ASELACINS, NOVEL COMPOUNDS THAT INHIBIT BINDING OF ENDOTHELIN TO ITS RECEPTOR

I. THE PRODUCING ORGANISM, FERMENTATION AND BIOLOGICAL ACTIVITY

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A radioligand test to detect inhibitors of endothelin-1 binding to its receptors in bovine atrial and porcine cerebral membranes was used to screen fungal metabolites from stationary fermentations. Inhibitory activity, observed in culture extracts of two *Acremonium* species, led to the discovery of aselacins A, B and C. Aselacin A inhibits binding to both membrane fractions with IC_{50}s of approximately 20 μg/ml.

The endothelins (ET-1, ET-2 and ET-3) are a family of 21-amino acid residue peptides. They are hormones produced by endothelial cells which have been shown to constrict arterial blood pressure and decrease cardiac output[1]. Agents that block the binding of endothelin to its natural receptors would be expected to produce beneficial effects. In fact, endothelin receptor antagonists have been the subject of research directed toward the treatment of hypertension, congestive heart failure, myocardial infarction, reperfusion injury, coronary angina, cerebral vasospasm, acute renal failure, asthma and atherosclerosis[2]. Distinct ET receptors termed ET_A (ET-1 selective) and ET_B (equally sensitive to ET-1 and ET-3) have been cloned and sequenced[3,4]. Membranes prepared from atria and cerebellum have been shown to represent the ET_A and ET_B receptor subtypes, respectively[5,6]. A screen for agents which block the binding of [^{125}I]ET-1 was established using natural receptors present in membranes prepared from bovine heart and porcine cerebellum. From this screen, we have discovered novel compounds, named the aselacins, which inhibit the binding of ET-1 to receptor subtypes ET_A and ET_B. The aselacins are different from previously described microbially-produced endothelin receptor antagonists[7~11].

The traditional method for growing microorganisms for screening of metabolites has been submerged fermentation. However, in this environment, microorganisms usually do not differentiate as they do in nature. We grew fungi in stationary fermentations, where the relatively dry atmosphere and exposure to air would encourage the formation of aerial hyphae, fruiting bodies, sclerotia and other structures. Metabolites produced during these growth stages would be available for screening. This paper describes the screen, the producing microorganisms, their fermentation and the biological activity of the isolated compounds. The isolation and the structure elucidation are presented in a companion paper[12].

Materials and Methods

The Screen

ET-1 receptors of ET_A subtype were prepared from bovine atrial membranes. Bovine hearts were

transported on ice from the abattoir. Atrial tissue was dissected free and homogenized in buffer A in a Waring blender. Buffer A consisted of 5 mM Tris, 1 mM $MgCl_2$, 250 mM sucrose, 0.5 mg/ml phosphoramidon and 0.5 mg/ml leupeptin at pH 7.4. The homogenate was centrifuged at $3,000 \times g$ for 15 minutes at 4°C to pellet the debris. The supernatant, containing the plasma membrane fraction, was centrifuged at $72,000 \times g$ for 30 minutes. The pellet was washed twice in buffer B, which consisted of 75 mM Tris, 25 mM $MgCl_2$, 0.5 mg/ml phosphoramidon and 0.5 mg/ml leupeptin at pH 7.4. The washed pellet was resuspended at 1 g tissue per ml in buffer B containing 250 mM sucrose. Aliquots were frozen immediately at −20°C. The non-specific binding of [^{125}I]endothelin-1 to crude bovine atrial membranes is $14 \pm 1\%$.

ET-1 receptors of ET_B subtype were prepared from porcine brain membranes. Porcine brains were transported on ice from the abattoir. Cerebral tissue was dissected free and homogenized with a Virtis Tempest Virtishear in buffer A. The homogenate was centrifuged at $3,000 \times g$ for 15 minutes at 4°C to pellet the debris. The supernatant, containing the plasma membrane fraction, was centrifuged at $72,000 \times g$ for 30 minutes at 4°C. The pellet was washed twice in buffer B. The washed pellet was resuspended at 1 g of tissue per ml in buffer B containing 250 mM sucrose. Aliquots were frozen immediately. The non-specific binding of crude porcine cerebral membranes is $15 \pm 1\%$.

Fungal cultures for the screen were grown for 21 days in solid state fermentations (30 ml medium plus 25 g Spoon Size Shredded Wheat in a 500-ml Erlenmeyer flask). At harvest, 25 ml of acetone was added to the flask. Six hours later, 30 ml of toluene-ethyl acetate 1:1 mixture was added and the flasks were placed at 4°C for 20 hours. Twenty-five ml of the extract was removed and the solvent evaporated. The residue was used for the radioligand assays.

