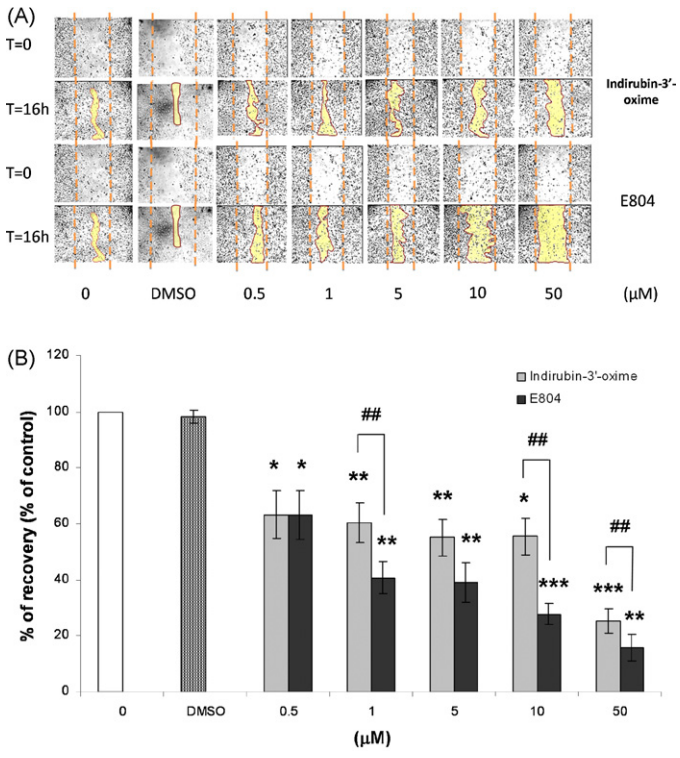

Fig. 3. Inhibition of HUVECs migration by indirubin-3′-oxime and E804. At time zero ($T = 0$ h), an artificial wound was created by mechanical scratching, and captured. Cells were incubated with indirubin-3′-oxime (A, upper panel, first row) and E804 (A, lower panel, first row) (0.5–50 μM) for 16 h. After treatment ($T = 16$ h) (A, upper and lower panel, second row), cells were captured again and denuded area was measured. (B) The cell motility in each group was calculated from triplicate experiments and presented as the percentages of recovery. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with medium alone; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ compared with indirubin-3′-oxime.

in a concentration-dependent manner. The 50% and 80% growth inhibition were achieved at the concentrations of about 60 and 85 μM for the indirubin-3′-oxime, and 15 and 53 μM for the E804, respectively. Under high concentration treatment (90 μM), E804 could inhibit almost 99% cell proliferation ($p < 0.001$). When comparing the proliferation inhibition between the two indirubin analogs throughout the testing concentrations, analog E804 was found to be more potent than indirubin-3′-oxime.

3.2. Inhibition of in vitro HUVECs migration

To test the influence of indirubin-3′-oxime and E804 on cell motility, two dimensional cell migration/wounding assay was employed. As shown in Fig. 3A, 100% confluent monolayer of HUVECs was denuded using the home-made mechanical scratch wounder ($T = 0$ h). A consistent "wound" was created in each well and the images were captured. Upon treatment with indirubin-3′-oxime and E804 (0.5–50 μM) for 16 h ($T = 16$ h), HUVECs migration ability was found to be reduced in a concentration-dependent manner. When comparing the percentage of recovery area in each trial between the two analogs, anti-migration ability of E804 on HUVECs was found to be more effective than indirubin-3′-oxime (Fig. 3B). For indirubin-3′-oxime, except for the 50 μM treatment group, all the tested concentrations (0.5, 1, 5, 10 μM) could only result in about 55–63% wound recovery; meanwhile, in E804 group, except the 0.5 μM treatment group, all the tested concentrations were able to significantly reduce cell migration and resulted in the wound recovery in less than 50%.

