

RK-95113, a New Angiogenesis Inhibitor Produced by *Aspergillus fumigatus*

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Abstract We found that the strain *Aspergillus fumigatus* var. *fumigatus* RK95-113 produced a new angiogenesis inhibitor, RK-95113. Spectroscopic analyses identified the structure of RK-95113 as a new fumagillin-related small molecule. RK-95113 preferentially inhibited the growth of human umbilical vein endothelial cells (HUVECs) rather than that of human normal fibroblasts in cell proliferation assays and blocked endothelial cell migration induced by vascular endothelial growth factor (VEGF).

Keywords angiogenesis inhibitor, RK-95113, endothelial cells, migration, vascular endothelial growth factor (VEGF), *Aspergillus fumigatus*

Angiogenesis is a complex process involving several distinct and sequential steps, such as membrane degradation, migration (chemotaxis), proliferation, and the formation of capillary tubes in endothelial cells. Angiogenesis inhibition is a promising strategy for the treatment of cancer and several other diseases, including diabetic retinopathy and rheumatoid arthritis. Several angiogenesis inhibitors are undergoing clinical trials, including a neutralizing antibody against vascular endothelial growth factor (VEGF). Avastin (bevacizumab), in combination with 5-fluorouracil-based chemotherapy regimens, is now used to treat patients with metastatic colorectal cancer in the United States and Europe. However, it is of great interest to identify new

angiogenesis inhibitors, as several small molecules with antiangiogenic activity are not clinically useful for toxicity and efficacy [1–5].

In this regard, we have commenced a screening program to identify and develop new angiogenesis inhibitors derived from microbial metabolites. Recently, we reported the identification of novel angiogenesis inhibitors, epoxyquinolins A and B, epoxytwinol A, azaspirene, and RK-805, isolated from fungal metabolites [6–10]. In the course of our phenotypic screening for angiogenesis inhibitors, RK-95113 (Fig. 1a) was discovered from the fermentation broth of *Aspergillus fumigatus* var. *fumigatus* RK95-113 (JCM 12527, FERM P-19233). The strain was isolated from soil collected in Wako, Saitama, Japan, and identified according to the methods of Klich and Pitt [11, 12] as well as by comparison with the *Aspergillus* Reference Cultures (<http://www.cbs.knaw.nl/ICPA/>) maintained in the Japan Collection of Microorganisms (JCM; <http://www.jcm.riken.jp/JCM/aspref.html>). The strain was inoculated into two 30-liter jar fermenters, each containing 15 liters of the culture medium, as previously described [10]. Further work on the culture broth led to the isolation of RK-95113 [13].

The molecular formula of RK-95113 was determined to be $C_{24}H_{34}O_5$ based on HREI-MS data (M^+ ; m/z 402.2393, calcd. for $C_{24}H_{34}O_5$: 402.2407). All one-bond 1H - ^{13}C connections in RK-95113 were elucidated by the PFG-HMQC and DEPT experiments. The NMR data and the molecular formula indicated the presence of one methoxy, four methyls, four methylenes, four sp^3 methines, seven sp^2