To assess the ability of extracts to block [^{125}I]ET-1 binding, an aliquot of atrial or cerebral membrane preparation was thawed and diluted 1 to 20 in binding buffer, which consisted of 50 mM Tris, pH 7.4, 25 mM $MgCl_2$, 1 mM EDTA, 0.5 mg/ml phosphoramidon, 0.5 mg/ml leupeptin, 0.1% CHAPS and 0.5% bovine serum albumin. Fermentation extract samples for testing were dissolved in 95% ethanol at a 50-fold concentration of the first extraction. Five μl was transferred to wells of a 96-well microtiter tray. The ethanol was removed under vacuum and 100 μl diluted membrane was added for a two hour pre-incubation period. A 100 μl aliquot of a solution of [^{125}I]ET-1 (20,000 dpm, Amersham) in binding buffer was added to each well. The trays were incubated for an additional two hours. Membranes were collected on glass fiber filters (Packard self-aligning RG filters) with a 96-well harvester (Inotech Biosystems). The filters were washed twice with binding buffer, removed from the harvester and dried in a vacuum oven. The radioactivity associated with the membranes was determined with a Packard Matrix 9600 β-detector. Non-specific binding was defined as the radioactivity associated with membranes in the presence of 50 ng/ml of unlabeled ET-1 (Peninsula Laboratories, Belmont, U.S.A.).

Microorganisms

The producing microorganisms were isolated from soil samples collected in Asela, Ethiopia and at Cape Canaveral, U.S.A. Both cultures were morphologically recognized by light microscopy as *Acremonium* and were described by criteria suggested by ONIONS and BRADY[13]. The strains have been deposited in the Agricultural Research Service Collection at the National Center for Agricultural Utilization Research in Peoria, U.S.A. The accession number for the producer of aselacins A and B in NRRL 21047. The accession number for the producer of aselacin C is NRRL 21062.

Fermentation

Stationary fermentations (incubated on solid substrate without agitation) were used for screening fungi and for scale-up to quantities sufficient to isolate and characterize the active components. Small Shredded Wheat biscuits were used as a matrix for increasing the surface area available for fungal growth (Spoon Size Shredded Wheat, Nabisco Brands Inc., East Hanover, U.S.A.). After selection for their bioactivity from the screen, *Acremonium* sp. AB 2093T-194 and *Acremonium* sp. AB 2086L-51 were maintained as frozen vegetative inoculum at −70°C and used at 1% to inoculate seed flasks containing 100 ml of a tomato paste-oat flour medium described by GOETZ *et al.*[14]. The seed flasks were incubated on a rotary shaker (225 rpm) at 28°C for 72 hours. The fermentation was conducted in four glass 20-liter carboys. Spoon Size Shredded Wheat was dispensed at 1,200 g per carboy and the carboys were sterilized

for 45 minutes at 121°C. The medium used to produce aselacins A and B consisted of dextrin 1.6%, glucose monohydrate 0.8%, dried distillers solubles (Brown, Foreman Distillers, Louisville, U.S.A.) 0.4%, primary whole yeast 0.4% and $CaCO_3$ 0.16%. The pH was adjusted to 7.0 with NaOH. The medium for the production of aselacin C consisted of starch 2.4%, molasses 1.6%, spray-dried lard water (Inland Molasses Co., Dubuque, U.S.A.) 0.8%, primary whole yeast 0.4% and $CaCO_3$ 0.16%. The pH was adjusted to 7.0. Media were dispensed at 1,440 ml into 2-liter Erlenmeyer flasks and sterilized for 45 minutes at 121°C. The inoculation process consisted of mixing vegetative seed growth at 5% with the liquid medium. The mixture was poured onto and mixed with the Shredded Wheat, which had been pre-sterilized in the carboys. Incubation of the carboys was at 20°C for 21 days.

Biological Activity

The concentration of the isolated compounds, aselacins A and C, which inhibited 50% of the binding activity of ET-1 (IC_{50}) to each of the receptor membranes, was determined in the assay described for screening.

Results and Discussion

Taxonomy

The producer of aselacins A and B (strain AB 2093T-194) and the producer of aselacin C (strain AB 2086L-51) have similar microscopic morphology. The conidiophores are smooth and colorless, 21 ~ 30 μm long, 2.5 ~ 3.5 μm at the base tapering to 0.9 ~ 1.2 μm at the tip. Conidiophores are abundant on most media and are formed at regular intervals along the vegetative hyphae. The conidiogenous cells are holoblastic arising as terminal cells from relatively undifferentiated conidiophores. The conidia are

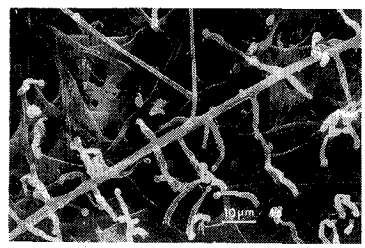
Table 1. Differential cultural characteristics of *Acremonium* strains AB 2093T-194 and AB 2086L-51.

Characteristic	AB 2093T-194	AB 2086L-51
On 2% malt extract agar		
Mycelial mat	Azonate, appressed	Zonate, appressed
Colony color	White to translucent	Light tan to translucent
Reverse	Colorless	Colorless
Soluble pigment	Absent	Absent
Colony diameter	18 mm	18 mm
On potato flakes agar		
Mycelial mat	Azonate, slightly raised	Zonate, raised
Colony color	White to translucent	White to light tan
Reverse	Light yellow tan	Tan
Soluble pigment	Absent	Light tan
Colony diameter	19 mm	16 mm

Incubation conditions: 7 days at 22°C.

