RES-701-1, A NOVEL AND SELECTIVE ENDOTHELIN TYPE B RECEPTOR ANTAGONIST PRODUCED BY *Streptomyces* sp. RE-701

I. CHARACTERIZATION OF PRODUCING STRAIN, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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RES-701-1, a novel cyclic peptide endothelin antagonist, was isolated from the culture broth of *Streptomyces* sp. RE-701. RES-701-1 selectively inhibited the ET-1 binding to type B endothelin receptor (ET_B receptor) with an IC_{50} of 10 nM expressed in CHO cells and blocked the ET-1-induced elevation of intracellular free Ca^{2+} concentration in ET_B receptor-expressing COS-7 cells. Characterization of producing strain, fermentation, isolation, structure, physico-chemical and biological properties of RES-701-1 are described.

Endothelins (ETs) are a family of three related peptides, endothelin 1, 2, and 3 (ET-1, ET-2 and ET-3), which have a variety of biological activities in both vascular and non-vascular tissues, including hemodynamic, cardiac, pulmonary and renal effects. Circulating levels of ET-1 have been found to be elevated in some pathophysiological conditions such as systemic hypertension, cardiac ischemia, asthmatic attacks and cyclosporin-induced renal failure. These observations suggest that specific blockage of endothelin actions at receptor level can be potential treatment of disease states caused by elevated levels of ETs[1,2].

In our microbial screening for endothelin antagonists using bovine lung membranes, we isolated a novel cyclic peptide RES-701-1 from the fermentation broth of *Streptomyces* sp. RE-701. RES-701-1 selectively inhibited ET-1 binding to type B endothelin receptor (ET_B receptor) expressed in CHO cells and blocked the ET-1-induced elevation of intracellular free Ca^{2+} concentration in ET_B receptor-expressing COS-7 cells. This paper describes the characterization and fermentation of the producing strain and the isolation, physico-chemical properties and biological activities of RES-701-1. Details of the structure determination are described in the succeeding paper[3].

Materials and Methods

Materials

(3-[^{125}I]Iodotyrosyl^{13})endothelin-1 was purchased from Amersham Japan. Endothelin-1 (ET-1) was purchased from Peptide Institute, Inc., Osaka, Japan. Bovine lung was obtained from a local slaughter-house. The human B type endothelin receptor gene was the generous gift of Dr. HIROSE (Department of Biological Science, Tokyo Institute of Technology, Tokyo, Japan.) All other chemicals were of analytical grade.

Characterization of the Producing Strain

Strain RE-701 that produced RES-701-1, endothelin antagonist, was isolated from a soil sample collected in Aichi Prefecture, Japan. For cultural and physiological characterization of the producing strain, the methods of SHIRLING and GOTTLIEB[4] were employed. The cultural and physiological characterization except a liquefaction test of gelatin were determined after incubation at 28°C for 2 weeks. Liquefaction of gelatin was determined after incubation at 20°C for 2 weeks. The temperature range for growth of the strain was determined after submerged cultivation for 1 week. For analysis of the configuration of diaminopimelic acid in whole-cell hydrolysate of strain RE-701, the method of HASEGAWA, TAKIZAWA, and TANIDA[5] was employed.

