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Streptochlorin, a Marine Natural Product, Inhibits NF-κB Activation and Suppresses Angiogenesis *In Vitro*

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Abstract Angiogenesis is an essential step in tumor progress and metastasis. Accordingly, small molecules that inhibit angiogenesis would appear to be a promising way to cure angiogenesis-related diseases, including cancer. In the present study, we report that streptochlorin, a small molecule from marine actinomycete, exhibits a potent antiangiogenic activity. The compound potently inhibited endothelial cell invasion and tube formation stimulated with vascular endothelial cell growth factor (VEGF) at low micromolar concentrations where it showed no cytotoxicity to the cells. In addition, streptochlorin inhibited TNF-α-induced NF-κB activation in the newly developed cell-based reporter gene assay. These data demonstrate that streptochlorin is a new inhibitor of NF-κB activation and can be a basis for the development of novel anti-angiogenic agents.

Keywords: Antiangiogenic agent, NF-κB, streptochlorin

Angiogenesis, the formation of new blood vessels from an existing vascular bed, is a crucial step in the progression of tumor growth, invasion, and metastasis. Therefore, its inhibition is a putative therapeutic target toward cancer [5, 6]. Angiogenesis is a multistep process that includes endothelial cell (EC) proliferation, migration, basement membrane degradation, and new lumen organization [1, 7]. These processes are tightly controlled through a balance of positive and negative regulatory factors [8, 12]. The principal growth factors driving angiogenesis are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor.

Although the endothelial cell receptors and signaling pathways activated by proangiogenic factors such as VEGF have been extensively studied, less is known about the downstream transcription factors activated by these factors. Nuclear transcription factors likely integrate these upstream signals, activating and repressing downstream batteries of genes, to produce an angiogenic global gene expression profile, resulting in the angiogenic phenotype. In this sense, the role of NF-κB during angiogenesis is very crucial. In addition to the IL-8 gene, NF-κB regulates many angiogenesis-related genes, including those encoding tumor necrosis factor-alpha (TNF-α), vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 [2]. Consequently, understanding the transcriptional mechanisms by which endothelial cells become activated is likely to provide new therapeutic strategies for inhibiting the process at a very distal point in its signaling cascade, with potential applications toward the antiangiogenic therapy of cancer.

Antiangiogenic therapy designed to block neovascularization is an evolving anticancer strategy [3, 19]. Antiangiogenic tumor therapies have recently attracted intensive interest because of their broad spectrum of action, low toxicity, and absence of drug resistance [3]. A number of therapeutic agents having antiangiogenic potential have been developed and several antiangiogenic agents are now in clinical trials [4, 9, 11, 16, 26]. Although several small molecules and antibodies are being developed as angiogenesis inhibitors, antiangiogenic small molecules with novel structures can be valuable tools for both deciphering the molecular mechanism of angiogenesis and develpiing new antiangiogenic therapeutic drugs [10].

In our continuing efforts for discovery of new antiangiogenic agents from natural products, we have found a new antiangiogenic agent, designated streptochlorin, from marine-derived actinomycete. Streptochlorin was originally isolated from *Streptomyces* sp. as a new antibiotic, SF2583A [22, 25]. However, there has been no report on the antiangiogenic activity of the compound. Herein, we report a new biological activity of streptochlorin as being an antiangiogenic agent and a potent inhibitor of NF-κB transcription factor. Given the facts that activation of NF-κB activity in endothelial

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cells plays a key role as an proangiogenic factor [13–15, 17, 18, 20, 21, 23], the inhibition of NF-κB by the compound may represent one potential mechanism for its antiangiogenic activity.

MATERIALS AND METHODS

Isolation of Streptochlorin

Streptochlorin was obtained from the fermentation broth of marine *Streptomyces* sp. isolated from marine sediment collected at Ayajin, Korea. The culture broth was centrifuged (2,000 ×g for 15 min at 4°C) and then the supernatant was extracted with ethyl acetate. The extract was subjected to ODS flash chromatography with a stepwise gradient of $MeOH/H_2O$ as eluant. The fraction eluted with 60% MeOH in water was purified by reversed-phase HPLC (YMC ODS-A column, 10×250 mm; 55-70% MeOH; flow rate, 1.5 ml/min; UV detection at 210 nm) to yield streptochlorin [22].