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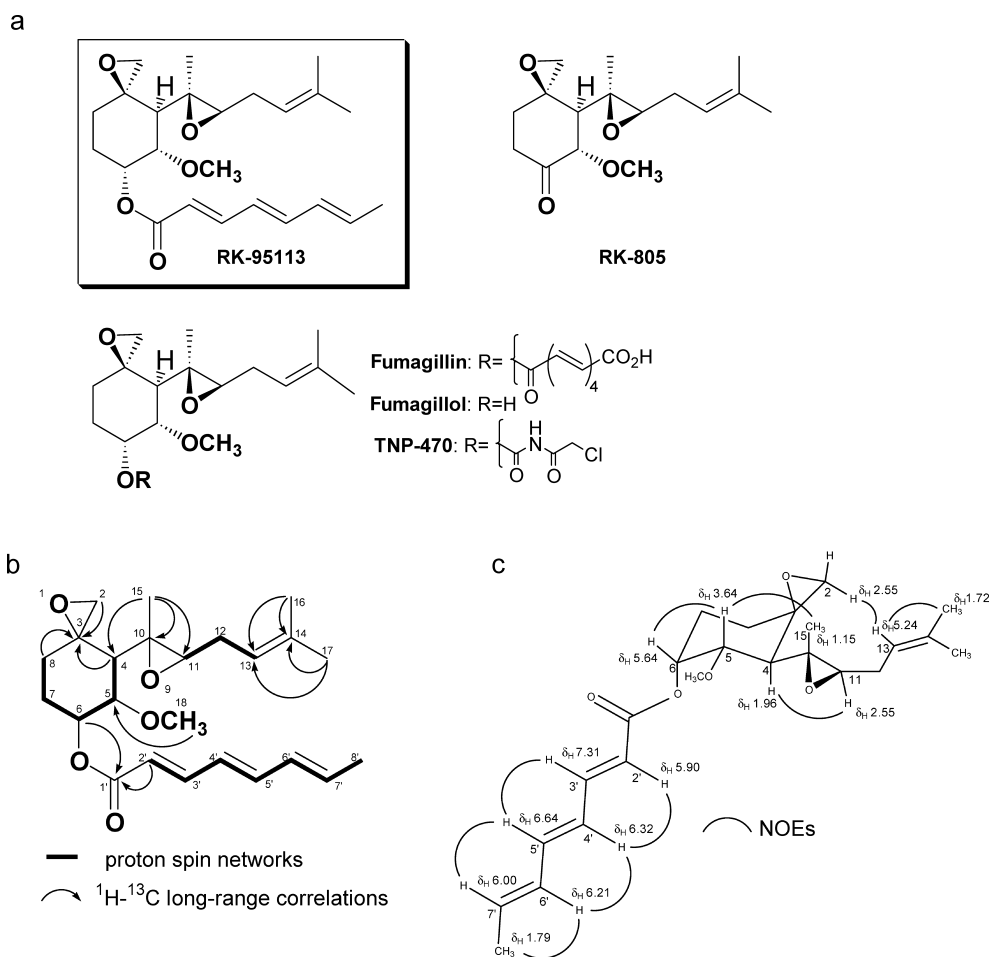


Fig. 1. (a) Structures of RK-95113, RK-805, fumagillin, fumagillol, and TNP-470. (b) Key correlations in PFG-DQFCOSY and PFG-HMBC spectra in RK-95113. (c) Key NOE data for RK-95113.

methines, and four quaternary carbons including a carbonyl carbon [14]. ^1H - ^1H correlations in PFG-DQFCOSY (Fig. 1b), H-11 through H-13 and H-2' through H-8', indicated the presence of an alkenyl moiety including a terminal methyl proton. Also, long-range couplings from H-15 to C-4, C-10, and C-11; from H-16 and H-17 to C-13 and C-14; and from H-2' to carbonyl carbon at C-1' were observed in the PFG-HMBC experiment (Fig. 1b). Thus, the linkages from C-10 to C-17 and from C-1' to C-8' were established. The cyclohexanol skeleton was confirmed by ^1H - ^1H correlations in PFG-DQFCOSY: H-4 through H-8, as well as by ^1H - ^{13}C correlations in the PFG-HMBC spectra: the strong two- and three-bond ^1H - ^{13}C correlations from H-18 of the methoxy proton signal to C-5 and from the proton at H-6 to the carbonyl carbon at the C-1' position. The exocyclic epoxide ring on cyclohexanol was confirmed by the chemical shifts, coupling constants, and two strong correlations from H-2, H-4, and H-8 to C-3 in the PFG-HMBC experiment. Thus, the planar structure of RK-