Culture and Medium Conditions

A loopful of *Streptomyces* sp. RE-701 from a mature slant was inoculated into 10 ml of the seed medium composed of glucose 1%, soluble starch 1%, Bacto-Tryptone (Difco) 0.5%, Yeast extract (Nihon-Seiyaku) 0.5%, Beef extract (Kyokuto) 0.3%, KH_2PO_4, 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.2% (pH 7.2 before sterilization) in a test tube (21 mm × 200 mm). The inoculated tube was incubated at 28°C for 4 days. A 10%-inoculation from the above seed medium was added to a 300-ml Erlenmeyer flask containing 50 ml of the same medium. After incubation for 2 days on a rotary shaker (200 rpm) at 28°C, a 10%-inoculation from the above seed medium was added to the third stage seed in a 300-ml Erlenmeyer flask containing 50 ml of the seed medium and incubated for 2 days on a rotary shaker (200 rpm) at 28°C. The 800 ml of seed culture, prepared as above, was transferred to 8 liters of the fermentation medium containing soluble starch 4%, soy bean meal 1%, corn steep liquor 0.5%, dry yeast (Asahi Brewery) 0.5%, KH_2PO_4 0.05%, HP-20 resin (Mitsubishi-Kasei) 10%, $ZnSO_4 \cdot 7H_2O$ 10 μg/ml, $CoCl_2 \cdot 6H_2O$ 1 mg/ml, $NiSO_4$ 1 mg/ml, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% (pH 7.0 before sterilization) in one hundred and eighty of 300-ml Erlenmeyer flasks. The fermentation was carried out at 28°C for 4 days on a rotary shaker (200 rpm). The production of RES-701-1 was monitored by measuring an inhibitory activity against [^{125}I]ET-1 binding to bovine lung membranes. For this measurement, the cultured broth was sampled and mixed with an equal volume of methanol. The resultant mixture was stirred vigorously and centrifuged. An aliquot (10 μl) of the supernatant was provided for the assay.

Preparation of Membranes

For preparation of bovine lung membranes, lung parenchyma were dissected and homogenized with a polytron (settings 8 for 30 seconds × 2) in 5 volumes of buffer A containing 1 mM $NaHCO_3$, 5 mM EDTA (pH 8.3), 5 μg/ml Leupeptin, 5 μg/ml Pepstatin A, and 40 μM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 8,000 × *g* for 10 minutes. The supernatants were then centrifuged at 40,000 × *g* for 1 hour at 4°C. The pellets were homogenized in buffer A and recentrifuged at 40,000 × *g* for 1 hour. The resulting pellets were homogenized in buffer A supplemented with 130 mM NaCl, 5 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 and stored at −70°C. For preparation of CHO cell membranes, transfected cells were washed with PBS and removed from the culture dish with a rubber policeman and collected by centrifugation. The resulting cell pellets were used to obtain the membrane preparation as described above.

ET-1 Receptor Assay

The reaction mixture (1 ml) containing 0.74 kBq/ml [^{125}I]ET-1, 50 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, 0.2% bovine serum albumin (BSA), 0.02% bacitracin, 14 μg of lung membrane protein or CHO cell membranes, and various concentration of samples was incubated at room temperature for 2 hours, then filtered through GF/B glass filters. The glass filters were washed three times with cold 50 mM Tris-HCl buffer containing 1 mM EDTA by a Brandel M-24R cell harvester. The radioactivity on the washed filter was measured by using a Packard γ counter. Non specific binding was measured in the presence of 0.1 μM of unlabeled ET-1.

Expression of Cloned Human type A and type B Endothelin Receptors in COS-7 and CHO Cells

Human ET_A receptor genes were obtained from human placenta mRNA by reverse transcription and PCR methods. Human ET_A and ET_B receptor genes were cloned into pcDNA I vector (Invitrogen) for transient expression in COS-7 cells or were cloned into pAGE107 whose promoter was replaced by the one of Momulv for stable expression in CHO cells as described[6]. Resulting ET_A or ET_B receptor gene

was introduced to COS-7 cells as described[7] and cultured in DULBECCO's modified EAGLE's medium supplemented with 10% fetal calf serum. After 3 days cultivations, the COS-7 cells were provided for the studies on intracellular Ca^{2+} concentration measurement. For stable expression of ET_A and ET_B receptors, the genes were introduced to CHO cells, and ET_A or ET_B receptors-expressing cells were selected as described[6]. Then, the cells were cultured in a minimal essential medium supplemented with 10% dialysed bovine calf serum, but without ribonucleosides and deoxyribonucleosides. Binding experiments were performed with membranes prepared from the transfected CHO cells.