Cell Culture

HT1080 (fibrosarcoma) cells were grown in MEM supplemented with 10% fetal bovine serum (FBS, Life Technology, Grand Island, NY, U.S.A.). HT29 (colon carcinoma), HepG2 (hepatocarcinoma), and HeLa (cervical carcinoma) cells were maintained in DMEM containing 10% FBS. Human umbilical vein endothelial cells (HUVECs) (2–5 passages) were grown in a type I collagen (2%, v/v)-coated flask in EGM-2 medium (Cambrex, Walkersville, MD, U.S.A.) supplemented with 10% FBS, 0.04% (v/v) hydrocortisone, 0.4% (v/v) human fibroblast growth factor (hFGF)-B, 0.1% (v/v) VEGF, 0.1% (v/v) long R3-human insulin-like growth factor (IGF-1), 0.1% (v/v) ascorbic acid, 0.1% (v/v) human epidermal growth factor (hEGF), 0.05% (v/v) gentamicin, 0.05% (v/v) amphotericin-B, and 0.1% (v/v) heparin. CellSensor NF-κB-*bla* HEK293T (Invitrogen, Carlsbad, CA, U.S.A.) cells were grown in standardized assay medium containing DMEM, 10% FBS, 0.1 mM nonessential amino acids (NEAA), 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin, 5 μg/ml blasticidin. All cell lines were grown in a humidified atmosphere of 5% CO_2 in air at 37°C. When cells reached confluence, they were passaged by detaching with 0.025% trypsin/0.01% EDTA.

Cell Growth Assay

The viability of endothelial cells was determined by trypan blue exclusion assay. HUVECs were seeded at a density of 1×10^4 cells/well in a 24-well plate. The cells were incubated in growth media for 24 h. Various concentrations of streptochlorin were added to the wells and incubation was continued for 48 h. The cells in each well were trypsinized and pelleted by centrifugation. Each pellet was resuspended in 10 μl of phosphate-buffered saline (PBS) and trypan blue dye was added. Cells were observed under an inverted microscope (IX71, Olympus, Japan) and counted with a hemocytometer. Cell viability was accessed as unstained cells/total cells×100.

Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were inoculated at a density of 5×10^3 cells per well in 96-well culture plates and incubated for 24 h for stabilization. Various concentrations of streptochlorin were added to each well and incubated for 2 days; thereafter, 50 μl of MTT (2 mg/ml stock solution, Sigma, St. Louis, MO, U.S.A.) was added, incubated for an additional 4 h, medium was removed, and then 150 μl of DMSO was added. The plate was read at 595 nm by a GENios plate reader (Tecan).

Chemoinvasion Assay

The invasiveness of human umbilical vein endothelial cells (HUVECs) was examined *in vitro* using a transwell chamber system with 8.0 μm pore-sized polycarbonate filter inserts (Corning Costar, Cambridge, MA, U.S.A.). The lower side of the filter was coated with 10 μl of gelatin (1 mg/ml), whereas the upper side was coated with 10 μl of Matrigel (3 mg/ml). Serum-starved cells (1×10^5 cells) were placed in the upper part of the filter and compounds were added in lower parts in the presence of VEGF (50 ng/ml, KOMA Biotech, Seoul, Korea). The chamber was then incubated at 37°C for 18 h. The cells were fixed with methanol and stained with hematoxylin/eosin. The cell invasion was determined by counting the whole-cell numbers in a lower side of the filter using an inverted microscope (IX71, Olympus) at ×100 magnification and photographed at a ×100 magnification with a camera (DP70, Olympus). Assays were performed in triplicate independently, and data are presented as mean values±SD.

Tube Formation Assay

Matrigel (150 μl, 10 μg/ml; Collaborative Biomedical Products, Bedford, MA, U.S.A.) was coated in a 48-well culture plate and polymerized for 2 h at 37°C. The HUVECs (1×10^5 cells) were seeded on the surface of the Matrigel, treated with VEGF (50 ng/ml), streptochlorin was added, and then incubated for 6–18 h. The morphological changes of the cells and tubes formed were observed under an inverted microscope (IX71, Olympus) and photographed at a ×100 magnification with a camera (DP70, Olympus).