3.3. Inhibition of in vitro tubular formation of HUVECs

Using the Matrigel-based tube formation assay, the effect of indirubin-3′-oxime and E804 on the organization of endothelial cells into tubular structures was studied. When HUVECs were seeded onto the Matrigel substratum, they migrated, aligned, and subsequently formed the tubular structures (Fig. 4A, left). Upon drug treatment (0.5–50 μM), both analogs induced a concentration-dependent inhibition on tube formation (Fig. 4A, right), with E804 exhibiting a stronger inhibitory effect than indirubin-3′-oxime. As shown in Fig. 4B, the activity differences between the two analogs in the tested ranges were about 6.7–37% ($p < 0.05$, $p < 0.001$); especially at the concentration range of 10–50 μM, E804 completely abolished the tubular formation, and the cells remained interspersed throughout the gel; while indirubin-3′-oxime could only achieve 64.4% and 79.8%, respectively.

3.4. Inhibition of ex vivo microvascular sprouting

The angiosuppressive effect of indirubin-3′-oxime and E804 were also compared in the *ex vivo* aortic ring sprouting assay. Rat aortic explants were embedded in GFR-Matrigel and cultured with various concentrations of indirubin-3′-oxime and E804 (0.4, 4, 40 μM). After incubation for 8 days, extensive microvascular sprouting was developed from the explants in the presence of ECGS (200 μg/ml) (Fig. 5A, left). In the presence of indirubin-3′-oxime, microvascular sprouting was significantly inhibited in a concentration-dependent manner ($p < 0.01$ and $p < 0.001$) (Fig. 5A (right, top panel) and B). However, the inhibition in E804 was even more dramatic; E804 at the concentration of 0.4 μM could largely reduce 85.3% of the microvascular sprouting ($p < 0.001$), while at higher concentrations (4 and 40 μM), E804 could completely stop the *ex vivo* outgrowth.

3.5. Inhibition of MMP-2 and MMP-9 gelatinolytic activities

To determine the effect of indirubin-3′-oxime and E804 on the production of proteinases by endothelial cells, culture supernatants collected from aortic explants culture were subjected to

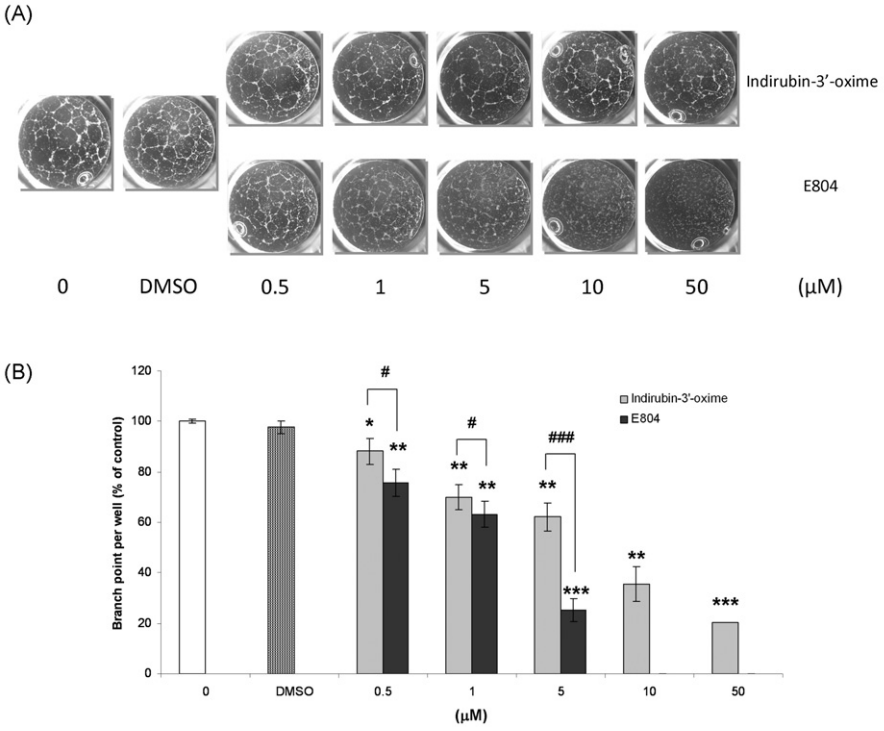

Fig. 4. Inhibition of HUVECs tube formation by indirubin-3′-oxime and E804. HUVECs were plated onto GRF-Matrigel precoated plate and incubated with indirubin-3′-oxime and E804 (0.5–50 μM) for 16 h. (A) Photomicrographs depict the alignment of HUVECs in the presence of indirubin-3′-oxime (A, upper panel) and E804 (A, lower panel). (B) Numbers of branch point in each well were counted for each treatment. The data represented mean ± S.D. from triplicate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with medium alone; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ compared with indirubin-3′-oxime.