Fig. 1. Scanning electron micrograph of the mycelium of *Acremonium* sp. strain AB 2093T-194 showing conidiophores spaced at regular intervals along the vegetative hyphae.

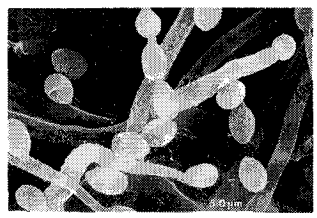
Bar represents 10 μm.



Growth was on corn meal agar with incubation for 1 week at 25°C.

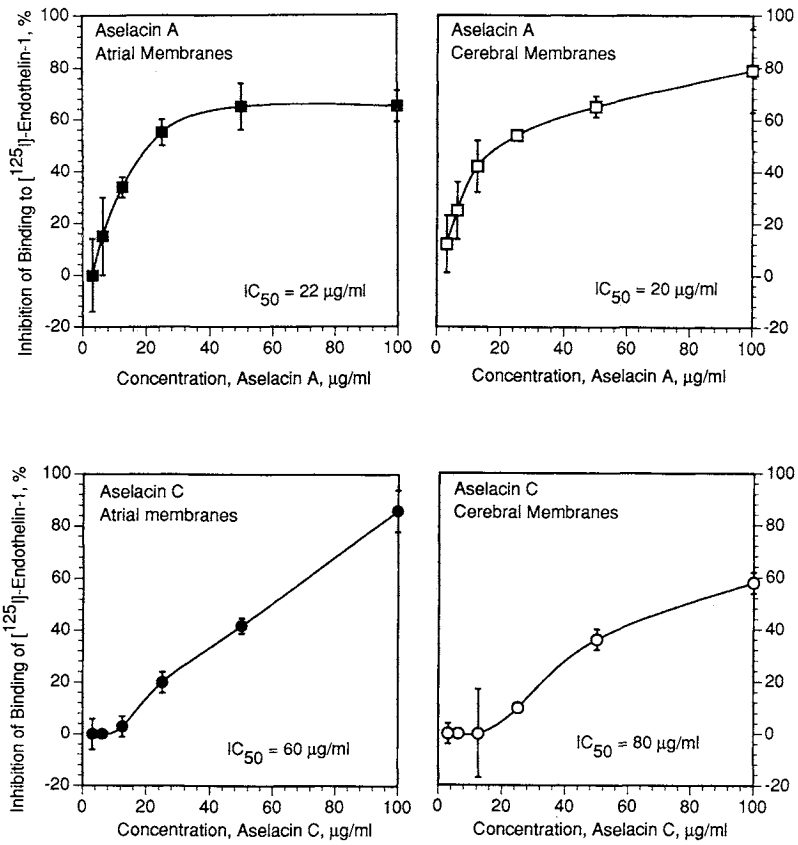
Fig. 2. Scanning electron micrograph of *Acremonium* sp. AB 2093T-194 illustrating tapered tips of the conidiophores and smooth surface of the conidia.

Bar represents 5 μm.



Growth was on corn meal agar with incubation for 1 week at 25°C.

Fig. 3. Inhibition of binding of [^{125}I]endothelin-1 to receptors by aselacins A and C.



smooth, colorless and elliptical. They typically measure $3\sim4\,\mu m \times 3.5\sim5\,\mu m$ and are produced singly, in long false chains and occasionally in loose heads. However, the two strains differ in gross colony morphology and color. These cultural differences, appearing on malt extract agar and on potato flakes agar[15], are summarized in Table 1. The microscopic morphology indicated that these strains are closely related hyphomycetous fungi belonging to the genus *Acremonium*. Scanning electron micrographs show the *Acremonium*-like morphology of strain AB 2093T-194 (Figs. 1 and 2).

Fermentation

The solid state fermentations were not monitored during incubation. Aselacin production was improved by decreasing the amount of medium and Shredded Wheat in the carboys, presumably improving aeration. It was possible to detect aselacins in liquid, agitated fermentations but attempts to optimize yields in fermenters have not been undertaken.

Biological Activity

The inhibition of binding results for aselacin A and C are shown in Fig. 3. The IC_{50} of aselacin A with atrial membranes was determined to be 22 µg/ml and 20 µg/ml with cerebral membranes. The IC_{50} of aselacin C was 60 µg/ml against atrial membranes and 80 µg/ml with cerebral membranes. There was no selectivity from aselacins A and C for receptor membranes of ET_A or ET_B subtypes. Insufficient quantity of aselacin B was isolated for IC_{50} determination, however, the compound was demonstrated to inhibit

binding during the activity-driven isolation process.

Conclusions

A radioligand assay was successfully used to identify fermentation extracts that inhibited binding of [^{125}I]ET-1 to its receptors. Purification, followed by the bioassay, led to the isolation of aselacins A, B and C. Aselacin A inhibits binding to receptors in both atrial and cerebral membranes with IC_{50}s of approximately 20 μg/ml. Stationary fermentations appear to be an excellent source of interesting fungal metabolites.

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