Fusarielin A as an Anti-angiogenic and Anti-proliferative Agent: Basic Biological Characterization

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Fusarielin A shows anti-angiogenic activity in the human umbilical vein endothelial cell (HUVEC) tube formation assay. Structural development studies indicated the importance of the hydroxyl groups in this molecule. A 3H-labeled derivative and a fluorescent affinity-labeling agent were prepared and used to examine the cellular distribution and biological behavior of fusarielin A.

Key words fusarielin A; anti-angiogenic activity; tritium-labeling; human umbilical vein endothelial cell; HL-60; binding protein

Natural products that influence cell proliferation/differentiation and signal transduction systems are of great interest as candidate anti-tumor agents. Generally, isolation of bioactive natural products based on some specific biological activity requires a specific bioassay system that reflects the corresponding biological activity.1–6) For example, tubulin function disruptors (inhibitors of tubulin polymerization or depolymerization) have generally been isolated with the aid of microtubule assembly assay,1–4) while signal transduction inhibitors have generally been isolated with the guidance of inhibition assays of specific enzymes, such as protein kinases and phosphatases.5,6) On the other hand, a bioassay system that could simultaneously detect various kinds of cell function modulators, including cell proliferation inhibitors, cell differentiation modulators, signal transduction modulators, and so on, would be a superior tool to discover unique bioactive compounds, even if it provided no information as to their specific molecular targets/mechanisms.

One such broad-spectrum bioassay method utilizes the conidia of Pyricularia oryzae (P. oryzae) P-2b (Fig. 1).7,8) Germination of conidia of P. oryzae P-2b is sensitive to various kinds of bioactive compounds (details will be published elsewhere). For example, tubulin polymerization inhibitors, including rhizoxin,1) griseofulvin,2) nocodazole3) and phomopsidin,4) generally show morphological effects, such as curling, on mycelia germinated from conidia of P. oryzae P-2b [Fig. 1, panels (c)—(f)].

Using the P. oryzae P-2b assay method, we have isolated an antifungal antibiotic, fusarielin A (1: FSA) (Fig. 2), from a culture of Fusarium sp. K432.7) The effect of FSA (1) in this bioassay [Fig. 1, panel (b)] was similar to those elicited by compounds that interfere with microtubule function, including rhizoxin [panel (c)],1) griseofulvin [panel (d)],2) nocodazole [panel (e)],3) and phomopsidin [panel (f)]4) (Fig. 1). However, FSA (1) did not inhibit the assembly or disassembly of microtubule protein prepared from porcine brain.7)

Further investigation revealed that FSA (1) shows cell type-selective anti-proliferative activity, i.e., it inhibited proliferation of a leukemia cell line HL-60 with an IC50 value of 23.6 μM, while it inhibited proliferation of the epidermal cell line HeLa with an IC50 value of 54.6 μM.1)

On the other hand, ICM0301A (3), which is a natural product isolated from the culture broth of Aspergillus sp. F-1491 and structurally similar to FSA (1), has recently been reported to possess anti-angiogenic activity [the anti-angiogenic activity of ICM0301A (3) was assessed by using the in

Fig. 1. Curling on Mycelia Germinated from Conidia of Pyricularia oryzae P-2b

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vitro angiogenesis model of culturing fragment of rat aorta in three-dimensional fibrin gels, and it is reported to show significant inhibitory activity of 52% inhibition at 1 μg/ml). This reported result together with the above-mentioned findings prompted us to examine the effect of FSA (1) on the growth and tube formation of human umbilical vein endothelial cells (HUVEC).

Here, we describe the anti-angiogenic activity of FSA (1) in HUVEC tube formation assay, a preliminary study of the structure–activity relationship, an analysis of the cellular uptake and distribution of FSA (1) with biosynthetically prepared 3H-labeled FSA (Fig. 7), and the preparation of an affinity-labeling agent, CAFSA (Coumarin- and Azidophenyl-substituted FSA derivative: 6, Fig. 9).