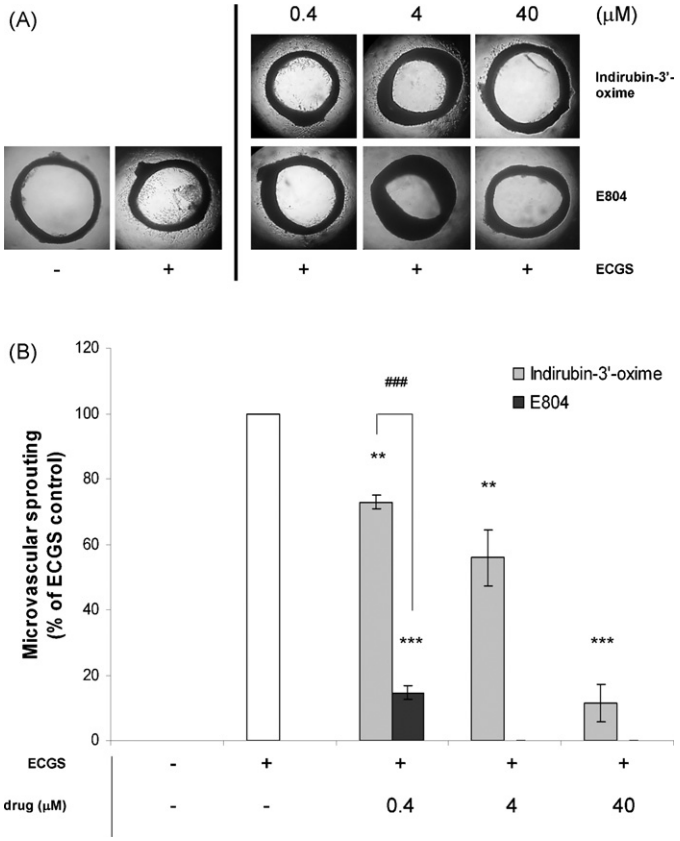

Fig. 5. Inhibition of *ex vivo* endothelial sprouting by indirubin-3′-oxime and E804. (A) Rat aortic rings were embedded in GFR-Matrigel and cultured in serum-free medium supplemented with or without ECGS (200 μg/ml) or various concentrations of indirubin-3′-oxime or E804 (0.4–40 μM) for 8 days. Photomicrographs depict microvascular sprouting in defined conditions. (B) The area of microvascular sprouting was measured and analyzed. The data represented mean ± S.D. from triplicate experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ compared with ECGS-control; $^{\#\#\#}p < 0.001$ compared with indirubin-3′-oxime.