NF-κB Reporter Assay

CellSensor NF-κB-*bla* HEK293T cell-based assay (Invitrogen, Carlsbad, CA, U.S.A.) was used to monitor the inhibition of NF-κB activation by compounds. This cell line contains a β-lactamase (BLA) reporter gene under control of the NF-κB response element stably integrated

into HEK293T cells. NF-κB-*bla* HEK293T cells were seeded into 96-well black-wall/clear-bottom plates (Costar) at 6.25×10^4 cells/well in 100 μl of the indicated medium. Cells were then treated for 1 h with compounds before treatment with 25 ng/ml tumor necrosis factor alpha (TNF-α) for 5 h. Following incubation, expression of β-lactamase (BLA) was monitored in 96-well plates by loading with CCF4-AM loading solution (PanVera LLC, Madison, WI, U.S.A.). After incubation in the dark at room temperature for 120 min, plates were read on a GENios plate reader (TECAN) using 409 nm excitation; emission was detected *via* 460 nm (cleaved CCF4, blue) and 530 nm (CCF4, green) band filters.

Statistical Analysis

Results are expressed as the mean±standard error (SE). Student's *t* test was used to determine statistical significance between control and test groups.

Results and Discussion

Streptochlorin as a Potent Inhibitor of Tube Formation

We undertook a phenotypic screen for small molecules with antiangiogenic activity among marine natural products. Tube formation assay was applied for monitoring the phenotypic activity of the libraries, because tube formation is one of the typical angiogenic properties. During the course of screening, we found that streptochlorin from a marine natural product caused a remarkable inhibition of tube formation at low micromolar concentrations (Fig. 1A).

The Growth Inhibitory Effect of Streptochlorin on Endothelial Cells

We next investigated the effect of streptochlorin on the growth of various cell lines. Both normal (HUVECs) and tumor cell lines (HT1080, HepG2, and HeLa cells) were treated with various concentrations of streptochlorin (0–20 μM) for 48 h and the cell growth was assessed by MTT colorimetric assay. As shown in Fig. 1B, streptochlorin inhibited the proliferation of each cell line with a different growth inhibitory spectrum. Although the cytotoxicity of streptochlorin was found to be greatly elevated at a concentration above 20 μM (data not shown), streptochlorin showed the most potent growth inhibitory activity against HUVECs. Other cancer cell lines (HeLa, HT1080, HepG2) also showed a degree of different sensitivities to streptochlorin. These data demonstrate that streptochlorin may exhibit the antiangiogenic activity by inhibition of specific growth-related signals of endothelial cell proliferation.

Antiangiogenic Activity of Streptochlorin

To investigate the antiangiogenic activity of streptochlorin in detail, the optimum dose of streptochlorin on the endothelial cell growth was first determined. Various concentrations of streptochlorin (0–15 μM) were applied to HUVECs and the effect of streptochlorin on the cell viability assay was determined by trypan blue exclusion assay. As shown in Fig. 1C, streptochlorin (1–10 μM) for 48 h did not significantly affect the viability of HUVECs. However, the viability of HUVECs was slightly decreased at high concentrations of streptochlorin (15 μM) for 48 h. Therefore, angiogenesis assays were performed in a concentration range of 1 to 10 μM of streptochlorin at which no cytotoxicity was observed.

We next investigated the effect of streptochlorin on the angiogenic phenotypes of endothelial cells, such as cell invasion and tube formation, using *in vitro* assays. Vascular endothelial growth factor (VEGF) was used as a chemoattractant or an angiogenic factor. For *in vitro* invasion assay with endothelial cells, HUVECs were starved for 24 h and stimulated by VEGF in the presence or