Results and Discussion

Large-Scale Preparation of FSA (1) To obtain a sufficient amount of FSA (1) for our studies, the culture conditions were optimized. Our previous studies indicated that the production of FSA (1) by Fusarium sp. K432 is temperature-dependent, i.e., FSA (1) was produced at 20 °C, but not at 27 °C. Therefore, we investigated the time-course of FSA (1) production by the fungus in standing culture in a potato dextrose medium at 20 °C. Quantification of FSA (1) in the culture medium by means of HPLC indicated that the production of FSA (1) was time-dependent and saturated after 21 d. Based on the results, a total of 2.4 l of culture was kept at 20 °C for 21 d, then the medium was extracted with a mixture of acetone and benzene. Successive column chromatographies using three types of resins gave 550 mg of FSA (1) as a white powder (Fig. 3, see Experimental). The structure and purity of the isolated FSA (1) were confirmed by HPLC, NMR spectroscopy and mass spectrometry.

Growth-Inhibitory Activity and Anti-angiogenic Activity of FSA toward HUVEC Next, we investigated the growth-inhibitory activity of FSA (1) on HUVEC. FSA (1) inhibited the growth of HUVEC with the IC50 value of 19.3 μM after 72 h incubation, which is in good accordance with the IC50 value for HL-60 cells (IC50 = 23.6 μM) (Fig. 4a, Table 1). Anti-angiogenic activity of FSA (1) was assessed by means of HUVEC tube formation assay. FSA (1) exhibited moderate anti-angiogenic activity with the IC50 value of 7.1 μM (Figs. 4b, c, Table 1).

In our previous studies, fusarielin B (2: FSB) (Fig. 2), in which one of the two epoxy groups of FSA (1) was opened, was co-isolated with FSA (1). FSB (2) did not induce curling of mycelia of P. oryzae P-2b, suggesting that the two epoxy groups of FSA (1) are essential for the activity. On the other
hand, it was not clear whether or not the two hydroxyl groups at positions 1 and 3 (numbering is shown in Fig. 7) are necessary for the activity. To obtain preliminary information about the structure–activity relationships of FSA (1), derivatives with one or two benzylated hydroxyl group(s) were prepared (Fig. 5), i.e., 3-Bn-FSA (4) in which the 3-secondary alcohol group is protected with a benzyl group and 1,3-Bn₂-FSA (5) in which the 1-primary and 3-secondary alcohol groups are both protected with benzyl groups (Fig. 5). Because azido-functionalized benzyl group was successfully applied in identifying the molecular weight of target protein of dantrolene analog by Hosoya and co-workers,¹²) we made a choice for the benzyl group not only to obtain preliminary information of SAR study of FSA (1), but also to determine the site of FSA (1) to introduce azido-functionalized benzyl group for a planned photoaffinity labeling (vide infra),¹²) The structures of the synthesized compounds were confirmed by ¹H-NMR spectroscopy and mass spectrometry.

The two derivatives, 3-Bn-FSA (4) and 1,3-Bn₂-FSA (5) (Fig. 5), were investigated for anti-angiogenic activity, growth-inhibitory activity against HUVEC/HL-60 cells (Table 1, Fig. 6A), and malformation-inducing activity towards mycelia of *P. oryzae* P-2b (Fig. 6B). 3-Bn-FSA (4) showed anti-angiogenic activity (Fig. 6A), but lacked growth-inhibitory activity against HUVEC/HL-60 cells. It did not show curling-inducing activity on mycelia or growth-inhibitory activity against *P. oryzae* P-2b (Fig. 6B). The results suggest that (i) the free hydroxyl groups are critical for the curling-inducing activity towards mycelia germinated from conidia of *P. oryzae* P-2b, (ii) the free hydroxyl groups are not necessary for the anti-angiogenic activity, and (iii) the free hydroxyl groups at the 1-position and the 3-position are necessary and unnecessary, respectively, for the HUVEC/HL-60 cell growth-inhibitory activity. These findings indicate that it may be possible to separate each activity, i.e., cell growth inhibition, anti-angiogenic activity and curling-inducing activity, by the structural development of FSA (1). In other words, different structural features, especially the presence of the free hydroxyl groups, appear to be critically recognized by putative target molecules involved in the three biological activities.