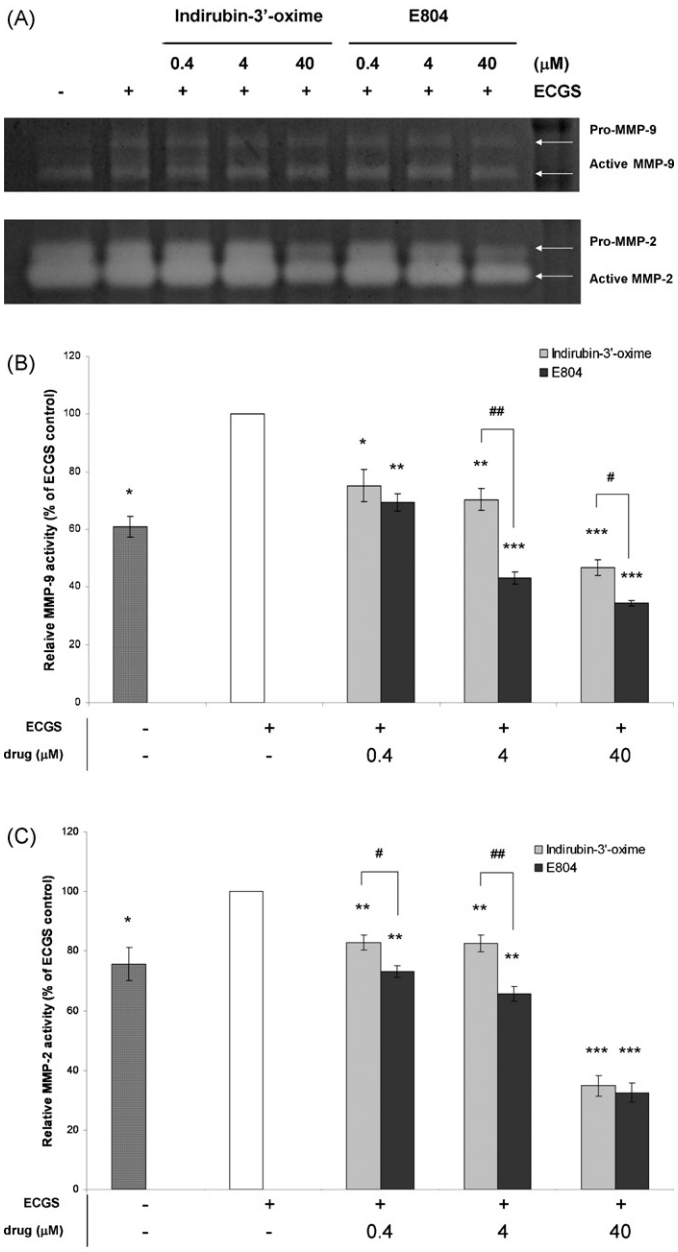

Fig. 6. Inhibition of MMP-2 and MMP-9 activities by indirubin-3′-oxime and E804. Conditioned medium from rat aortic ring *ex vivo* culturing were collected on day 8 and MMP activities were analyzed by gelatin zymography. (A) Gelatinolytic activities of MMP-2 (lower panel) and 9 (upper panel) were indicated by clear bands in the zymograms. The relative percentage of (B) MMP-9 (active-form) and (C) MMP-2 (active form) activities were determined, respectively. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with ECGS-control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ compared with indirubin-3′-oxime.

gelatin zymography, in which the presence of MMPs was detected by the gelatin-containing gel. For MMP-2 and MMP-9 analysis, 8% and 10% polyacrylamide gels were used, respectively. The clear bands at 86 and 66 kDa bands were assigned to the active forms MMP-9 and MMP-2, respectively (Fig. 6A). As shown in Fig. 6B and C, both indirubin derivatives could reduce the MMPs activities in a concentration-dependent manner ($p < 0.05$, $p < 0.01$, $p < 0.001$). Similarly, E804 were found to be more potent than indirubin-3′-oxime.

3.6. Inhibition of in vivo neovascularization in mice model

The angiosuppressive effect of indirubin-3′-oxime and E804 was also tested in the Matrigel plug assay and DIVAA test. For the former assay, indirubin-3′-oxime or E804 were mixed with Matrigel and endothelial growth supplements (bFGF and heparin) and the mixture was injected s.c. into mice abdomen. As shown in Fig. 7A (left panel), neovascularization in the Matrigel plug was developed inside the mice upon induction of angiogenic factors 5 days after incubation; and the plug was reddish in color. In the presence of indirubin derivatives, the Matrigel plugs were light yellow (0.4 μM) or clear (4 and 40 μM) in appearance indicating the reduction of neovessel formation. The morphological judgment was validated by measuring the hemoglobin content in each plug. As shown in Fig. 7B, indirubin-3′-oxime and E804 could reduce the hemoglobin content dramatically; the neovascularization was almost completely attenuated ($p < 0.001$). However, no difference was observed between these two indirubin derivatives. To quantitatively measure the invaded endothelial cells in the *in vivo* implants during neovascularization, DIVAA test was employed. During the neovessels formation, endothelial cells under the induction of angiogenic factors (FGF/VEGF/heparin) invaded into the angioreactors containing Matrigel. Same as the Matrigel plug assay, after incubation, extensive neovessels formation was observed in the angioreactors containing angiogenic factors; the gel inside were found to be reddish in color (containing blood) (Fig. 7C). On the other hand, implants containing either indirubin-3′-oxime or E804 appeared in white or pale yellow color, which