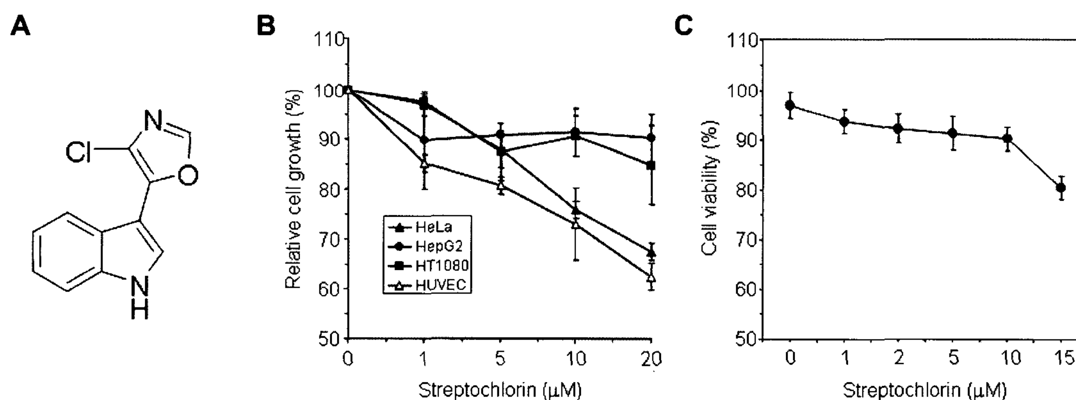

Fig. 1. Effect of streptochlorin on endothelial cell viability and the cell proliferation.
A. Chemical structure of streptochlorin. **B**. The cell proliferation was measured using MTT assay. Symbols indicate HeLa (▲), HepG2 (●), HT1080 (■) cells, and HUVEC (△). **C**. Effect of streptochlorin on the viability of HUVECs. HUVECs were treated with various concentrations of streptochlorin (0–15 μM) and incubated for 48 h. Cell viability was measured by trypan blue assay. Each value represents the mean±SE from three independent experiments.

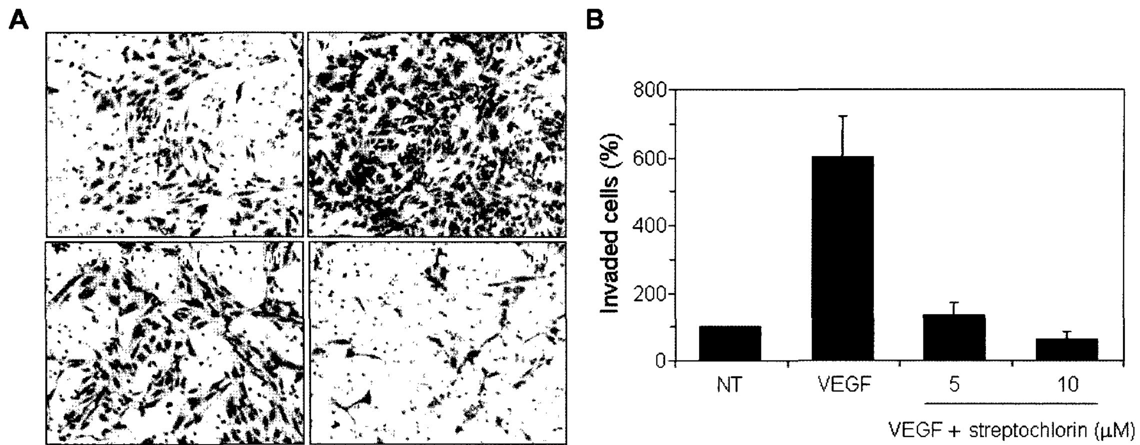

Fig. 2. Effect of streptochlorin on VEGF-induced cell invasion of HUVECs.
A. HUVECs starved with serum-free media for 12 h were treated with various concentrations of streptochlorin in the presence or absence of VEGF and invasion assay was performed. Figures were selected as representative results from three independent experiments. **B**. The bar graph represents the quantitative analysis of the invasion assay from three independent experiments. Each value represents the mean±SE from three independent experiments.

absence of streptochlorin. The assay was performed using polycarbonate-filter transwells coated with the Matrigel to prevent the migration of noninvasive cells. Streptochlorin potently inhibited VEGF-induced invasion of HUVECs in a dose-dependent manner (Figs. 2A and 2B). We next examined the effect of streptochlorin on capillary tube formation. In the presence of VEGF, cultured HUVECs on the Matrigel formed an extensive network of thick tubes (Fig. 3). Treatment of HUVECs with streptochlorin resulted in dose-dependent inhibition of tube formation induced by VEGF. These results suggest that streptochlorin efficiently inhibits angiogenesis *in vitro*, and the antiangiogenic activity of the compound may mostly originate from a specific effect on the angiogenic differentiation of endothelial cells, rather than antiproliferative activity.