Measurement of Intracellular Ca^{2+} Concentration

The transfected COS-7 cells were plated on a glass coverslip with a silicon rubber wall (Heraeus, Flexiperm). The culture was maintained for 3 days with the DULBECCO's modified EAGLE's medium supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 in air at 37°C. After cultivation, culture medium was removed, and the cells on the coverslip were washed at least three times with a basal salt solution (BSS: NaCl 140 mM, KCl 4 mM, $CaCl_2$ 1.25 mM, D-glucose 11 mM, $MgCl_2 \cdot 6H_2O$ 1 mM, $Na_2HPO_4 \cdot 12H_2O$ 1 mM, BSA 1 mg/ml, HEPES-NaOH 5 mM; pH was adjusted to 7.4). Fura-2/AM (10 μM) in BSS was then incubated with the cells for 60 minutes at 37°C and washed extensively with BSS. The coverslip with transfected COS-7 cells that had been loaded with fura-2 was filled with 1 ml BSS, ET-1 and/or RES-701-1 were then added to the cells. Fluorescence measurements were carried out at 37°C using an ARUGAS 2000 system (Hamamatsu Photonics). Excitation was at 340 or 380 nm, and emission intensity was measured at 510 nm. The concentration of Ca^{2+} was estimated by comparison with the fluorescence intensity ratios of Ca^{2+}-EGTA mixtures in MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer added to 10 μM fura-2 and excited at the two wavelengths.

Results

Characterization of the Producing Strain

Vegetative hyphae of strain RE-701 were well developed, branched, and did not fragment into bacillary or coccoid elements. Aerial mycelia were formed on various agar media. Spore chains were born on the aerial mycelia in spiral form with rows of 10 to 50 spores. The spores were smooth-surfaced and non-motile. The formation of sporangia or synnemata was not observed. The configuration of diaminopimelic acid in whole-cell hydrolysate of strain RE-701 was LL-form. Based on taxonomic characteristics described above, strain RE-701 is considered to belong to the genus *Streptomyces*. The cultural characteristics of the producing strain are shown in Table 1. The aerial mass color was in the

Table 1. Cultural characteristics of strain RE-701.

Medium	Substrate mycelium	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP No. 2)	Good, mustard gold (2ne)	Moderate, white (a)	None
Oatmeal agar (ISP No. 3)	Moderate, golden olive (1½lg)	Moderate, lamp black (p)	None
Inorganic salts-starch agar (ISP No. 4)	Good, mustard (2le)	Abundant, white~lamp black (a~p)	None
Glycerol-asparagine agar (ISP No. 5)	Poor, yellow tint (1ba)	Scanty, white (a)	None
Tyrosine agar (ISP No. 7)	Poor, honey gold (2ic)	Scanty, white (a)	None
Sucrose-nitrate agar	Moderate, honey gold (2ic)	Moderate, white~gray (a~f)	None
Glucose-asparagine agar	Moderate, putty (1½ec)	Moderate, white (a)	None
Nutrient agar	Moderate, light wheat (2ea)	Moderate, white (a)	None

Color names and numbers used in this table were based on Color Harmony Manual (Container Corporation of America).

Table 2. Physiological characteristics of strain RE-701.

Temperature for growth	10~40°C
Optimum temperature	25~30°C
Liquefaction of gelatin	+
Hydrolysis of starch	+
Coagulation of milk	+
Peptonization of milk	+
Formation of melanin	−
Utilization of:	
D-Glucose	+
L-Arabinose	−
D-Xylose	d
D-Fructose	+
Sucrose	+
Inositol	+
L-Rhamnose	−
Raffinose	+
D-Mannitol	+

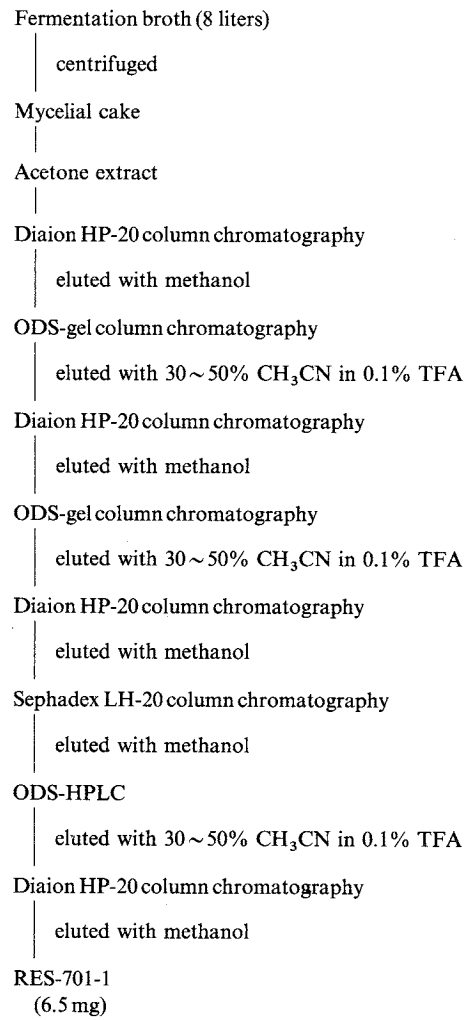
+, Positive; −, negative; d, doubtful.