**Preparation of [³H]FSA** As mentioned above, the anti-angiogenic activity of FSA (1) seems to be specific, at least in part, and the cell growth-inhibitory activity elicited by FSA (1) seems to be cell type-selective. These results suggest that FSA (1) may elicit its biological activity by binding to a specific target molecule(s). To examine this working hypothesis, we planned to prepare [³H]-labeled FSA ([³H]FSA). FSA (1) is a decaketide, and previous experiments indicated the incorporation of both carbons of acetate and the methyl carbon of methionine (Fig. 7).¹³ To prepare [³H]FSA, we first

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**Table 1.** HL-60/HUVEC Cell Growth Inhibitory Activity and HUVEC Tube Formation Inhibitory Activity of FSA (1), 3-Bn-FSA (4) and 1,3-Bn₂-FSA (5)

<table>
<thead>
<tr>
<th></th>
<th>FSA (IC₅₀: μM)</th>
<th>3-Bn-FSA (IC₅₀: μM)</th>
<th>1,3-Bn₂-FSA (IC₅₀: μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell growth</strong></td>
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<tr>
<td>inhibitory activity</td>
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<td>15.3</td>
<td>63.4</td>
</tr>
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<td>HL-60</td>
<td>19.3</td>
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<td>&gt;100</td>
</tr>
<tr>
<td>HUVEC (IC₅₀: μM)</td>
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<td>98</td>
<td>34</td>
</tr>
<tr>
<td>HUVEC (IC₅₀: μM)</td>
<td>48</td>
<td>46</td>
<td>49</td>
</tr>
<tr>
<td><strong>HUVEC tube</strong></td>
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<td></td>
<td></td>
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<tr>
<td>formation inhibitory activity</td>
<td>7.1</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>HUVEC: after 6 h (IC₅₀: μM)</td>
<td></td>
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</tbody>
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N.D. = not determined.

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³H-NMR spectroscopy and mass spectrometry.

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Fig. 5. Synthesis of 3-Bn-FSA (4) and 1,3-Bn₂-FSA (5)

Reagents and conditions: (a) tert-butyl(dimethyl)silane, Et₃N, DMAP, CH₂Cl₂, r.t., 23 h (86%); (b) BnBr, NaH, DMF, r.t., 20 h (39%); (c) 0.13 M tetra-n-butylammonium fluoride, THF solution, r.t., 25 h (89%); (d) BnBr, NaH, THF, 45 °C, 14 h (74%).
examined the incorporation of methionine-$d_3$, using cultures of the K432 strain. After standing culture for 21 d, FSA ($1$) was extracted and its structure was confirmed by $^1$H-NMR and mass spectrometry. The results showed that methionine-$d_3$ was incorporated into FSA ($1$). Therefore, to prepare $[^3$H]$1$, we added L-methionine, [methyl-$^3$H] (185 MBq) to a culture medium which had been pre-cultured for 6 d. The culture was continued for a further 8 d, then FSA ($1$) was extracted from the medium and purified by HPLC to give $[^3$H]$1$ with the specific activity of 75 MBq/mmol.

Distribution of $[^3$H]$1$ in HL-60 Cells To examine the cellular uptake and distribution of FSA ($1$), we selected HL-60 cells because the HL-60 cell growth inhibition assay could discriminate FSA ($1$) and 3-Bn-FSA ($4$) [active] from 1,3-Bn$_2$-FSA ($5$) [inactive]. This raises the possibility of developing affinity-labeling agents using the 3-hydroxyl group as a functional group on which a photolabile group can be introduced ($vide$ $infra$).

