RK-270D and E, Oxindole Derivatives from Streptomyces sp. with Anti-Angiogenic Activity

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Introduction

Many small molecules derived from natural products have enabled drug discovery. Over the last four decades, 66% of approved small-molecule drugs were naturally derived or inspired by natural products [1]. Particularly, secondary metabolites produced by actinomycetes with their unique structural scaffolds have been prolific chemical sources of bioactive organic compounds [2]. More than half of all naturally derived antibiotics were isolated from actinomycetes, and 70% of these were found in Streptomyces [3]. We recently reported novel metabolites isolated from Streptomyces species, such as jejucarbazoles A, B, and C [4], aturanosides A and B [5], pentaminomycins A and B [6], and octaminomycins A and B [7].

Cell migration is involved in a number of physiological processes including ovulation, embryonic development, tissue regeneration (wound healing), and inflammation. These migration activities of cells in vitro to be related to many in vivo cellular behaviors such as tumor angiogenesis and metastasis [8]. Then, cell migration is attracting much attention as one of the alternative strategies for development of anti-cancer chemotherapies. Several natural products such as withaferin A [9], prodigiosin [10], and migrastatin [11] have been reported to inhibit cell migration.

We have previously reported the isolation and structural elucidation of three new oxindole derivatives, RK-270A, B, and C from the Streptomyces sp. RK85-270 in our search for novel and bioactive secondary metabolites from actinomycetes [12]. Further, an investigation of minor fractions from this fermentation resulted in the isolation of new oxindole derivatives, RK-270D (1) and E (2). In this study, we describe the isolation, structural elucidation, and anti-angiogenic activity of these compounds.

Materials and Methods

General Experimental Procedures

The specific rotations were measured on a JASCO P-1020 polarimeter (JASCO Corporation, Japan) that uses a 100-mm glass microcell. UV spectra were recorded on an Optizen 2120 UV spectrophotometer (Mecasys, Korea). The IR spectra were recorded on a Bruker VERTEX80V FT-IR spectrometer (Bruker, Germany). The NMR spectra were recorded on Bruker AVANCE HD 800 NMR spectrometer (Bruker, USA) at the Korea Basic Science Institute (KBSI) in Ochang, South Korea. Chemical shifts were referenced to a residual solvent signal (DMSO-d6 δH 2.50, δC 39.51). High-resolution electrospray ionization mass spectrometry (HRESIMS) data were acquired...
using Q-TOF mass spectrometer (Waters, USA) on a SYNAPT G2 at KBSI in Ochang, South Korea. Column chromatography was performed on reversed-phase silica gel (0.075 mm; Cosmosil, Japan) and semipreparative C18 (10 μm, 10 × 250 mm; Cosmosil, Japan) columns were used for reversed-phase HPLC on a YL900 HPLC system (Young Lin, Korea) equipped with a YL9120 UV/vis detector (Young Lin, Korea) that used HPLC grade solvents (Burdick & Jackson, USA). Open column chromatography was performed using a silica gel column (silica gel 60 (0.063-0.200 mm); Merck, USA). Vacuum liquid column chromatography was conducted with an ODS (Cosmosil, 75 μm). Semi preparative C18 (Cosmosil 5C18, 5 μm, 10 × 250 mm) columns were used for HPLC on a YL9100 HPLC system equipped with a photodiode array detector (YL9160) that uses HPLC grade solvents.

Fermentation and Isolation of Compounds
RK85-270 was cultured in a medium consisting of soluble starch (10 g), yeast extract (1 g), NZ-amine (1 g), and agar (15 g) in 1.0 L of distilled water at pH 7.0. The stock culture was cultured in a 250 ml Erlenmeyer flask containing 50 ml of seed culture medium (soluble starch 1%, yeast extract 0.1%, and tryptone 0.1%) for 3 days at 28°C on a rotary shaker with agitation at 165 rpm [12]. For a large culture (10 L), 1% of the preculture broth was inoculated into 40 × 1,000 ml baffled Erlenmeyer flasks containing 250 ml of modified CDY broth (glucose 2%, soluble starch 1%, meat extract 0.3%, yeast extract 0.25%, K2HPO4 0.005%, NaCl 0.05%, CaCO3 0.05%, and MgSO4·7H2O 0.05%), which was cultured for 8 days at 28°C on a rotary shaker agitated at 165 rpm. The mixture was then centrifuged, and the supernatant was extracted with EtOAc, while the mycelium was extracted with acetone. After concentrating the residual solvents under reduced pressure, the two portions were combined and dried to yield an extract of 1.1 g of the *Streptomyces* sp. RK85-270 of extract. The dried extract (1.1 g) was then fractionated by reversed phase C18 vacuum column chromatography with a stepwise solvent system of MeOH-H2O (20:80 to 100:0, each × 1 L) to yield nine fractions. The compounds 1 (2.4 mg, tR 25.9 min) and 2 (2.8 mg, tR 28.6 min) were obtained when the fraction was eluted with MeOH-H2O (60:40, 65.8 mg) and purified via reversed phase HPLC (Cosmosil, semipreparative C18, 45% CH3CN, 3 mL/min, UV detection at 210 and 270 nm).