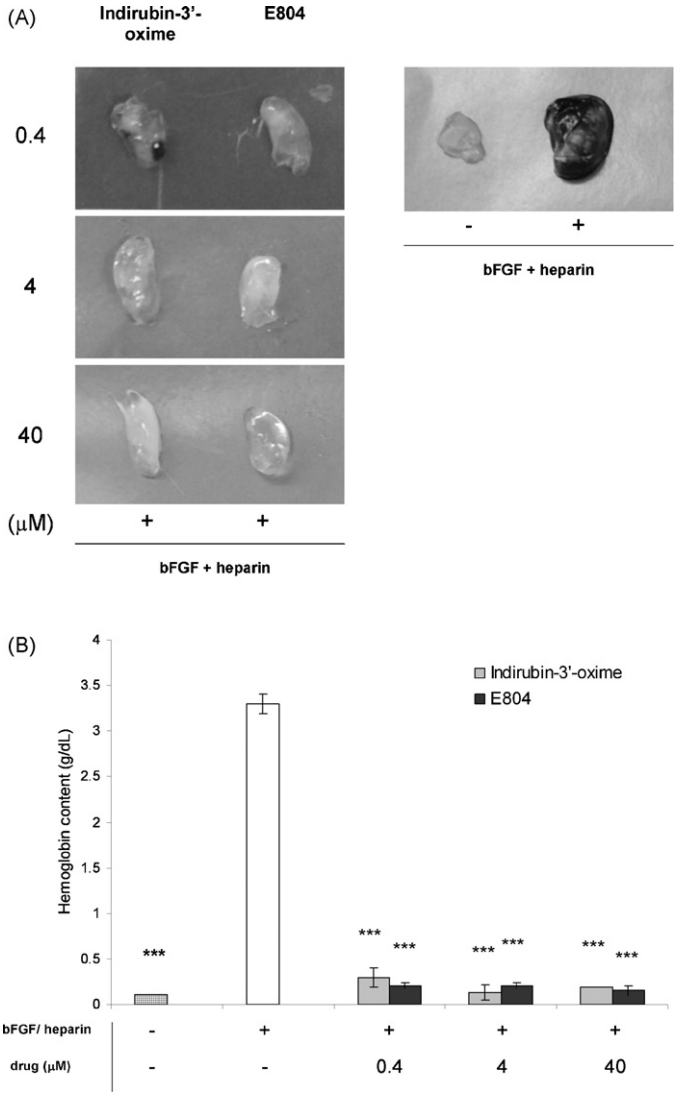

Fig. 7. Inhibition on bFGF or VEGF/FGF-induced *in vivo* angiogenesis by indirubin-3′-oxime and E804. For the Matrigel plug assay (A and B), GFR-Matrigel containing drugs and supplements were injected into the ventral abdominal of Balb/c mice. After 5 days, mice were sacrificed and the plugs were removed. (A) Pictomicrographs show gross morphologies of vehicle control (Matrigel alone plug; right column), positive control (bFGF and heparin; right column), indirubin-3′-oxime and E804 (0.4, 4 and 40 μM plus bFGF and heparin; left column). (B) The extent of angiogenesis was quantitated by measuring the hemoglobin contents in the Matrigel plug. At least two mice were used for each group and the experiments were repeated at least twice. $^{***}p < 0.001$ compared with positive control group. For the DIVAA test (C and D), angioreactor containing Matrigel constituted with angiogenic factors or mixed with indirubin-3′-oxime or E804 (0.4 and 40 μM) were implanted into the dorsal flanks of Balb/c mice. After 9 days, mice were sacrificed and the tubes were removed. (C) Pictomicrographs show gross morphologies of angioreactors of Matrigel alone, VEGF/FGF/heparin, VEGF/FGF/heparin + indirubin (0.4 and 40 μM) and VEGF/FGF/heparin + E804 (0.4 and 40 μM). (D) The extent of angiogenesis was determined by measuring the FITC-labeled invaded endothelial cells. Two mice were used for each group and the experiments were repeated at least twice. $^{***}p < 0.001$ compared with VEGF/FGF/heparin group; $^{\#}p < 0.05$ compared with indirubin-3′-oxime.

indicating that neovessels formation was inhibited significantly. As shown in Fig. 7D, invaded endothelial cells in each angioreactors were determined, both indirubin-3′-oxime and E804 (0.4 and 40 μM) could significantly suppress *in vivo* angiogenesis ($p < 0.001$). Furthermore, the data indicated that E804 at 40 μM could completely abolish FGF/VEGF/heparin-induced neovessel formation.

