RES-701-2, -3 and -4, Novel and Selective Endothelin Type B
Receptor Antagonists Produced by *Streptomyces* sp.

I. Taxonomy of Producing Strains, Fermentation,
Isolation, and Biochemical Properties

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RES-701-2, -3 and -4, novel cyclic peptide endothelin antagonists, were isolated from the culture broths of *Streptomyces* sp. RE-701 and RE-896. RES-701s selectively inhibited the ET-1 binding to endothelin type B receptor (ET_B receptor) with IC_{50} values ranging from 5 to 20 nM. Taxonomy of the producing strains, fermentation, isolation and biochemical properties of RES-701s are described.

Endothelins (ETs), which consist of 21 amino acid residues, are a family of potent vasoactive peptides termed endothelin -1, -2, and -3 (ET-1, ET-2 and ET-3)[1]. ETs induce numerous biological responses in both vascular and non-vascular tissues by binding to at least two distinct receptor subtypes, ET_A and ET_B. The ET_A receptor shows a high affinity for ET-1 and mediates vasoconstriction, whereas the ET_B receptor is non-selective for isopeptides and mediates vasoconstriction as well as vasodilatation[2,3].

In the course of screening of endothelin antagonists, we have recently isolated a novel cyclic peptide RES-701-1 from the fermentation broth of *Streptomyces* sp. RE-701[4,5]. RES-701-1 selectively inhibited ET-1 binding to ET_B receptor and blocked ET_B receptor-mediated responces[6]. In the present investigation, we have found that many strains of *Streptomyces* produce RES-701-1-related compounds and isolated three novel compounds, designated RES-701-2, RES-701-3 and RES-701-4 from the culture broths. In this paper, we describe taxonomy of the producing strains, fermentation, isolation and biological properties of RES-701-2, -3 and -4. Studies on structural determination are described in the succeeding paper.

Materials and Methods

Materials

(3-[^{125}I]iodotyrosyl^{13})Endothelin-1 was purchased from Du Pont-New England Nuclear. Other radioligands used for binding assays were purchased from Du Pont-New England Nuclear and Amersham. Endothelin-1 (ET-1) was purchased from Peptide Institute, Inc.,

Osaka, Japan. BQ-123 was purchased from American Peptide Co., Santa Clara, CA. Fura-2/AM, calcium fluorescence indicator, was purchased from Dojin Chemical Institute, Inc., Kumamoto, Japan. Bovine cerebellum and lung tissues were obtained from a local slaughterhouse. All other chemicals were of analytical grade.

Microorganism

The producing organisms, *Streptomyces* sp. RE-896 and RE-701[4], were isolated from soils collected in Aichi Prefecture, Japan.

Method for Taxonomical Characterization

Cultural and physiological characteristics of strains RE701[4] and RE896 were determined by the methods of the International *Streptomyces* Project[7] (ISP). Color codes were assigned to the pigments of substrate and aerial mycelium according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago). Morphology of the strains was ascertained by light microscopy and electron microscopy with a HITACHI S-570 scanning electron microscope. The temperature ranges for growth of the two strains were determined after submerged cultivation with ISP No. 5 medium for 7 days. The chemotypes of the cell walls after whole-cell hydrolysates were determined as described previously[8].

Culture and Medium Conditions

A loopful of cells from the mature slant was inoculated into eight 250-ml Erlenmeyer flasks containing 40 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% and $CaCO_3$ 0.2% (pH 7.2 before sterilization). The inoculated flasks were

incubated at 28°C for 4 days on a rotary shaker (200 rpm). Forty ml of the above seed culture were added to a 2-liter Erlenmeyer flask containing 400 ml of the same medium. Eight inoculated flasks were incubated for 2 days on a rotary shaker (200 rpm) at 28°C. Three liters of seed culture, prepared as above, was transferred to 100 liters of the same medium in a 200-liter fermentor. The fermentor was operated for 2 days at 28°C with agitation at 200 rpm and aeration of 60 liters per minute. Whole broth of the 200-liter fermentor was transferred into a 2,000-liter fermentor containing 1,000 liters of a fermentation medium composed of soluble starch 4%, soy bean meal 1%, corn steep liquor 0.5%, dry yeast (Asahi brewery) 0.5%, KH_2PO_4 0.05%, $ZnSO_4 \cdot 7H_2O$ 10 μg/ml, $CoCl_2 \cdot 6H_2O$ 1 μg/ml, $NiSO_4$ 1 μg/ml and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% (pH 7.0 before sterilization). The fermentation was carried out for 4 days at 28°C with agitation of 120 rpm and aeration of 400 liters per minute.