grey color series. No soluble pigment was produced on tested agar media. The physiolosical characteristics of strain RE-701 are shown in Table 2. The strain has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as *Streptomyces* sp. RE-701 with the accession No. FERM-BP3624.

Isolation and Purification

The isolation procedure of RES-701-1 is outlined in Fig. 1. Fractionation was guided by inhibition of [^{125}I]ET-1 binding to bovine lung membranes. The mycelial cake was obtained by centrifugation (7,000 rpm, 20 minutes) of the fermentation broth (8 liters) and extracted with methanol (5 liters) followed by acetone (5 liters). [^{125}I]ET-1 displacing activity was mainly found in the acetone extract. The acetone extract was diluted with 5 liters of water and adsorbed on a Diaion HP-20 column (1 liter). After being washed with 25% aqueous acetone (4 liters), the active fractions were eluted with methanol (5 liters). The eluate was concentrated *in vacuo* to give a crude oily residue (2.45 g). This residue was dissolved in 100 ml of methanol and this solution was mixed with 40 ml of ODS gel. After the solvent of the slurry was evaporated, the resulting dry powder was added to the top of a ODS-gel column (260 ml) which was prepacked with 30% acetonitrile in 0.1% tifluoroacetic acid (TFA). [^{125}I]ET-1 displacing activity was eluted with a linear gradient of CH_3CN in 0.1% TFA (30~50% in 100 minutes, flow rate 9 ml/minute) and the active fractions were pooled, adjusted to pH 7, and then desalted on a Diaion HP-20 column (100 ml). This purification procedure was repeated and the desalted eluate from Diaion HP-20 column (100 ml) was concentrated and dissolved in 3.7 ml of methanol. This solution was chromatographed on a Sephadex LH-20 column equilibrated and eluted with

Fig. 1. Isolation procedure of RES-701-1.



methanol. The active fractions were pooled and purified by preparative HPLC using a YMC SH-363-10 column (ODS, 30×250 mm, YMC Co., Ltd.) with a 30~50% CH_3CN gradient elution in 0.1% TFA (0~10 minutes, from 30 to 50%, linear; 10~20 minutes at 50%; flow rate 20 ml/minute). The retention time of RES-701-1 was 16.3 minutes. The active eluate was diluted with water, adjusted to pH7 and then desalted on a HP-20 column (40 ml). The evaporation of the solvent gave 6.5 mg of RES-701-1 as a white powder.

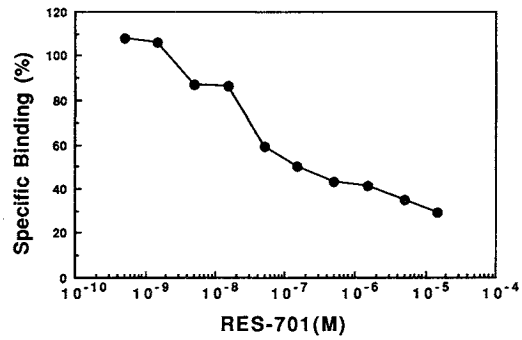
Physico-chemical Properties of RES-701-1

Physico-chemical properties of RES-701-1 are summarized in Table 3. RES-701-1 is readily soluble in methanol, acetonitrile, and dimethyl sulfoxide, and virtually insoluble in chloroform, acetone, and water. The molcular formula of RES-701-1 was determined to be $C_{103}H_{115}N_{23}O_{23}$ on the basis of HRFAB-MS. The structure of RES-701-1 was determined as shown in Fig. 2 on the basis of HRFAB-MS, peptide sequencing, and amino acid analysis. Details of structural elucidation studies are described in the succeeding paper[3].