3.7. Inhibition of in vivo neovascularization in zebrafish model

As zebrafish is a powerful *in vivo* model for angiogenic study, the formation of subintestinal vessel (SIV) basket was assessed. The embryos were collected and cultured at 28.5 °C in embryo medium. After 24 h, the embryos were dechorionated and incubated with various concentrations of indirubin-3′-oxime and E804 (0.04, 0.4, 4, 10 μM) for 48 h (Fig. 8). Embryos incubated in egg water and egg water containing DMSO acted as medium control and solvent control, respectively. Data showed that E804 could significantly inhibit the size of SIV basket and the neovessel formation. The appearance of the neovessels was less dense and with spikes projected from the SIV basket. At moderate concentration (4 and 10 μM), the SIV basket was almost diminished.

4. Discussion

Indirubin, a naturally occurring purple pigment, and its derivatives collectively refer to as indigoids, form a class of chemicals with cultural, historical, and pharmaceutical importance. Previously, indirubin was first recognized by its anti-inflammatory effects on the treatment of viral infection such as influenza and mumps [11]. Apart from that, indirubin and its derivatives have been shown to possess marked anti-tumor effects in various models [13]. Among these indigoids, indirubin-3′-oxime, is the most widely studied and has been demonstrated as a promising anti-cancer agent. Besides, both indirubin-3′-oxime (E231) and indirubin-3′-(2,3 dihydroxypropyl)-oximether (E804) were shown to inhibit the growth of different human cancer cells through cell cycle arrest and apoptosis induction [12,15–19].

Recently, various indigoids have been shown to possess anti-angiogenic ability [21,22,24,32]. Alex et al. showed that indirubin concentration-dependently inhibited HUVECs proliferation and intersegmental vessel formation in zebrafish embryos; while Kim et al. demonstrated that indirubin-3′-oxime not only inhibit *in vitro* proliferation and tubular formation of HUVECs, but also suppress neovascularization in mice Matrigel plug model and endothelial sprouting in *ex vivo* organ culturing of rat aortic fragments. Moreover, Xiao et al. showed that another indigoid, meisoindigo, could attenuate angiogenesis *in vitro* and VEGF secretion. These findings proposed the potential of indigoids to act as anti-angiogenic agents. In fact, emerging evidence suggested that aberrant angiogenesis plays significant role in the pathogenesis of many diseases including solid tumors, rheumatoid arthritis and psoriasis [4–6]. In the case of psoriasis, the disease pathogenesis is attributed to aberrant keratinocytes or endothelial cells signaling. Formation of new blood vessels and increase of dermal vascularity start the psoriatic changes that accompany with "red spots" which is a sign of excessive angiogenesis [33]. Yet, clinical studies showed that indirubin and indigo naturalis could significantly reduce psoriatic plaque lesions [34,35]. Definitely, such therapeutic efficacy would be partly attributed to the anti-angiogenic ability of the active ingredients – indirubin and indigo naturalis. However, there was no evidence yet to show the angiosuppressive effect of indirubin derivatives, E804.

In this study, we provide the first evidence that E804, another novel indirubin analog, exhibited potent angiosuppressive effects *in vitro* and *in vivo*. Given the solubility problem of indirubin, restricted therapeutic efficacy would be resulted during application. Phytochemists have embarked on the synthesis of indirubin analogs with improved solubility, selectivity and cellular efficacy [14,36,37]. Thus, this work emphasized the improved potency of E804 on angiosuppression when comparing with indirubin-3′-oxime. The data presented here demonstrated that E804 is more potent than indirubin-3′-oxime or even indirubin in its ability to suppress angiogenesis.