Analysis of RES-701s Production by HPLC

Strain RE-701 or the other producing strain was cultured in a 300-ml Erlenmeyer flask containing 50 ml of the same medium used in the 2,000-liter fermentor. One hundred ml of cultured broth was mixed with 60 ml of n-propanol, agitated, and filtered. The filtrate was diluted with 150 ml of water, and adsorbed on Diaion HP-20 column (20 ml). The column was washed with 55% aqueous methanol and then eluted with 85% aqueous methanol. The eluate was concentrated to dryness under reduced pressure. The dried residue was dissolved in 1 ml of methanol. Ten μl of each extract were analyzed by HPLC. HPLC analysis was performed on a YMC-pak, Protein-RP (4.6 mm i.d. × 250 mm, YMC Co., Ltd.). The column was eluted with a gradient from 30% CH_3CN containing 0.1% trifluoroacetic acid to 40% CH_3CN containing 0.1% trifluoroacetic acid over a period of 25 minutes, at a flow rate of 1.0 ml per minute. The effluent was monitored using a photodiode array detector (Waters 955J) throughout the wavelengths from 200 nm to 350 nm. The retention times of RES-701-1, -2, -3 and -4 were 17, 14, 16 and 13 minutes, respectively.

Receptor Binding Assay

ET-1 binding assays were performed as described previously[6]. Briefly, bovine cerebellum membranes were used as a source of the ET_B receptor. Bovine lung membranes, which express both ET_A and ET_B receptors, were used as a source of the ET_A receptor in the presence of 5 μM RES-701-1 (ET_B selective antagonist). The reaction mixtures (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 14 μg of cerebellum membrane protein, and various concentration of samples were incubated at room temperature for 2 hours and then filtered through GF/B glass filters. The glass filters were washed three times with cold 50 mM Tris-HCl buffer (pH

7.6) containing 1 mM EDTA, using a Brandel M-24R cell harvester. The radioactivity on the washed filters was measured by a Packard γ counter. Nonspecific binding was measured in the presence of 0.1 μM unlabeled ET-1.

$[^3H]WB4101$, $[^3H]$clonidine, $[^3H]$dihydroalprenol, $[^3H]SCH23390$, $[^3H]$spiperone, $[^3H]$pyrilamine, $[^3H]$tiotidine, $[^3H]$quinuclidinyl benzilate, $[^3H]$8-hydroxy-2-(di-n-propylamino)tetralin, $[^3H]$ketanserin, and $[^3H]$bradykinin binding to adrenaline α_1, α_2, β_1, dopamine D_1 and D_2, histamine H_1 and H_2, acetylcholine M_1, serotonin 5-HT_{1A} and 5-HT_2, and bradykinin B_2 receptors, respectively, were measured as described[9~18]. ^{125}I-Atrial natriuretic peptide and ^{125}I-Angiotensin II binding was performed by the methods described previously[6,19].

Measurement of Intracellular Ca^{2+} Concentration

Expression of cloned human ET_A and ET_B receptors in COS-7 cells was performed as described[6]. 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 DULBECCO's modified EAGLE's medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 in air at 37°C. After cultivation, the 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 adjusted to 7.4). Fura-2/AM (10 μM) in BSS was then incubated with the cells for 60 minutes at 37°C and the cells were then washed extensively with BSS. The coverslip with transfected COS-7 cells that had been loaded with fura-2 was filled with 1 ml BSS and ET-1 and / or RES-701-1 added. 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

Detection of RES-701-2, 3 and 4

During the HPLC analysis of the culture broth extracts which inhibit the binding of ET-1 to ET_B receptor, we found the presence of three novel compounds which have similar UV spectra to that of RES-701-1 (Figs. 1, 2). One was in the broth of strain RE-701 and we termed it RES-701-2; the other two were from strain RE-896 and we termed them RES-701-3 and RES-701-4.

Fig. 1. HPLC profile of an extract from strain RE-701.

