ICM0301s, New Angiogenesis Inhibitors from Aspergillus sp. F-1491

I. Taxonomy, Fermentation, Isolation and Biological Activities

HIROYUKI KUMAGAI*, TETSUYA SOMENO, KAZUYUKI DOBASHI†, KUNIO ISSHIKI‡,
MASAAKI ISHIZUKA and DAISHIRO IKEDA

Microbial Chemistry Research Center
Numazu Bio-Medical Research Institute
18-24 Miyamoto, Numazu-shi, Shizuoka 410-0301, Japan
†Bioresource Laboratories, Mercian Co.
1808 Nakaizumi, Iwata-shi, Shizuoka 438-0078, Japan

(Received for publication September 26, 2003)

In the course of screening program for inhibitors of angiogenesis, novel substances designated as ICM0301A–H (1–8) were isolated from the culture broth of Aspergillus sp. F-1491. ICM0301s inhibited the growth of human umbilical vein endothelial cells (HUVECs) induced by basic fibroblast growth factor (bFGF) with IC50 values of 2.2–9.3 μg/ml. ICM0301A (1) showed significant anti-angiogenic activity at lower than 10 μg/ml in the angiogenesis model using rat aorta cultured in fibrin gel. ICM0301s showed very low cytotoxicity against various tumor cells. Furthermore, ICM0301A did not show any toxic symptom in mice by intraperitoneal injection at 100 mg/kg.

Angiogenesis is the process of the formation of new blood vessels from preexisting blood vessels1,2). This process plays a key role in the development and wound healing. Furthermore, angiogenesis is essential for the development of solid tumor3), metastasis of tumors cells4) and chronic inflammation such as rheumatoid arthritis5). The process of angiogenesis6,7) consists of: (i) degradation of basement membrane by MMPs; (ii) migration of blood endothelial cells (ECs); (iii) growth of ECs induced by growth factors such as bFGF and vascular endothelial growth factor (VEGF); (iv) tube formation of ECs and (v) the maturation of tube to vessel. Thus, each process of angiogenesis should be a target for development of anti-tumor and anti-inflammatory agents. In fact, TNP-4708), which shows very strong inhibitory activity against growth of ECs, antibodies against various growth factors9,10), anti-αVβ3 integrin antibody11), mimic peptide of RGD motif12) contained in integrins and kinase inhibitors of VEGF receptors13) have been developed in clinical trials.

We have screened for angiogenesis inhibitors, which exhibit inhibitory activity against the growth of HUVECs induced by bFGF, among metabolites of microorganisms. In the course of screening, ICM0301A (1), B (2), C (3), D (4), E (5), F (6), G (7) and H (8) (Fig. 1) were isolated from the culture broth of Aspergillus sp. F-1491. In this paper, we describe the taxonomy of the producing organism, and the fermentation, isolation and biological activities of ICM0301s.

Materials and Methods

Materials
Inertsil ODS-3 columns and silica gel (Wako gel C-200) were obtained from GL Science (Tokyo, Japan) and Wako Chemical (Osaka, Japan), respectively. HUVECs and bFGF were obtained from Dainippon Pharmaceuticals (Osaka, Japan) and PEPRO TECH EC Ltd. (London, UK), respectively. Culture plate coated with collagen Type I was obtained from Sumitomo Bakelite Co. (Tokyo, Japan).

* Corresponding author: kumagaih@bikaken.or.jp
RPMI1640, DMEM medium and HANK's balanced salt solution were obtained from Nissui Seiyaku Co. (Tokyo, Japan), and MCDB-131 medium was obtained from Kurorera Kogyo Co. (Tokyo, Japan), respectively. Bovine thrombin and fumagillin were obtained from Sigma (St. Louis, MO, USA). Bovine fibrinogen was obtained from Ito Ham (Hyogo, Japan). Lys- and gelatin-Sepharose 4B were obtained from Amershan Bioscience Co. (Piscutaway, NJ, USA).

Animals
Female ICR mice and male SD rats were obtained from Charles River Japan (Kanagawa, Japan), and were maintained under specific pathogen-free conditions at 23±1°C and 55±5% humidity.

Taxonomic Study
The producing strain, F-1491 was isolated from a soil sample collected at Kanagawa prefecture. The taxonomic studies of strain F-1491 were carried out according to the methods of Pitt and Carmichael et al. The color guide of Kornerup and Wanscher was used for determining and standardizing colors. Morphological observation of strain F-1491 was carried out using a light microscope and a scanning electron microscope.