95113 with the fumagillol nucleus and alkenyl moiety was established. The relative stereochemistry of RK-95113 was elucidated by NOE experiments (Fig. 1c). Significant NOEs between H-5 (δ_{H} 3.64) and H-15 (δ_{H} 1.15), H-5 and H-6 (δ_{H} 5.64), H-4 (δ_{H} 1.96) and H-11 (δ_{H} 2.55), and H-13 (δ_{H} 5.24) and H-2a (δ_{H} 2.55) were observed, indicating that H-4 and H-5 were both axial and H-6 was equatorial. The geometry of the three conjugated double bonds was determined as all-*E* on the basis of the coupling constants ($J_{2',3'} = 15.2$ Hz, $J_{4',5'} = 14.2$ Hz, and $J_{6',7'} = 14.4$ Hz) and the NOE correlations between H-2' and H-4', H-3' and H-5', H-4' and H-6', H-5' and H-7', and H-6' and H-8'. Therefore, the relative stereochemistry of RK-95113 was unambiguously determined as shown in Fig. 1a.

We tested the effects of RK-95113 on the growth of human umbilical vein endothelial cells (HUVECs) and normal human fibroblast WI-38 cells in a proliferation assay. The IC_{50} values in the HUVECs of RK-95113, RK-805, fumagillin, and TNP-470 were 0.1, 0.03, 0.5, and

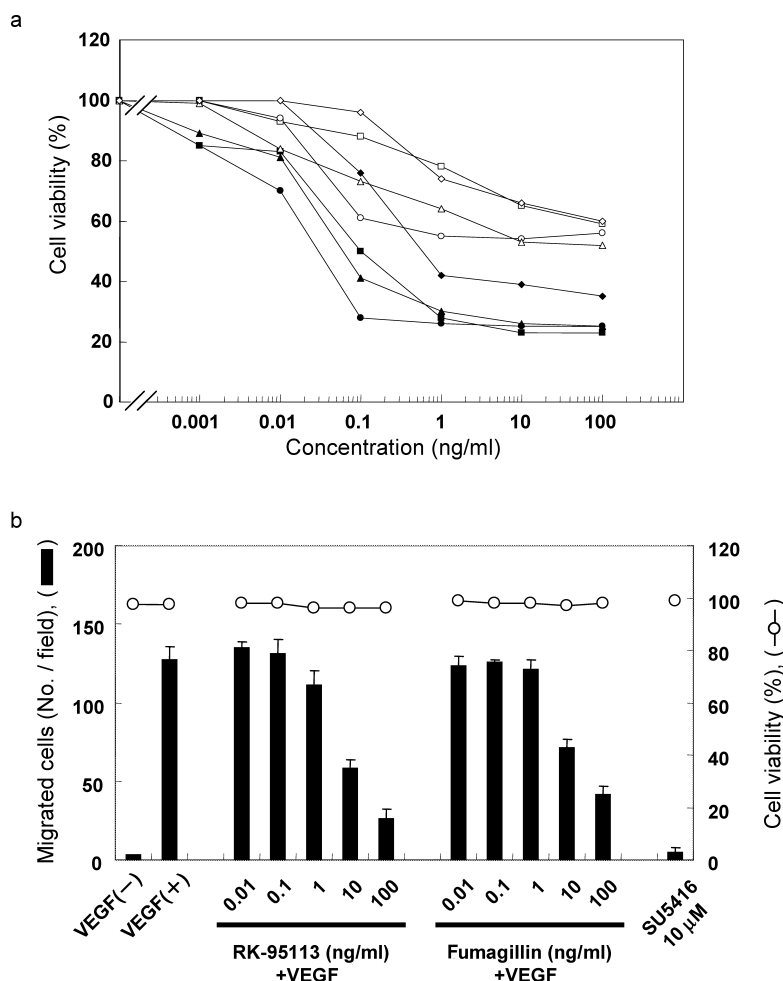


Fig. 2. RK-95113 effectively inhibits the growth and VEGF-induced migration of HUVECs.