A, UV spectra of peak 1 (RES-701-1); B, UV spectra of peak 2 (RES-701-2); C, chromatogram of the extract at 220 nm.
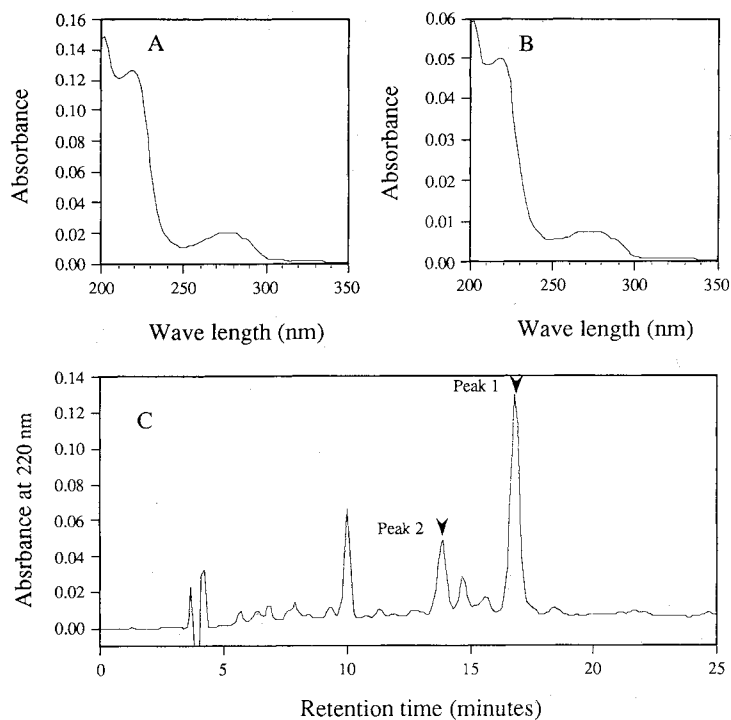
The extraction of culture broth was performed as described in materials and methods. 10 μl of extract was analyzed by HPLC.

Fig. 2. HPLC profile of an extract from strain RE-896.

A, UV spectra of peak 1 (RES-701-3); B, UV spectra of peak 2 (RES-701-4); C, chromatogram of the extract at 220 nm.
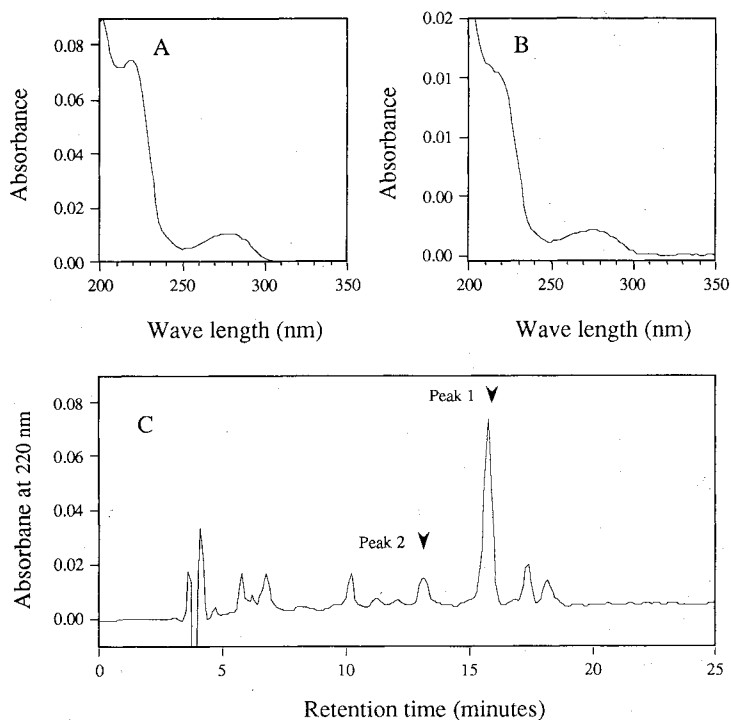
The extraction of culture broth was performed as described in materials and methods. 10 μl of extract was analyzed by HPLC.
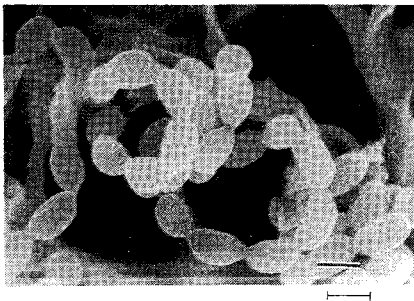
Table 1. Cultural characteristics of strain RE-896.