Fermentation
The seed medium was composed of glycerin 2%, potato starch 2%, soy bean meal 2%, KH2PO4 0.1% and MgSO4·7H2O 0.005%. The seed culture was incubated at 25°C for 3 days on a rotary shaker at 225 rpm using 50 ml of medium containing 5 glass beads in a 500 ml Erlenmeyer flask. The production medium was composed of glycerin 5%, potato extract (hot water extract of 20% minced potato) 25%, malt extract 0.5%, yeast extract 0.5%, tryptone peptone 1% and Span 20 (antifoam) 0.025%, and adjusted to pH 6.5. The production culture was incubated at 25°C for 4 days on a rotary shaker at 225 rpm using 50 ml of medium in a 500 ml Erlenmeyer flask.

HPLC Analysis and Preparative HPLC
Inertisil ODS-3 columns were used for HPLC analysis (4.6×150 mm, mobile phase: 50% acetonitrile) and preparative HPLC (20×250 mm, mobile phase: 35 or 70% acetonitrile). The detection of IMC0301s was performed using ultra violet absorption at 280 nm.

Growth Inhibitory Activity against HUVECs
The inhibitory activities of ICM0301s against the growth of HUVECs were assessed as follows. HUVECs were cultured in MCDB-131 medium supplemented with 10%
FCS and 10 ng/ml of bFGF at 2 × 10^3 cells/100 μl in 96 wells culture plate coated with collagen Type I, and then test samples dissolved in DMSO were added to the culture. Cells were cultured for 36 hours at 37°C in 5% CO₂-air, and were further pulsed with 3[H]TdtR (7.4 KBq/well) for 12 hours. Proliferation of the cells was assessed by measuring incorporated radioactivity of [H]TdtR into cells using a β-ray counter.

Cytotoxicity against Tumor Cells

The cytotoxic activities of ICM0301s against human tumor cell lines including chronic myelogenous leukemia K562, non-small cell lung carcinoma H226, prostate carcinoma DLD-1 and fibrosarcoma HT1080 were assessed. These cells were cultured at 5 × 10^3 cells/100 μl in RPMI1640 or DMEM medium supplemented with 10% FCS for 3 days with the test samples, and proliferation of these cells was measured by the MTT method.

Anti-angiogenic Activities in Rat Aorta Organ Culture

Rat aorta organ culture was done by the methods reported by NICOSIA R. F. et al. with some modifications. Thoracic aortas were removed from male SD rats under anesthesia using pentobarbital, and immediately transferred to a culture dish containing ice-cold serum-free HANK's balanced salt solution. The peri-aortic fibroadipose tissue was carefully removed with fine microdissecting forceps and iridectomy scissors paying special attention not to damage the aortic wall. Aortas were sectioned within small fragments (2 × 2 mm), and embedded in 0.5 ml of ice-cold 0.3% bovine fibrinogen (passed through gelatin- and Lysine-Sepharose) gel-MCDB131 solution on 24-wells culture plate. Clotting was obtained by adding 20 μl of a 50 NIH units/ml bovine thrombin solution to 1 ml of fibrinogen solution. The fibrin gels formed within 30 seconds at room temperature. After polymerization, 0.5 ml of MCDB 131 medium containing e-aminocaproic acid was added to the gels, and then compound 1 or fumagillin, as a positive control, dissolved in DMSO was added to the culture. The concentration of e-aminocaproic acid was 300 μg/ml during the first 2 days of culture followed by 50 μg/ml for the remainder of the experiment. The cultures were kept at 37°C in 5% CO₂-air. The culture medium was changed every another day. At 7 days after the start of culture, the number of tubes derived from aorta fragment was measured by light microscope observation.

Anti-microbial Activity and Toxicity in Mice

Anti-fungal activities of ICM0301s were measured by the agar dilution method. Compound 1 was dissolved in 5% DMSO-saline solution and injected to female ICR mice intraperitoneally. Body weight changes of mice were monitored for 2 weeks.

Results and Discussion

Taxonomic Studies

The fungal strain F-1491 was cultured on various media at 25 or 37°C for 7 days. The cultural characters are summarized in Table 1. For media tested the growth rates of the strain F-1491 were greatest on medium CYA. Colony surfaces on every media were flat to centrally raised, cottony to felty and white to reddish gray color. Soluble pigment was not found in the culture on every media.