Characterization of Compounds
RK-270D (1): a yellowish powder; [α]D25 0.5 (c 0.05, MeOH); IR (ATR) νmax (cm⁻1) 3400, 3200, 2917, 1683, 1614, 1556; UV (MeOH) λmax (log ε) 228 (3.37), 258 (4.20) 264 (4.11), 297 (3.55); 1H and 13C NMR data, Table 1; HRESIMS m/z 293.1268 [M + Na]+ (calcd for C16H18N2O2Na, 293.1266).

RK-270E (2): a yellowish powder; [α]D25 16 (c 0.05, MeOH); IR (ATR) νmax (cm⁻1) 3385, 2900, 1683, 1622, 1446, 1380; UV (MeOH) λmax (log ε) 230 (3.37), 258 (4.20) 264 (4.11), 297 (3.55); 1H and 13C NMR data, Table 1; HRESIMS m/z 295.1427 [M + Na]+ (calcd for C16H20N2O2Na, 295.1422).

Cell Viability Assay
HUVECs were cultured in EGM-2 bulletkit medium (Lonza, CC-3156 & CC-4176) supplemented with 10% fetal bovine serum (Welgene, S001-07), 100 units/mL penicillin, and 100 μg/ml streptomycin (Gibco, 15140-122). HUVECs were seeded in 96-well cell culture plates (1 × 104 cells/well) and treated with various concentrations of 1 or 2 for 24 h. The cell viability was measured using the EZ-Cytox colorimetric assay (Daeil Lab service, 0793) according to the manufacturer's protocol.

Table 1. NMR data for compounds 1 and 2 in DMSO-d6.

| Position | 1          | 2         | 1          | 2         |
|----------|------------|-----------|------------|-----------|
| 1-NH     | 10.36, s   | 10.35, s  | 10.35, s   | 10.35, s  |
| 2        | 169.3      | 169.3     | 169.3      | 169.3     |
| 3        | 123.1      | 123.2     | 123.2      | 123.2     |
| 3a       | 122.1      | 121.9     | 121.9      | 121.9     |
| 4        | 124.0      | 124.0     | 124.0      | 124.0     |
| 5        | 121.5      | 121.3     | 121.3      | 121.3     |
| 6        | 140.7      | 142.4     | 142.4      | 142.4     |
| 7        | 109.6      | 109.6     | 109.6      | 109.6     |
| 7a       | 141.1      | 141.0     | 141.0      | 141.0     |
| 8        | 153.1      | 153.0     | 153.0      | 153.0     |
| 9        | 25.2       | 25.2      | 25.2       | 25.2      |
| 10       | 22.6       | 22.5      | 22.5       | 22.5      |
| 1’       | 35.4       | 33.6      | 33.6       | 33.6      |
| 2’       | 2.29, s    | 2.29, s   | 2.29, s    | 2.29, s   |
| 3’       | 133.3      | 39.5      | 39.5       | 39.5      |
| 4’       | 22.6       | 22.5      | 22.5       | 22.5      |
| 5’       | 21.2       | 18.4      | 18.4       | 18.4      |
| 4’-NH₂   | 7.15, brs  | 7.15, brs | 7.15, brs  | 7.15, brs |

1H and 13C data were recorded at 800 and 200 MHz, respectively.
Wound Healing Assay

HUVECs were seeded in a confluent monolayer in 24-well cell culture plates (6 x 10^4 cells/well). Cells were scratched with a yellow tip, washed with PBS to remove nonadherent cells, and then treated with 1 μg/ml mitomycin C (Roche, 1010749001) for 3 h. After incubation, cells were treated with 1 or 2 for 12 h and fixed in 4% paraformaldehyde for 10 min. Cells stained with 0.2% crystal violet were observed under a microscope at 100× magnification.