Medium	Amount of growth	Color of:		
		Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP No.2)	good	white(a)	yellow maple(3le)	brown
Oatmeal agar (ISP No.3)	poor	beige brown(3ig)	Lt mustard tan(2ie)	brown
Inorganic salt-starch agar (ISP No.4)	moderate	white(a)	Lt mustard tan(2ie)	brown
Glycerol-asparagine agar (ISP No.5)	good	white(a)	dusty yellow (1.1/2gc)	light brown
Tyrosine agar (ISP No.7)	good	white(a)	camel(3ie)	light brown
Sucrose-nitrate agar	good	gray(f)	oatmeal sand(2ec)	none
Glucose-asparagine agar	good	white(a)	LT wheat (2ea)	none
Nutrient agar	moderate	white(a)	LT wheat (2ea)	none

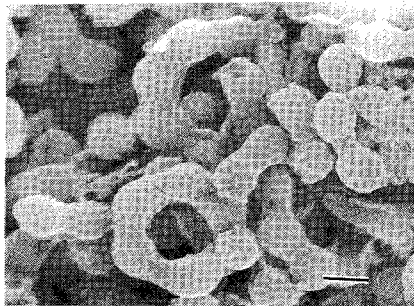Fig. 3.   Scanning electron micrograph of strain RE-701 and strain RE-896.

A, strain RE-701; B, strain RE-896. Bar represents 0.75 μm.

A

B

Table 2.   Physiological properties of strain RE-896.

Characteristics	RE-896
Temperature for growth	15 ~ 45°C
Optimum temperature	25 ~ 30°C
Liquefaction of gelatin	negative
Hydrolysis of starch	positive
Coagulation of milk	negative
Peptonization of milk	positive
Formation of melanin	negative
Utilization of	
D-Glucose	positive
L-Arabinose	doubtful
D-Xylose	positive
D-Fructose	positive
L-Rhamnose	negative
Sucrose	positive
Raffinose	positive
D-mannitol	positive
Inositol	positive

Characterization of the Producing Microorganisms

Strain RE-701[4] grew well or moderately on various ISP agar media but growth was poor on media containing glycerol as carbon source and tyrosine as nitrogen source, whereas strain RE-896 grew well on various ISP media except ISP No. 3 medium. The color of the aerial myceria of both strains ranged from white to gray or lamp black on tested agar media. Strain RE-896 produced a brown or light brown soluble pigment during cultivation on various ISP media after 14 days, whereas strain RE-701 did not form soluble pigment (Table 1)[4]. The aerial mycelium of these strains was moderately short and simple branches arranged in spiral chains of 10 or more

ellipsoidal spores of 0.5 to 0.6 mm by 0.7 to 0.9 mm. The surface of the spores of both strains was smooth (Fig. 3)[4]. No coccoid and/or bacillary fragments produced by division of substrate mycelium were observed in cultures of either strain. No fragmentation of substrate mycelium was observed, and no sclerotia, sporangia, or flagellated spores were formed. The physiological characteristics of strain RE-896 is shown in Table 2. Analysis of whole-cell hydrolysates of these strains revealed that their cell walls contained LL-diaminopimelic acid. These taxonomic observations indicate that strain RE-896 belongs to the genus *Streptomyces*.
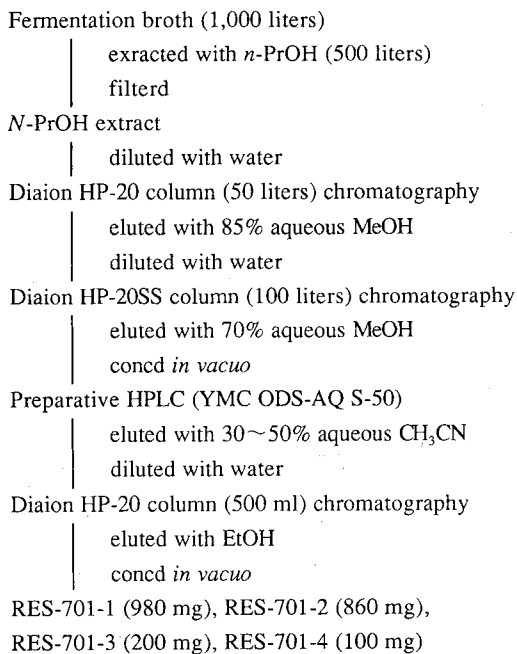
Isolation and Purification