Morphological characteristics (Fig. 2) of the strain were

Table 1. Cultural characteristics of strain F-1491.

<table>
<thead>
<tr>
<th>Media</th>
<th>Diameter of Colony (mm)</th>
<th>Surface</th>
<th>Color</th>
<th>Reverse</th>
<th>Surface Characteristics</th>
<th>Pigment or Exudate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYA**</td>
<td>24-26</td>
<td>White ~ Reddish Gray (8A~B1-2)</td>
<td>White ~ Reddish Gray (8A~B1-2)</td>
<td>Cottony to Felty</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

*: Strain F-1491 was cultured at 25 °C for 7 days.
**: Strain F-1491 was cultured at 37 °C for 7 days.
***: Strain F-1491 was cultured after several passages.
as follows. Conidial structure of the strain was biseriate and similar to that of *Aspergillus*. Sterigmata were shaped like an ampule with short a neck. Conidiophores were smooth, extremely short (40–80 × 2.5–3.0 μm), colorless, with foot cells and with vesicle (sub-rounding to flask shape, 10–15 μm i.d.) at the apex. Metulae were not found. Phialides grew from upper half of the vesicle to upper side, and were 6.0–7.5 × 1.8–2.5 μm in size. Conidia were one-celled, rounded, 2.5–3.5 μm i.d., smooth in the surface and formed connected to each other like a chain. Hüle cells and chlamydoospores were not observed. Sexual reproduction organs such as cleistothecium were not found when the culture was observed for over four weeks.

These cultural and morphological characteristics suggest that the strain should be included in the genus *Aspergillus*. However, the properties mentioned above did not agree with those of any known species in the genus. Then, we classified this isolate as one strain of *Aspergillus*, and named it *Aspergillus* sp. F-1491. It was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial science and Technology, Japan as FERM P-18549.

Isolation and Purification

The isolation procedure of ICM0301s is shown in Fig. 3. After the fermentation, broth (130 liters) was filtered and the mycelium was extracted with MeOH (15 liters). The filtrate was adsorbed on a Diaion HP-20 column (5 liters) and washed with 20% MeOH (10 liters). Active ingredients were eluted by MeOH (15 liters) and combined with the mycelial extract. The solution was concentrated, and resulting aqueous solution was extracted with EtOAc (5 liters). The organic layer was washed with water, dried over Na₂SO₄ and concentrated *in vacuo* to give an oily material (80 g). This was dissolved in a small volume of CHCl₃, and applied on a silica gel column (3 liters, dry volume). After washing with CHCl₃ (5 liters), 1, 2, 3, 4, 5, 6 and 7 were eluted with CHCl₃-MeOH (25:1, 5 liters), and 8 was eluted with CHCl₃-MeOH (15:1, 5 liters).
further eluted with CHCl₃-MeOH (15:1, 5 liters). The fractions containing 1 to 7 were dissolved in a small volume of toluene and applied on a silica gel column (400 ml). After washing with toluene-acetone (20:1, 1 liter), active ingredients were eluted with toluene-acetone (10:1, 1 liter). This fraction was concentrated in vacuo to give a yellow powder (1.2 g). This powder was dissolved in a small volume of MeOH and applied on a preparative HPLC column. Active ingredients were eluted with 70% acetonitrile at a flow rate of 7 ml/minute. Compounds 1, 2 and 3 were separately eluted in this order to give 1 (110 mg), 2 (100 mg), 3 (10 mg) as white powders, respectively. Compounds 4 and 5, 6 and 7 were eluted as mixture. Further purification was carried out by preparative TLC (toluene-acetone 3:1) to give 4 (10 mg), 5 (10 mg), 6 (10 mg) and 7 (10 mg) as white powders, respectively. The fraction containing 8 was dissolved in a small volume of toluene and applied on a silica gel column (400 ml). After washing with toluene-acetone (5:1, 1 liter), active ingredients were eluted with toluene-acetone (3:1, 1 liter) to give a yellow powder (0.8 g). This powder was applied on a preparative HPLC, and eluted with 35% acetonitrile at a flow rate of 7 ml/minute to give 8 (15 mg) as a white powder.

The retention time and Rf value of ICM0301s are summarized in Table 2.

Physico-chemical properties and structure elucidation of ICM0301s will be described in a following paper.