HL-60 cells were incubated for 20 h under 5% CO$_2$ at 37 °C in the presence of $[^3$H]$1$ ($10 \mu M$). After the incubation, the mixture was centrifuged at 2000 rpm for 5 min at 4 °C to separate the supernatant and cell pellet. The cell pellet contained about ten times higher concentration of radioactivity than the supernatant, suggesting efficient uptake of $[^3$H]$1$ into the cells. Separation of subcellular fractions of HL-60 cells and the measurement of $[^3$H]$1$ uptake were performed as described previously.$^{13-15}$ Briefly, HL-60 cells, which had been incubated with $[^3$H]$1$ ($10 \mu M$), were lysed with hypotonic solution (20 mM Tris–HCl), and the nuclear fraction, soluble cytosolic fraction and membrane fraction were separated by successive centrifugation. Approximately 92% of $[^3$H]$1$ was distributed to the soluble cytosolic fraction (Fig. 8A). Next, 90% ammonium sulfate was added to the soluble cytosolic fraction, and the mixture was centrifuged to separate the supernatant and pellet. More than 90% of $[^3$H]$1$ was found in the pellet, i.e., the salted-out protein fraction (Fig. 8B). Therefore, FSA ($1$) might have been bound to specific protein(s) existing in the soluble cytosolic fraction.

Photoaffinity Label Based on the above results, we designed a photoaffinity probe to detect the putative target molecule(s) of FSA ($1$) in HL-60 cells. As the free hydroxyl group at the 3-position of FSA ($1$) was considered to be unnecessary for cell growth-inhibitory activity toward HL-60 cells, we designed and synthesized an FSA derivative with a
fluorescent and photoreactive group(s) at the 3-secondary alcohol group, i.e., an FSA derivative bearing a coumarin and an azidophenyl group \(6\): CAFSA, Fig. 9). The coumarin skeleton was chosen as a fluorescent chromophore, and an azidophenyl moiety was introduced as the photoreactive part.16—18) The probe CAFSA \(6\) was synthesized as shown in Fig. 9.

A preliminary photoaffinity labeling experiment using CAFSA \(6\) was performed (Fig. 10) with the soluble cytosolic fraction of cultured HL-60 cells, using a method similar to that described previously.19—21) The HL-60 cell extract was incubated with CAFSA \(6\) [bars (a—c) in Fig. 10] in the presence [bar (b) in Fig. 10] or absence [bars (a) and (c) in Fig. 10] of an excess amount of FSA \(1\), and then the mixture was irradiated at 254 nm with a compact UV lamp (UVP, 4 W). The irradiated mixture was subjected to gel filtration column chromatography to obtain the macromolecular (protein) fraction, and the fluorescence intensity of this fraction was measured (relative fluorescence intensity is indicated by the length of the bars in Fig. 10). As shown in Fig. 10, the macromolecular fraction and non-reacted CAFSA \(6\) were efficiently separated by the gel filtration column chromatography [bars (c) and (d) in Fig. 10]. The high and low levels of relative fluorescence intensity shown by the bars (a) and (c) in Fig. 10, respectively, indicate that the covalent binding of CAFSA \(6\) to the macromolecular fraction occurred irradiation-dependently. The covalent binding of CAFSA \(6\) was inhibited in the presence of an excess of FSA \(1\) (compare bars (a) and (b) in Fig. 10), suggesting that CAFSA \(6\) binds specifically at the FSA \(1\) binding site(s). These results suggest that CAFSA \(6\) is a suitable photoaffinity labeling agent to identify the target molecule(s) of FSA \(1\).

Further work to identify the macromolecule(s) photoaffinity-labeled with CAFSA \(6\) is under way.

**Experimental**

**Large-Scale Purification of FSA \(1\)** Fusarium sp. K432 strain was cultured in dishes each containing 30 ml of potato dextrose medium (potato 200 g, dextrose 20 g per liter of water) at 20 °C for 21 d. The acetone–benzene extract of a total of 2.4 l of culture was separated by silica gel column chromatography. The column was eluted with ethyl acetate–hexane (3 : 2). The solution was concentrated, and the residue (2.8 g) was subjected to Sephadex LH-20 column chromatography. The column was eluted with MeOH, and the residue was applied to a column of activated charcoal. The column was eluted with MeOH, and the eluate was concentrated to give FSA \(1\) as a white powder (550 mg).