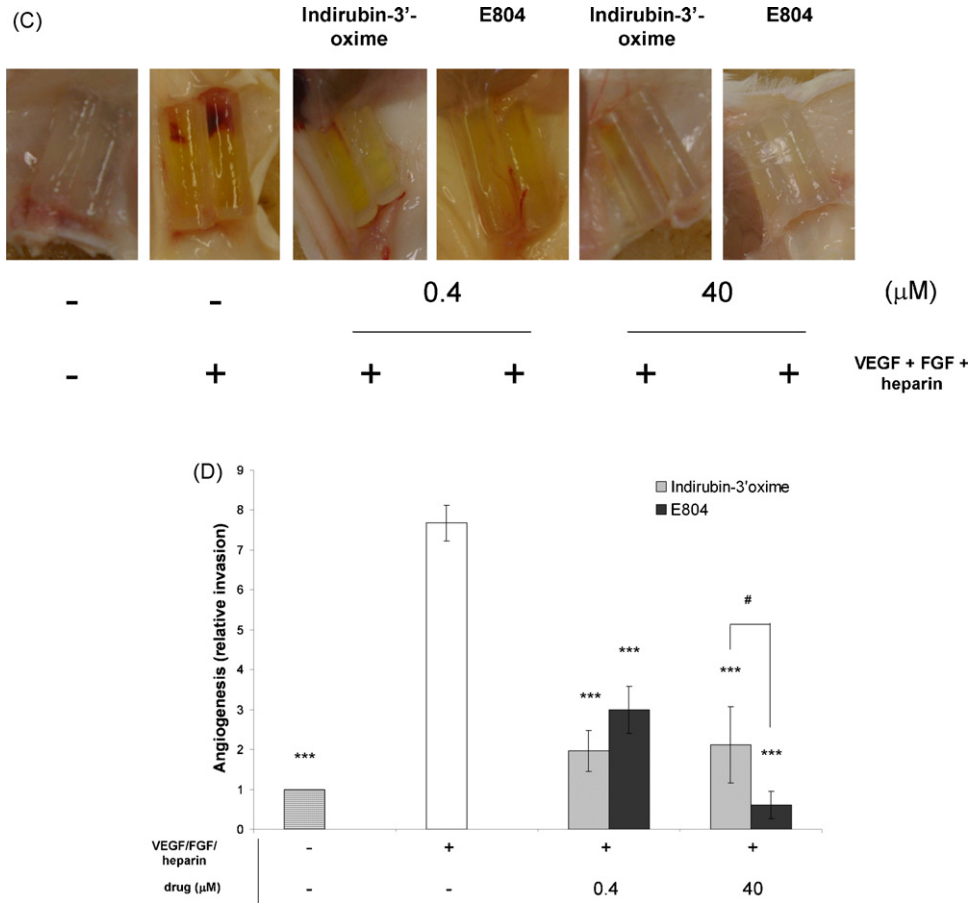

Fig. 7. (*Continued*).

In vitro, both indirubin derivatives inhibited HUVECs proliferation, migration, and tubular formation in a concentration-dependent manner. Beginning with 1 μmol, E804 exhibited significant inhibition than indirubin-3′-oxime. For the growth inhibition, the IC_{50} were achieved by indirubin-3′-oxime and E804 at the concentration of 20 and 10 μM, respectively. Similar distinction was obtained in cell migration and tube formation assay. *In vivo*, the angiosuppressive effect of E804 was further elucidated in Matrigel plug assay and DIVAA models which closely mimicked the multistep of angiogenesis. The injected Matrigel matrix acted as scaffold for the endothelial cells infiltration and neovessel formation. Once neovessels are become mature and functional, blood flow can be initiated. Data showed that both infiltrated endothelial cells and hemoglobin content were reduced by both indirubin derivatives at the range of 0.4–40 μM. Furthermore, using rat aortic sprouting assay, the influence of indirubin-3′-oxime and E804 on microvessels outgrowth from aortic fragments was assessed. In this *ex vivo* organotypic culture model, certain numbers of endothelial cells and smooth muscle cells normally appeared on the endothelium (inner lining) and muscle layer of the rat aortic fragments, respectively. Endothelial cells invaded into the surrounding Matrigel in the presence of ECGS, and finally form a filamentous like microvessel outgrowths. Unlike indirubin-3′-oxime, E804 could completely attenuate these endothelial sprouting at moderate (4 μM) and high concentration (40 μM). Besides, it is well-known that MMPs are critical in the initial step of angiogenesis; they regulate ECM re-organization, break down the stroma and ECM proteins, and allow the endothelial cells to invade into the matrix and migrate towards the growth factors [38,39]. Thus, the condition medium of aortic ring assay was collected for the measurement of gelatinolytic activities of MMP-2 and -9. Concomitant result was observed that E804 could suppress MMP-2 and -9 activities. In order to demonstrate the angiosuppressive ability of E804 more clearly, zebrafish model was employed. Through this assay, the morphology and number of newly formed blood vessels in fish embryo could be clearly observed after staining. Data showed that the subintestinal vessel basket was greatly reduced in size in the presence of E804 (0.04–10 μM), and the neovessels were less dense and spiked. This indicated that E804 could effectively inhibit *in vivo* angiogenesis.