Growth Inhibitory Activities against Human Cells

Growth inhibitory activities of ICM0301s against HUVECs and human tumor cell lines were assessed. As shown in Table 3, ICM0301s showed potent inhibitory activities against HUVECs. Among them, ICM0301A (1) showed the strongest inhibitory activity against HUVECs with IC₅₀ value of 2.2 μg/ml.

On the other hand, ICM0301s had no significant cytotoxic activities at 100 μg/ml against human tumor cell lines including H226, DLC-1, HT-1080. ICM0301s showed weak cytotoxicities against K562 cells.

Anti-angiogenic Activities in Rat Aorta Organ Culture

Anti-angiogenic activity of ICM0301A (1) was assessed by using the in vitro angiogenesis model of culturing fragment of rat aorta in three-dimensional fibrin gels. As shown in Fig. 4, 1 exhibited dose dependent anti-angiogenic activity, and showed significant inhibitory activity of 52% inhibition at 1 μg/ml. Angiogenesis was completely inhibited at 10 μg/ml. Fumagillin, a potent angiogenesis inhibitor through methionine amino-peptidase inhibition, showed complete inhibition at 10 ng/ml. It is known that anti-proliferative agents against ECs, MMP inhibitors, hydrocortisone, and endogenous

Table 2. Retention times and Rf values of ICM0301s.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention time (min.)*</th>
<th>Rf value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICM0301A</td>
<td>10.8</td>
<td>0.67</td>
</tr>
<tr>
<td>B</td>
<td>7.3</td>
<td>0.61</td>
</tr>
<tr>
<td>C</td>
<td>11.3</td>
<td>0.46</td>
</tr>
<tr>
<td>D</td>
<td>7.7</td>
<td>0.42</td>
</tr>
<tr>
<td>E</td>
<td>7.8</td>
<td>0.37</td>
</tr>
<tr>
<td>F</td>
<td>5.3</td>
<td>0.48</td>
</tr>
<tr>
<td>G</td>
<td>5.1</td>
<td>0.56</td>
</tr>
<tr>
<td>H</td>
<td>2.8</td>
<td>0.38</td>
</tr>
</tbody>
</table>

* Mobile phase: 50 % acetonitrile-H₂O
** TLC plate: Merck Art. 5715
Solvent systems: toluene-acetone 4:1 (A–E), 2:1 (F–H)

Table 3. Growth inhibitory activities of ICM0301s against HUVECs and human tumor cell lines.

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC₅₀ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A  B  C  D  E  F  G  H</td>
</tr>
<tr>
<td>HUVEC</td>
<td>2.2 3.2 7.4 4.6 3.2 9.3 7.7 5.6</td>
</tr>
<tr>
<td>K562</td>
<td>39  36 52 80 68 72 95 61</td>
</tr>
<tr>
<td>H226</td>
<td>&gt;100 &gt;100 &gt;100 &gt;100 &gt;100 &gt;100 &gt;100</td>
</tr>
<tr>
<td>DLC-1</td>
<td>&gt;100 &gt;100 &gt;100 &gt;100 &gt;100 &gt;100 &gt;100</td>
</tr>
<tr>
<td>HT1080</td>
<td>&gt;100 &gt;100 &gt;100 &gt;100 &gt;100 &gt;100 &gt;100</td>
</tr>
</tbody>
</table>
angiogenesis inhibitors\textsuperscript{21}) exhibit anti-angiogenic activity in this model. Anti-angiogenic activity of 1 may be mainly exhibited through its anti-proliferative activity against ECs. Since angiogenesis in this model has occurred without supplement of serum and growth factors, this assay bridges the gap between in vitro and in vivo models combining advantages of both systems. Then, effectiveness of 1 in the in vitro model may be expected to follow through into in vivo angiogenesis models.

Fig. 4. Anti-angiogenic activity of ICM0301A on rat aorta organ culture.

Antimicrobial Activity and Toxicity in Mice

Because fusarielin A\textsuperscript{19)}, a compound structurally related to ICM0301s, was reported to show an MIC value of 3.1 \(\mu\)g/ml against Aspergillus fumigatus 11268, ICM0301s were expected to have anti-fungal activities. However, ICM0301s had no anti-fungal activity at 100 \(\mu\)g/ml.

Toxicity of 1 against mice was assessed. Body weight changes of mice given 100 mg/kg of 1 was equal to that of control mice for 2 weeks after intraperitoneal injection.

From the results mentioned above, ICM0301s may be useful in human diseases such as solid tumor and rheumatoid arthritis by virtue of its anti-angiogenic activity.

References