Transwell Chamber Invasion Assay

The cell invasion assay was performed using 6.5 mm transwells with 8.0 μm pore polycarbonate membrane inserts (Corning, 3422). Starved HUVECs (1 x 10^5 cells/well) with EBM-2 basal medium without serum and growth factors were seeded in the Matrigel-coated upper chamber with 1 or 2 in basal medium for 24 h, whereas EGM-2 medium containing VEGF (30 ng/ml) (Sigma, V7259) was loaded in the lower chamber. The invasive cells were fixed in 4% paraformaldehyde for 10 min, stained with 0.2% crystal violet, and observed under a microscope at 100× magnification.

Capillary Tube Formation Assay

Starved HUVECs (2 x 10^5 cells/well) were treated with 1 or 2 in the presence of VEGF (30 ng/ml) in Matrigel (Corning, 356234)-coated 96-well cell culture plates. After 3 h incubation, the tubular capillary structures were captured using a microscope at 100× magnification.

Western Blot Analysis

Starved HUVECs were incubated with 1 for 12 h, then treated with VEGF (30 ng/ml) for 5 min. Cells were directly lysed with the 2× SDS sample buffer, and the proteins were separated via SDS-PAGE. The membranes were blocked with 5% skim milk and probed with the indicated primary antibodies at 4°C overnight, after transferring onto nitrocellulose membranes (0.2 μm; Bio-Rad) of the proteins. Primary antibodies against VEGF receptor 2 (#2479), p-VEGF receptor 2 (Y1175, #2478), p38 (#9212), and p-p38 (Thr180/Tyr182, #4631) were purchased from Cell Signaling Technology. A primary antibody against β-actin (sc-47778) was obtained from Santa Cruz Biotechnology. After incubation for 1 h with horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (Cell Signaling Technology, #7074 / #7076), protein bands were detected using the SuperSignal West Pico chemiluminescent substrate or SuperSignal West Femto maximum sensitivity substrate (ThermoFisher Scientific, #34080 / #34095).

Results and Discussion

Structural Determination of Compounds

Compound 1 (1) was isolated as a yellowish powder and its molecular formula was established as C_{16}H_{20}N_{2}O_{2} based on HRESIMS analysis. The characteristic UV absorptions at 206, 254, and 265 nm suggested the presence of an indole chromophore in 1. The 1H NMR spectrum in DMSO-d_{6} showed resonances for three methyls (δ_{H} 1.85, s; 2.29, s; and 2.49, s), an olefinic multiplet (δ_{H} 5.26, m), and a methylene (δ_{H} 3.48, d, J = 7.3 Hz). It also showed an exchangeable NH proton (δ_{H} 10.35, s) and an aromatic ABX spin system for a 1,2,4-trisubstituted aromatic ring at δ_{H} 7.42 (d, J = 8.0 Hz), 6.80 (dd, J = 8.0, 1.2 Hz), and 6.68 (d, J = 1.2 Hz). The 13C NMR data of 1 (Table 1) showed 16 carbon resonances assigned by HSQC-DEPT data, which can be attributed to three methyls, one methylene, four methines, six quaternary carbons, and two carbonyls. The NMR data of 1 were almost similar to those of RK-270C (3), a known oxindole metabolite from Streptomyces sp. RK85-270. Small differences were observed in the 13C NMR chemical shifts for C-5′ (δ_{C} 12.1 in 1; δ_{C} 13.3 in RK-270C) [12]. The planar structure of 1 was supported by interpretation of COSY and HMBC spectra (Fig. 2). The geometry of Δ^{2} was assigned as Z based on the 13C NMR chemical shift value of Me-5′ at 21.2 ppm. Therefore, 1 was a new geometric isomer of RK-270C at Δ^{2}, namely, RK-270D (Fig. 1).

Compound 2 (2) was obtained as a yellowish powder. The molecular formula C_{16}H_{18}N_{2}O_{2} of 2 was determined by HRESIMS. The difference of two mass units compared to 1 (C_{16}H_{20}N_{2}O_{2}) indicated saturation in one of the double bonds. The 1H and 13C NMR resonances were similar to those of 1 except for the resonances of a methine proton at δ_{H} 2.24 (1H, dd, J = 7.3 Hz, H-3′) and a methylene protons at δ_{C} 1.78, 1.51 (2H, m, H-2′). The NMR data of 2 (Table 1) were similar to those of 1. The main difference was the presence of a methine (δ_{H} 2.24, dd, H-3′, J = 7.3 Hz). The structure of 2 was determined to be a saturated analogue of 1. The proposed structure was fully supported by 2D NMR experiments (Fig. 2). The absolute configuration at C-3′ as determined by the negative optical value of 2 ([α]_{D}^{25} - 16) in comparison with the literature [13]. This established that the C-3′ position in 2 was supposed to be R-configuration, and the structure of 2 was designated as RK-270E.