The isolation procedure of RES-701s is schematically shown in Fig. 4. Strain RE-701 and RE-896 were cultured

according to the method described in materials and methods. Both of cultured broth were extracted with n-propanol and filtered with the aid of diatomaceous earth. The filtrates were diluted with 2000 liters of water and adsorbed on a Diaion HP-20 column (50 liters). After washing with 55% aqueous methanol (200 liters), the active principles were eluted with 85% aqueous

methanol (200 liters). The active fractions were combined and diluted with water. This aqueous solution was applied to a column containing 100 liters of Diaion HP-20SS. The column was eluted stepwise from 60% to 70% aqueous methanol. In the case of strain RE-701, RES-701-2 was eluted before RES-701-1. In the case of strain RE-896, RES-701-4 was eluted before RES-701-3. Each active fraction was concentrated in vacuo to give crude materials. These residues were dissolved in 1 liter of 25% aqueous CH_3CN and were purified by preparative HPLC using a column (ODS AQ-S50, 150 mm i.d. × 500 mm, YMC Co., Ltd.) with a CH_3CN gradient elution (from 0 to 10 minutes at 30%; from 10 to 90 minutes from 30 to 50%, linear; from 90 to 120 minutes at 50%, flow rate 300 ml per minute). The active eluates were diluted with water, and then adsorbed on an HP-20 column (200 ml). The active fractions were eluted with

Fig. 4. Isolation procedure of RES-701s.

Fermentation broth (1,000 liters)
  | exracted with n-PrOH (500 liters)
  | filterd
N-PrOH extract
  | diluted with water
Diaion HP-20 column (50 liters) chromatography
  | eluted with 85% aqueous MeOH
  | diluted with water
Diaion HP-20SS column (100 liters) chromatography
  | eluted with 70% aqueous MeOH
  | concd in vacuo
Preparative HPLC (YMC ODS-AQ S-50)
  | eluted with 30～50% aqueous CH_3CN
  | diluted with water
Diaion HP-20 column (500 ml) chromatography
  | eluted with EtOH
  | concd in vacuo
RES-701-1 (980 mg), RES-701-2 (860 mg),
RES-701-3 (200 mg), RES-701-4 (100 mg)

Fermentations of strain RE-701 and strain RE-896 were performed as discribed in materials and methods. RES-701-1 and RES-701-2 were isolated from broth of strain RE-701. RES-701-3 and RES-701-4 were isolated from broth of strain RE-896.

Fig. 5. Structures of RES-701s.
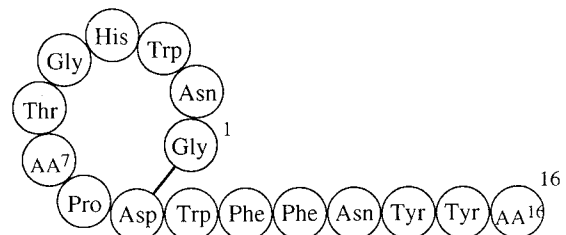
RES-701-1 : AA[7] = Ala, AA[16] = tryptophan
RES-701-2 : AA[7] = Ala, AA[16] = 7-hydroxytryptophan
RES-701-3 : AA[7] = Ser, AA[16] = tryptophan
RES-701-4 : AA[7] = Ser, AA[16] = 7-hydroxytryptophan

Fig. 6.   Inhibition of ^{125}I-ET-1 binding to ET_A(A) or ET_B(B) receptor by RES-701s and BQ-123.