(a) HUVECs and WI-38 cells were seeded on 96-well microplates (1.5×10^3 cells per well), respectively. Test compounds were dissolved in methanol at appropriate concentrations and were treated for 96 hours (both HUVECs and WI-38 cells). Cell proliferation assays were carried out by the WST-8TM (Nacalai Tesque, Kyoto, Japan) protocol. The growth of HUVECs: ■, RK-95113 (IC_{50} : 0.1 ng/ml); ●, RK-805 (IC_{50} : 0.03 ng/ml); ▲, TNP-470 (IC_{50} : 0.06 ng/ml); ◆, fumagillin (IC_{50} : 0.5 ng/ml). The growth of WI-38 cells: □, RK-95113 (IC_{50} : >100 ng/ml); ○, RK-805 (IC_{50} : >100 ng/ml); △, TNP-470 (IC_{50} : >100 ng/ml); ◇, fumagillin (IC_{50} : >100 ng/ml). (b) HUVECs (1×10^5 cells per well) suspended in Humedia-EG2 medium (Kurabo, Osaka, Japan) with various concentrations of test compounds were added to the upper compartment of a Chemotaxicell chamber (Kurabo) and incubated with HuMedia-EG2 medium containing 12.5 ng/ml of VEGF in the lower compartment for 18 hours at 37°C in a 5% CO_2 atmosphere. The filter was fixed with MeOH and stained with Wright solution. The cells on the upper surface of the filter were removed by wiping with cotton swabs. Cells that migrated through the filter to the areas of the lower surface were counted manually under a microscope at a magnification of $\times 100$. Values are means \pm S.D. for triplicate samples.

0.06 ng/ml, respectively, whereas they did not exhibit remarkable inhibitory activity in WI-38 cells even at 100 ng/ml, indicating that all these compounds exhibited preferential inhibitory activities in HUVECs (Fig. 2a).

Next we performed the cell migration assay. VEGF significantly induced cell migration, where as 10 μ M of SU5614, an inhibitor of VEGF receptor tyrosine kinase, inhibited the VEGF-induced cell migration (Fig. 2b) [15]. RK-95113 also inhibited cell migration induced by VEGF in a dose-dependent manner without showing significant

cell toxicity; this result was estimated by trypan blue dye exclusion assay [10]. The ED_{50} value of RK-95113 was *ca.* 10 ng/ml, as shown in Fig. 2b. Fumagillin exhibited almost the same inhibitory activity in this assay. These results suggested that RK-95113 blocked the function of endothelial cell migration activated by VEGF.

RK-95113 belongs to the fumagillin family including FR-111142 [16] and Sch528647 [17]. At the molecular level, one of the direct targets for fumagillin and its analogues, including RK-805 and ovalicin, has been

identified as methionine aminopeptidase-2 (MetAP2), a dimetalloprotease catalyzing the removal of *N*-terminal methionine in newly formed polypeptide chains [10, 18–20]. In particular, the spiro-epoxide moiety plays a crucial role by creating a covalent bond with a histidine residue (His231) of the active site in human MetAP2. The X-ray crystal structure of fumagillin with human MetAP2 also reveals that a water molecule forms hydrogen bonds with the oxygens of the methoxy group and the epoxide of the alkyl side chain in fumagillin, which plays a role in stabilizing His231 [21]. RK-95113 has a common moiety with fumagillin and its analogue for the above binding with human MetAP2, while the alkenyl side chain, including an octa-2,4,6-trienoic acid ester of RK-95113, is different from the others, especially regarding in the hydrophobicity and polarity. This suggests that RK-95113 may directly inhibit MetAP2 and may have different rates of cellular uptake and metabolism in intact cells. Meanwhile, it is not fully established why endothelial cell growth requires MetAP2, although the ability of fumagillin and its analogues to inhibit endothelial cell growth is related to their ability to inhibit MetAP2 activity in a p53-dependent manner [22, 23]. Moreover, very recently we found that fumagillin and TNP-470 suppress human immunodeficiency virus I (HIV-1) infection of macrophages through the inhibition of HIV-1 viral protein R (VPR) activity, independently from the MetAP2 pathway [24]. Therefore, further comprehensive chemical and biological studies of fumagillin and its analogues, including RK-95113, might shed light on these pivotal events in relation to chemotherapy for angiogenesis-related diseases as well as for HIV-1.