Table 3. Physico-chemical properties of RES-701-1.

Appearance	Colorless powder
UV λ_{max} nm (MeOH)	221, 280
IR (KBr) cm^{-1}	3375, 2925, 1660, 1515, 1455, 1440, 1405, 1340, 1235, 1105
Molecular formula	$C_{103}H_{115}N_{23}O_{23}$
HRFAB-MS	
Calcd $(M+H)^+$	2,042.8614
Found $(M+H)^+$	2,042.8722
Rf value on TLC	0.4 Plate; Silica gel F_{254} (Merck, Art. No. 5629) Solvent; $CHCl_3$-MeOH-EtOH-H_2O (10:4:4:2) 0.3 Plate: RP-18 (Merck, Art. No. 13724) Solvent; 80% MeOH

Fig. 3. Effect of RES-701-1 on the specific binding of [^{125}I]ET-1 to bovine lung membranes.



Biological Properties

Fig. 3 shows an inhibitory effect of RES-701-1 on [^{125}I]ET-1 binding to bovine lung membranes. RES-701-1 inhibited [^{125}I]ET-1 binding in a dose-dependent manner with and IC_{50} of 0.15 μM. However, even at 15 μM, 100 times higher concentra-

Fig. 2. The structure of RES-701-1.

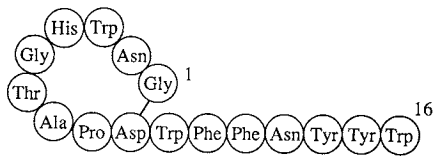


Table 4. Effect of RES-701-1 on [^{125}I]ET-1 binding to two distinct subtypes of endothelin receptor stably expressed in CHO cells.

Endothelin receptor subtype	IC_{50} (nM)
Human ET_A	>5,000
Human ET_B	10

Table 5. Effects of RES-701-1 on intracellular Ca^{2+} concentration ($[Ca^{2+}]^i$) induced by ET-1 in ET_B receptor expressing COS-7 cells.

RES-701-1 (μM)	$[Ca^{2+}]^i$ (nM)
0	61.7±5.58
0.49	24.8±3.64
4.9	3.67±1.08

tion than the IC_{50} value, RES-701-1 could not displace completely the specific binding of [^{125}I]ET-1, suggesting that RES-701-1 selectively recognized type A or type Bendothelin receptor in bovine lung membranes. Table 4 shows an inhibition of [^{125}I]ET-1 binding to cloned human type A and type B endothelin receptors expressed in CHO cells. RES-701-1 inhibited [^{125}I]ET-1 binding to type B receptor (ET_B) with an IC_{50} of 10 nM, but had no effect on [^{125}I]ET-1 binding to type A receptor (ET_A) even at 5 μM. Table 5 summarizes the effects of RES-701-1 on ET-1-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]^i$) in ET_B expressing COS-7 cells. At 4.9 μM, RES-701-1 completely abolished the elevation of $[Ca^{2+}]^i$ caused by 1 nM of ET-1. Taken together, these results indicate that RES-701-1, a novel cyclic peptide of microbial origin, is a selective antagonist for type B endothelin receptor.

Discussion

The recognition of the potential therapeutic benefit of controlling endogeneous endothelin activity has led to a search for endothelin antagonists by a random screening approach. Recently, several endothelin receptor antagonists have been found in microbial metabolites or plant extracts, namely, type A selective antagonist BE-18257B[8], 50-235[9], WS-7338[10] and nonselective antagonist cochinmicins[11]. Among these antagonists obtained from natural products, RES-701-1 is the first compound that selectively recognizes type B endothelin receptor.

Although two human subtypes of endothelin receptor have been cloned[7,12] and their differential mRNA distribution have been studied[13], there remains to be elucidated as to which subtype is responsible for the effects of endothelin in a given condition. RES-701-1, type B selective endothelin receptor antagonist, will be important tool for elucidating the relative contribution of two distinct subtypes of endothelin receptor in various physiological or pathophysiological conditions.

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