Peptide (logM)   Peptide (logM)
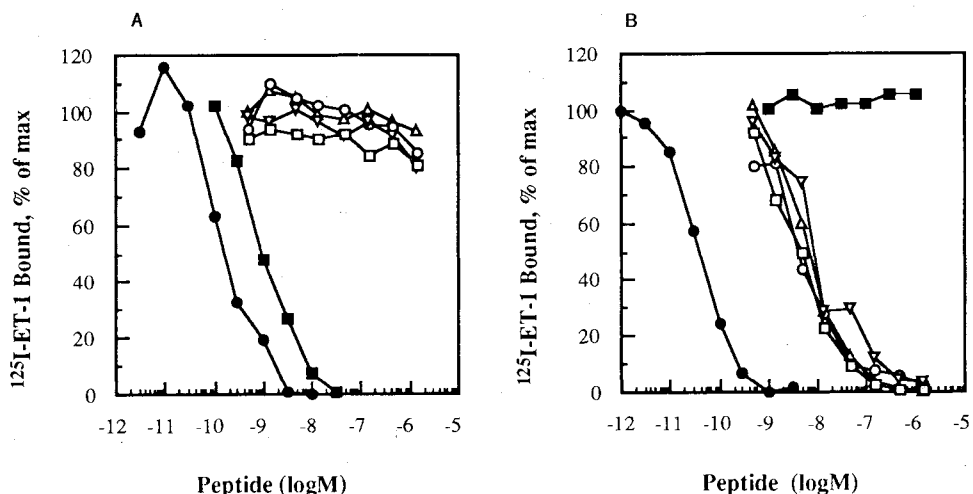
RES-701s and BQ-123 were dissolved in dimethylsulfoxide at various concentrations, and 10 μl were added to the reaction mixture. A, Specific binding of ^{125}I-ET-1 to ET_A receptor; B, specific binding of ^{125}I-ET-1 to ET_B receptor. One of the following unlabeled ligands was added to the reaction mixture: RES-701-1 (□), RES-701-2 (▽), RES-701-3 (○), RES-701-4 (△), BQ-123 (■), ET-1 (●). All experiments were performed in duplicate.

Table 3.   Comparison of the receptor specificity of RES-701s binding assays using various tissues.

Receptor	Subtype	Radioligand	Receptor source	IC$_{50}$ value (nM)			
				RES-701-1	RES-701-2	RES-701-3	RES-701-4
ET	ET$_A$	^{125}I-ET-1	Bovine lung	>1000	>1000	>1000	>1000
	ET$_B$	^{125}I-ET-1	Bovine cerebellum	5	22	3.5	8
Adrenaline	α_1	[3H]WB4101	Rat forebrain	>1000	>1000	>1000	>1000
	α_2	[3H]Clonidine	Rat cerebral cortex	>1000	>1000	>1000	>1000
	β_1	[3H]Dihydroalprenol	Calf cerebral cortex	>1000	>1000	>1000	>1000
Dopamine	D$_1$	[3H]SCH23390	Rat striatum	>1000	>1000	>1000	>1000
	D$_2$	[3H]Spiperone	Rat striatum	>1000	>1000	>1000	>1000
Histamine	H$_1$	[3H]Pyrilamine	Guinea pig cerebellum	>1000	>1000	>1000	>1000
	H$_2$	[3H]Tiotidine	Guinea pig cerebral cortex	>1000	>1000	>1000	>1000
Acetylcholine	M$_1$	[3H]QNB	Rat cerebral cortex	>1000	>1000	>1000	>1000
Serotonin	5-HT$_1$	[3H]8-OH-DPAT	Rat hipocampus	>1000	>1000	>1000	>1000
	5-HT$_2$	[3H]Ketanserin	Rat prefrontal cortex	>1000	>1000	>1000	>1000
ANP	NPR-A or B	^{125}I-rANP	Rabbit kidney cortex	>1000	>1000	>1000	>1000
Angiotensin II	AT$_1$	^{125}I-Angiotensin II	Bovine adrenal cortex	>1000	>1000	>1000	>1000
Bradykinin	BK$_2$	[3H]Bradykinin	Guinea pig ileum	>1000	>1000	>1000	>1000

Table 4.   Inhibition by RES-701s of 0.1 nM ET-1-induced [Ca^{2+}]$_i$ increase in ET$_B$ receptor expressing cells.

Compounds	Δ [Ca^{2+}]i (% of control)
RES-701-1	40.5 ± 5.5
RES-701-2	82.0 ± 5.6
RES-701-3	34.7 ± 4.8
RES-701-4	45.1 ± 5.4

Intracellular Ca^{2+} concentration was measured in the absence (control) or presence of one of RES-701s (1 μM). The values are means ± standard error of determinations on 16~42 cells.

ethanol and then evaporated to dryness. RES-701-1 (980 mg) and RES-701-2 (860 mg) were obtained as white powders from the broth of strain RE-701. RES-701-3 (200 mg) and RES-701-4 (100 mg) were also obtained as white powders from the broth of strain RE-896.