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13. The culture broth was centrifuged and the supernatant was adjusted to pH 3.0. The supernatant was extracted with an equal volume of ethyl acetate. The organic layer was concentrated *in vacuo* to dryness. The concentrate material was applied to silica gel column chromatography. The materials were eluted with a stepwise gradient of $CHCl_3$ - CH_3OH (0~100% MeOH in $CHCl_3$). The active fraction was concentrated *in vacuo* to yield a crude material. Further purification of RK-95113 was carried out by HPLC (PEGASIL ODS, 20 i.d.×250 mm, Senshu Scientific Co. Ltd., Tokyo) with a linear gradient solvent system of CH_3CN -water (from 20:80 to 0:100 for 40 minutes) at a flow rate of 9.0 ml/minute to give a yellow powder. Finally, 2.2 mg of RK-95113 was separated by preparative TLC with *n*-hexane-ethyl acetate (3:1). RK-95113 was obtained as a colorless amorphous powder, that is soluble in MeOH, acetone, and DMSO but not in *n*-hexane and H_2O . The R_f value in the solvent system ($CHCl_3$ -MeOH, 50:1) was 0.63 on a silica gel TLC (Merck 60 F254); $[\alpha]_D^{23}$ -40.0° ($c=0.035$, EtOH); IR; KBr ν_{max} 2920, 1700, 1610, 1265, 1225, 1125, 1005 cm^{-1} ; UV (MeOH) λ_{max} nm (ϵ) 300 (47830).
14. 1H NMR data of RK-95113: (500 MHz in acetone- d_6), δ_H 1.15 (s, 3H, H-15), 1.29 (m, 2H, H-8), 1.64 (s, 3H, H-16), 1.72 (s, 3H, H-17), 1.79 (d, 3H, $J=6.7$ Hz, H-8'), 1.88 (m,

- 2H, H-7), 1.96 (d, 1H, $J=10.40$ Hz, H-4), 2.22 (m, 2H, H-12), 2.55 (m, 1H, H-11), 2.55 (m, 1H, H-2a), 2.96 (d, 1H, $J=3.10$ Hz, H-2b), 3.35 (s, 3H, H-18), 3.64 (d, 1H, $J=10.40$ Hz, H-5), 5.24 (brs, 1H, H-13), 5.64 (brs, 1H, H-6), 5.90 (d, 1H, $J=15.2$ Hz, H-2'), 6.00 (dq, 1H, $J=14.4$ Hz, 6.7 Hz, H-7'), 6.21 (dd, 1H, $J=14.4$ Hz, 11.1 Hz, H-6'), 6.32 (dd, 1H, $J=14.2$ Hz, 11.8 Hz, H-4'), 6.64 (dd, 1H, $J=14.2$ Hz, 11.1 Hz, H-5'), 7.31 (dd, 1H, $J=15.2$ Hz, 11.8 Hz, H-3'). ^{13}C NMR data of RK-95113: (125 MHz in acetone- d_6), δ_{C} 14.53 (q, C-15), 17.98 (q, C-16), 18.53 (q, C-8'), 25.80 (q, C-17), 26.43 (t, C-7), 28.11 (t, C-12), 30.10 (t, C-8), 49.08 (d, C-4), 51.57 (t, C-2), 56.64 (q, C-18), 58.88 (s, C-10), 60.14 (s, C-3), 60.75 (d, C-11), 67.37 (d, C-6), 80.31 (d, C-5), 120.34 (d, C-13), 121.21 (d, C-2'), 128.58 (d, C-4'), 132.27 (d, C-6'), 134.67 (s, C-14), 135.57 (d, C-7'), 141.98 (d, C-5'), 145.59 (d, C-3'), 166.48 (s, C-1').
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