Fig. 1. Structures of compounds 1–3.
RK-270D (1) Inhibited the Migration, Invasion, and Capillary Tube Formation of Human Umbilical Vein Endothelial Cells (HUVECs)

Angiogenesis occurs sequentially in the following processes, including endothelial cell proliferation, sprouting cell migration, and invasion to generate new blood vessels [14]. Angiogenesis inhibitors are being used clinically to treat cancer, macular degeneration in the eye, and other diseases [15, 16]. The cell viability was tested in HUVECs before evaluating the anti-angiogenic effects of 1 and 2. Compounds 1 and 2 did not show cytotoxicity against HUVECs up to the concentration of 100 μg/ml (Figs. 3A and S13A). We first tested cell migration and invasion of HUVECs through wound healing and Matrigel invasion assays, respectively, to investigate the angiogenic effects of 1 (Figs. 3B and 3C). As shown in Figs. 3B and 3C, 1 inhibited cell migration and vascular endothelial growth factor (VEGF)-induced invasion of HUVECs in a dose-dependent manner. Further, we performed a capillary tube formation assay to investigate phenotypic effects of 1 as angiogenesis inhibitors (Fig. 3D). The tubular capillary structures on the Matrigel were effectively generated in HUVECs treated with VEGF, a signal protein that stimulates the formation of blood vessels. In this assay system, the potent anti-angiogenic effects were observed in the HUVECs treated with 1. On the contrary the anti-angiogenic activity of 2 was not observed (Figs. S13B–D). Our results suggest that 1 inhibits the VEGF-induced angiogenesis of HUVECs in vitro.
HUVECs were pretreated with the Korea Basic Science Institute, Ochang, Korea, for providing the NMR (700 and 800MHz), and HR-ESI-MS. Science Research Program (2021R1I1A2049704) of the Ministry of Education of the Republic of Korea. We thank the KRIBB Research Initiative Program (KGM5292221) funded by the Ministry of Science ICT (MSIT) and the Basic

p38 MAPK (Fig. 4B, S14). These data suggest that was decreased in HUVECs treated with activation of ERK and p38 mitogen-activated protein kinases [18]. The phosphorylation of p38 was decreased in HUVECs treated with 1, whereas the decrease in phosphorylation of ERK was not observed (Fig. 4B, S14). These data suggest that I inhibits the VEGF-induced angiogenesis via VEGFR2/p38 pathway in HUVECs.

Fig. 4. Effects of compound 1 on the phosphorylation of VEGFR2 and p38 in VEGF-induced HUVECs. (A, B) HUVECs were pretreated with 1 followed by stimulation with VEGF (30 ng/ml) for 5 min. Phosphorylation of VEGFR2 (A) and p38 MAPK (B) was analyzed via Western blot analysis using antibodies against VEGFR2, p-VEGFR2, p38, and p-p38. Cells not stimulated with VEGF were used as a negative control.

RK-270D (1) Inhibits the VEGF-Induced Angiogenesis of HUVECs via VEGFR2-Mediated Signaling
VEGFR receptor 2 (VEGFR2), a main VEGF receptor protein, is a key regulator of angiogenesis [17]. HUVECs were preincubated with 1 and treated with VEGF immediately to determine whether I inhibits the VEGF-induced angiogenesis via the VEGFR2-mediated signaling pathway. As expected, Western blot analysis showed that I efficiently inhibited the phosphorylation of VEGFR2 (Fig. 4A). VEGFR2 signaling has been reported to drive angiogenesis via the VEGFR2-mediated signaling pathway. As expected, Western blot analysis showed that I efficiently inhibited the phosphorylation of VEGFR2 (Fig. 4A). VEGFR2 signaling has been reported to drive angiogenesis via the VEGFR2-mediated signaling pathway. As expected, Western blot analysis showed that I efficiently inhibited the phosphorylation of VEGFR2 (Fig. 4A). VEGFR2 signaling has been reported to drive angiogenesis via the VEGFR2-mediated signaling pathway.

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Conflicts of Interest
The authors have no financial conflicts of interest to declare.

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