Structures of RES-701s

The structures of RES-701-2, -3 and -4 were determined as shown in Fig. 5 on the basis of high resolution fast atom bombardment-MS, peptide sequencing, and amino acid analysis. All of the RES-701s have an amide bound between the α-amino group of the amino-terminal Gly[1] and the β-carboxyl group of Asp[9]. Details of the structural elucidation studies are described in the next paper.

Biological Properties

RES-701s inhibited ^{125}I-ET-1 binding to ET$_B$ receptor of bovine cerebellum membranes in a dose-dependent manner (Fig. 6). A rank order of the potency was RES-701-3 > 1 > 2 > 4. On the other hand, RES-701s did not inhibit ^{125}I-ET-1 binding to ET$_A$ receptor of bovine lung membranes at concentrations up to 1 μM. IC$_{50}$ values of RES-701s in receptor binding assays are summarized in Table 3. RES-701s did not affect the binding of ligands to adrenaline α_1, α_2, β_1, dopamine D$_1$ and D$_2$, histamine H$_1$ and H$_2$, acetylcholine M$_1$, serotonin 5-HT$_{1A}$ and 5-HT$_2$, and bradykinin B$_2$ receptors at concentrations up to 1 μM. In order to test whether RES-701s were functional antagonists of the ET$_B$ receptor, an inhibitory effects on the increase in intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) elicited by 1 nM ET-1 in COS-7 cells expressing human ET$_A$ or ET$_B$ receptors were examined. RES-701s blocked the ET-1-induced elevation of [Ca^{2+}]$_i$ in human ET$_B$ receptor expressing COS-7 cells at concentrations of 1 μM (Table 4), they did not inhibit the [Ca^{2+}]$_i$ increase in human ET$_A$ receptor expressing COS-7 cells (data not shown). Taken together, these results indicate that RES-701s are selective antagonists of the ET$_B$ receptor.

Discussion

In the present study, we isolated three novel RES-701-1-related peptides from the culture broths of Streptomyces strains RE-701 and RE-896, termed RES-701-2, -3 and -4. When Trp[16] of RES-701-1, that is in the chain region of the peptide was replaced by 7-hydroxytryptophan (RES-701-2), the affinity for the ET$_B$

receptor was decreased. By contrast, substitution of Ala[7] of the ring region of RES-701-1 with Ser (RES-701-3) increased the affinity for the ET_B receptor. However, these substitutions did not affect their affinity for other receptors. These results indicate that both the ring and chain regions of peptides are important for binding to the ET_B receptor.

In this paper, we demonstrated that RES-701-1, -2, -3, and -4 have ET_B receptor-selective antagonistic activities. Recently, several compounds that selectively recognize the ET_B receptor have been reported; IRL-1038[20] is an ET-related peptide, BQ-788[21] is a derivative of BQ-123[22], which is a peptidic ET_A antagonist, and haloemodin[23] is a fungal metabolite. The structures of RES-701s are quite different from the other ET_B receptor antagonists.

We found that many Streptomyces species can produce RES-701-related compounds (data not shown). All of the amino acid residues of RES-701s have the L-configuration of the natural type, except for the 7-hydroxytryptophan. Probably, RES-701s are initially synthesized by translation of mRNAs as linear peptides, and then emzymaticlly cyclyzed.

Recently two types of ET_B receptors were reported, one is located on the smooth muscle and mediates direct vasoconstriction (ET_{B2}), the other is located on the vascular endothelium and mediates vasodilatation through the release of nitric oxide (ET_{B1})[24]. RES-701-1 inhibits the ET-3-induced relaxation in the rat aorta and guinea pig ileum[25]. These results indicate that RES-701-1 binds to the ET_{B1} receptor and blocks release of the vasodilater. Selectivity of RES-701-2, -3 and -4 between ET_{B1} and ET_{B2} will be classified by further pharmacological characterizations. In any case, RES-701s would be useful tools to elucidate the biological functions of ET_B receptors.

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