**Cell Culture** HL-60 cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL) at 37 °C under a 5% CO₂ atmosphere. HUVECs were cultured in EBM-2 medium supplemented with growth factors (hEGF, VEGF, hFGF-B, and R3IGF-1, as well as FBS) at 37 °C under a 5% CO₂ atmosphere.

**Growth Inhibitory Activity against HUVEC and HL-60** Each cell was placed in 12-well plates and treated with various concentrations of the test compounds at 37 °C for 6 or 72 h. After the incubation, the viability of the treated cells was measured by direct counting under a microscope.

**HUVEC Tube Formation Assay** HUVECs were plated on Matrigel and cultured in the presence of test compounds for 6 h, then tube formation...
was measured as previously reported. Briefly, six-well plates were coated with 1.5 ml of the Matrigel basement membrane matrix (Becton Dickinson) and allowed to gel at 37 °C under a 5% CO2 atmosphere for 30 min. Then, HUVECs were plated at 5.0 × 10^4 cells/well in Dulbecco's modified Eagle medium (DMEM) containing the vehicle (0.5% DMSO) in the presence or absence of various concentrations of the test compound, and incubation was continued at 37 °C under a 5% CO2 atmosphere for 4 h. Each well was photographed using a ×100 objective to analyze tube formation. The corresponding area was evaluated as the number of pixels using MetaMorph software (Universal Imaging, Downington, PA, U.S.A.).

Curling of Mycelia of P. oryzae 2B Conidia of P. oryzae 2B-grown was suspended in water at 30 °C. After 30 min, to this was added benzyl bromide (15.0 mmol) in DMF (0.4 ml) was added NaH (55—72% contained, 2.3 mg). The reaction was quenched by adding H2O and the mixture was extracted with ethyl acetate. The product was purified by silica-gel column chromatography (hexane/ethyl acetate = 5/1, 2000 rpm for 5 min at 4 °C). The radioactivity of the resulting pellet was measured.

Preparation of [1H]FSA α-Methionine, [methyl-3H] (185 MBq) in RIPA1640 medium in the presence or absence of 3H-FSA (10 μCi/ml) for 20 h. The reaction mixture was extracted with ethyl acetate. The product was purified by silica-gel column chromatography (hexane/ethyl acetate = 20/1, 2000 rpm for 5 min at 4 °C). The radioactivity of the resulting pellet was measured.

Distribution of [1H]FSA in HL-60 Cells HL-60 cells were incubated in RPMI1640 medium in the presence or absence of [1H]FSA (10 μCi/ml) for 20 h. Treated HL-60 cells were centrifuged at 3000 rpm for 5 min at 4 °C. The mixture was centrifuged at 2500 rpm for 15 min at 4 °C. The radioactivity of the resulting pellet was measured.

Preparation of 3-tert-Butyldimethylsilyloxymethyl)-5-nitrobenzoic Acid (11) Conidia of P. oryzae (583 mg, 1.87 mmol) in water suspension containing 0.02% yeast extract and were cultured at 27 °C in water suspension for overnight. The reaction mixture was applied directly to silica-gel and purified by silica-gel column chromatography (hexane/ethyl acetate = 3/1) to give 3-tert-Butyldimethylsilyloxymethyl)-5-nitrobenzoic acid (11) (583 mg, 1.87 mmol, 96%) as a colorless oil; 1H-NMR (DMSO-d6, 500 MHz) for 3-tert-Butyldimethylsilyloxymethyl)-5-nitrobenzoic acid (11) was measured as previously reported. Briefly, six-well plates were coated with 1.5 ml of the Matrigel basement membrane matrix (Becton Dickinson) and allowed to gel at 37 °C under a 5% CO2 atmosphere for 30 min. Then, HUVECs were plated at 5.0 × 10^4 cells/well in Dulbecco's modified Eagle medium (DMEM) containing the vehicle (0.5% DMSO) in the presence or absence of various concentrations of the test compound, and incubation was continued at 37 °C under a 5% CO2 atmosphere for 4 h. Each well was photographed using a ×100 objective to analyze tube formation. The corresponding area was evaluated as the number of pixels using MetaMorph software (Universal Imaging, Downington, PA, U.S.A.).