Effect of Streptochlorin on NF-κB Transcriptional Activity

To determine whether streptochlorin inhibits the NF-κB signaling pathway, we employed a recently developed CellSensor NF-κB-*bla* HEK293T cell-based assay (Invitrogen, Carlsbad, CA, U.S.A.). These cells respond to stimulation with tumor necrosis factor-alpha (TNF-α), which leads to activation of the NF-κB signaling pathway and subsequent β-lactamase (BLA) expression. Cytosolic BLA activity is subsequently detected by loading cells with CCF4-AM, a cell-permeant fluorescent BLA substrate that accumulates within cells by cleavage of the acetoxymethyl side chain by cytoplasmic esterases. Upon excitation of the coumarin ring at 409 nm, this substrate emits at green wavelengths because of fluorescence resonance energy transfer to the attached fluorescein acceptor group; however, upon cleavage by BLA, the emission spectrum shifts to blue because of

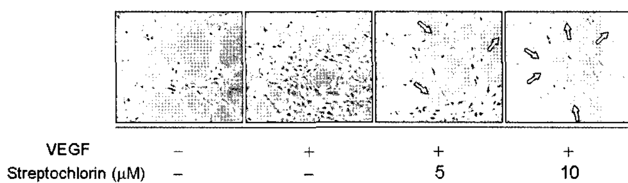

Fig. 3. Effect of streptochlorin on VEGF-induced tube formation of HUVECs.
Arrows indicate narrow or broken tubes formed by VEGF-stimulated HUVECs after drug treatment. Figures were selected as representative scenes from three independent experiments.

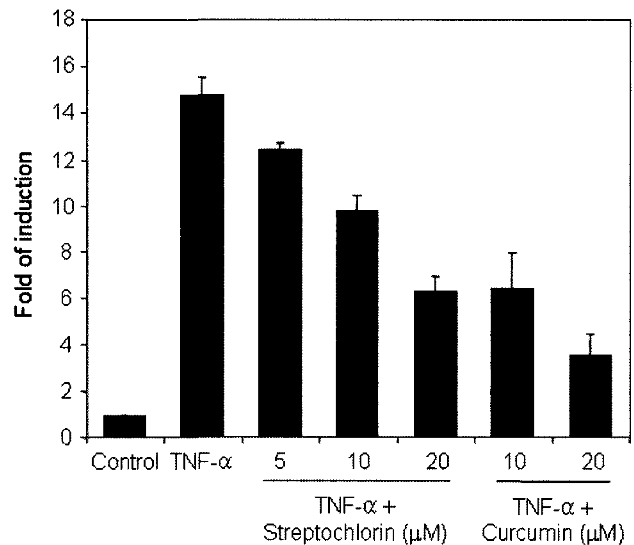

Fig. 4. Effect of streptochlorin on NF-κB transcriptional activity.
Stimulation with TNF-α (25 ng/ml) for 5 h induced reporter gene activity in NF-κB-*bla* HEK293T cells. Addition of streptochlorin inhibited TNF-α-stimulated reporter gene activity in a dose-dependent manner. Values are expressed as fold of basal activity of the reporter gene without TNF-α-stimulation.

the release of fluorescein and loss of fluorescence resonance energy transfer. The results revealed that streptochlorin exhibited dose-dependent inhibition of NF-κB signaling, which is nearly identical to curcumin, a phytochemical with potent NF-κB inhibitory activity (Fig. 4) [24]. These results suggest that streptochlorin could inhibit cell proliferation by interrupting NF-κB signaling, and thus applying streptochlorin treatment may be beneficial for controlling disease progression including angiogenesis. The exact mechanism of angiogenesis inhibition by streptochlorin is currently not understood. However, our results presented in this study provide a plausible clue that streptochlorin could inhibit angiogenesis by interrupting NF-κB signaling, which is required in tumor angiogenesis.

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