Although direct mechanism of the angiosuppressive effects induced by indirubin are still elusive, mechanistic studies revealed that indirubin and its derivatives are indeed potent inhibitor towards CDKs, STAT3, and GSK-3. The potent inhibitory effect of indirubin derivatives on ATP-binding pocket of various kinases such as CDKs may also lead to reduced cell proliferation or even cell death [40,41]. With these lines of evidence, the angiosuppressive effects of indirubin derivatives may be due to the anti-proliferative effects on endothelial cells. To date, there is little pharmacokinetic study demonstrated the blood concentration of indirubin derivatives; a report showed that the bioavailability of indirubin is relatively low [42], with the modification of the structure, indirubin derivatives may enhance solubility and affinity to its molecular target *in vivo*.

Angiogenesis is a complicated and obligatory process for the pathogenesis of many diseases. Several anti-angiogenic drugs have been applied in clinical purpose, meanwhile, many angiosuppressive agents are under different phases of tests and clinical trials. In fact, many of these potential drug candidates are natural in origin.

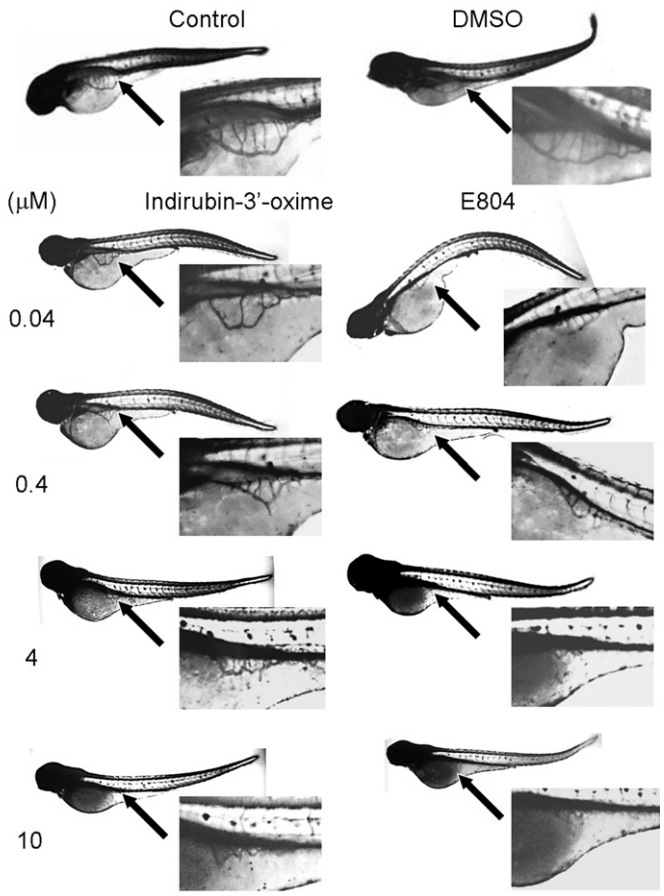

Fig. 8. Inhibition of *in vivo* SIVs formation in zebrafish embryos by indirubin-3′-oxime and E804. Dechorionated embryos were incubated with various concentrations of indirubin-3′-oxime and E804 (0.04, 0.4, 4, 10 μM) for 48 h. The SIV basket in zebrafish embryos was observed for morphological changes and images were captured and analyzed. Photomicrographs depict SIV basket in 3 dpf zebrafish embryos. Arrows indicate the location of SIV.

They would be original natural products, products derived semi-synthetically from natural products or synthetic products based on natural product models [43]. This was commonly found in the field of infectious diseases and cancers, in which, 60% and 75% of these drugs, respectively, were shown to be of natural origin [44,45]. There is no doubted that both indirubin-3′-oxime and E804 are both herbal origin, and exhibit differential angiosuppressive ability. Accumulated data showed that indirubin molecular template provides a fascinating perspective towards angiosuppressive agents, potentially leading to highly promising angiosuppressive drugs.

In conclusion, indirubin analog E804 was shown to exhibit angiosuppressive effects *in vitro*, *in vivo* and *ex vivo*. With the advancement in chemical structure, the angiosuppressive potency of E804 is largely increased. It would be a new potential candidate in the treatment of angiogenesis-dependent diseases.

Conflict of interest statement

None declared.

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