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mp 91—93 °C. 5H-NMR (CDCl₃) δ: 0.13 (6H, s), 0.95 (9H, s), 2.83 (2H, br s), 2.97 (2H, br s), 3.38 (2H, br s), 3.78 (2H, br s), 4.83 (2H, s), 7.69 (1H, s), 8.13 (1H, s), 8.23 (1H, s). MS (FAB): m/z 380 (M+H)⁺.

1-[1-(tert-Butoxymethyl)oxymethyl]-5-nitrobenzoyl]-4-[7-diethylamino-5-nitrobenzoyl]-4-[7-diethylaminocoumarin-3-carbonyl]piperazine (14) To a solution of 7-diethylaminocoumarin-3-carboxylic acid (209 mg, 0.880 mmol) in dimethylformamide (DMF) (4.0 ml) was added 1.1-carbonyls-tri-isopropylsilane (26.5 mg, 0.080 mmol) and tri-(tert-butyl)dimethylsilyloxymethyl triethylammonium (709 mg, 0.009 mmol) and the mixture was stirred at ambient temperature for 40 min. To this was added 13 (334 mg, 0.880 mmol) and the mixture was stirred at the same temperature for 2 h. The reaction was quenched by adding H₂O and brine, and the resulting precipitate was collected by filtration, and dried in vacuo. The product was purified by silica-gel column chromatography (CH₂Cl₂/ethyl acetate 10/1) to give 1-[1-(tert-butoxymethyl)oxymethyl]-5-nitrobenzoyl]-4-[7-diethylaminocoumarin-3-carbonyl]piperazine (14) (488 mg, 0.783 mmol, 98%) as a yellow foam; 1H-NMR (CDCl₃) δ: 1.21 (6H, s), 1.28 (6H, t, J = 7.0 Hz), 3.29—3.59 (10H, br), 3.41 (4H, q, J = 7.0 Hz), 3.49 (2H, br s), 4.65 (2H, br s), 6.58 (1H, d, J = 9.0 Hz), 6.59 (1H, s), 6.72 (2H, s), 7.28 (1H, d, J = 9.0 Hz), 7.87 (1H, s). MS (FAB): m/z 623 (M+H)⁺.

1-[1-Amino-(5-hydroxymethyl)benzoyl]-4-[7-diethylaminocoumarin-3-carbonyl]piperazine (15) To a solution of 14 (203 mg, 0.326 mmol) in ethyl acetate (3.0 ml) was added SnCl₂·2H₂O (210 mg, 0.931 mmol) and the mixture was refluxed for 7 h. The reaction was quenched by adding aqueous NaHCO₃ solution, and the resulting precipitate was removed by filtration through a pad of Celite. The filtrate was evaporated in vacuo and the residue was stirred at 0 °C. After 10 min, NaN₃ (84.6 mg, 1.30 mmol) was added, and the mixture was quenched by adding NaHCO₃ powder, and the resulting precipitate was stirred for 1 h at the same temperature. To this was added a solution of HCl (5.0 ml) was added NaNO₂ (89.8 mg, 1.30 mmol) and the mixture was stirred at ambient temperature. The reaction mixture was directly applied to silica-gel column chromatography (CH₂Cl₂/acetone 10/1) then canted onto gel (Sephadex G-25 Medium) and gel filtration was carried out using Tris-buffer (20 mm Tris–HCl, 600 mM KCl, 5 mM diithiothreitol (DTT), pH 7.5) as the eluent. The eluates were collected in fractions of 3 drops (about 100 μl) and the fluorescence intensity of each fraction was measured.

References