RES-1149-1 and -2, Novel Non-peptidic Endothelin Type B Receptor Antagonists Produced by *Aspergillus* sp.

III. Biochemical Properties of RES-1149-1, -2 and Structure-activity Relationships

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RES-1149-1 and -2, produced by *Aspergillus* sp. RE-1149, were found to be non-peptidic antagonists for endothelin type B receptor (ET_B receptor). RES-1149-1 and -2 selectively inhibited the endothelin-1 (ET-1) binding to ET_B receptor in a competitive manner with IC_{50} values of 1.5 μM and 20 μM, respectively. RES-1149-1 inhibited the increase in intracellular Ca^{2+} concentration elicited by 1 nM ET-1 in COS-7 cells expressing human ET_B receptor, but not in the case of cells expressing ET_A receptor. In addition, some structure-activity relationships are described.

ET-1 was originally isolated from the culture supernatant of porcine aortic endothelial cells[1]. After its discovery, two other isopeptides, ET-2 and ET-3, were identified by analysis of a human genomic library[2]. These peptides are widely distributed in many tissues and mediate numerous biological responses. The circulating levels of ET are increased in essential hypertension, pulmonary hypertension, acute myocardial infarction, renal failure, subarachnoid haemorhage, sepsis, and other conditions (reviewed in ref. 3). Therefore, ET may play an important role in the pathophysiology of these diseases and an ET antagonist could be useful in treatment of these diseases.

In the preceding paper, we described the isolation of RES-1149-1 and -2, novel sesquiterpenoids from the cultured broth of a fungus[4]. In this article, we report on the biochemical properties of RES-1149-1 and -2 and their structure-activity relationships.

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. RES-701-1 was purified from the cultured broth of *Streptomyces* sp. RE-701 in our laboratories[5]. Bovine and porcine tissues were obtained from a local slaughterhouse. Male Sprague-Dawley rats and male New Zealand white rabbits were obtained from SLC, Shizuoka, Japan. All other chemicals were of analytical grade.

Receptor Binding Assay

ET-1 binding assays were performed as described previously[6,7]. Briefly, bovine cerebellum membranes were used as a source of ET_B receptor. Bovine lung membranes, which express both ET_A and ET_B receptors, were used as a source of 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 washed filters was measured by a Packard γ counter. Nonspecific binding was measured in the presence of 0.1 μM unlabeled ET-1.

Binding assays with [^{3}H]bradykinin, ^{125}I-atrial natriuretic peptide and ^{125}I-angiotensin II binding were performed according to the methods previously described[6,8].

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 containing ET-1 and/or RES-1149-1. 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.

Synthesis of Derivatives

The synthesis of cinnamodial, KT-7619, KT-7624, KT-7766, KT-7913, KT-7914, KT-7915, and KT-7932 are discribed in the preceeding paper[9].

Results

Selectivities Based on Binding Assays

ET_A and ET_B receptor binding experiments using bovine tissue membranes were carried out (Fig. 1). RES-1149-1 and -2 inhibited the ^{125}I-ET-1 binding to ET_B receptor of cerebellum in a dose-dependent manner (Fig. 1A); IC_{50} values were calculated to be 1.5 μM and 20 μM, respectively. On the other hand, inhibitory effects of RES-1149-1 and -2 on 125I-ET-1 binding to ET_A receptor of lung membrane were weak (Fig. 1B); IC_{50} values for ET_A receptor were up to 15-fold lower than those determined for ET_B receptor.

In order to examine the selectivities of RES-1149-1 and -2, binding with various peptide ligand receptors was performed. IC_{50} values of RES-1149-1 and -2 in these assays are summarized in Table 1. RES-1149-1 and -2 have low or undetectable affinities for atrial natriuetic peptide, angiotensin II, and bradykinin receptors.

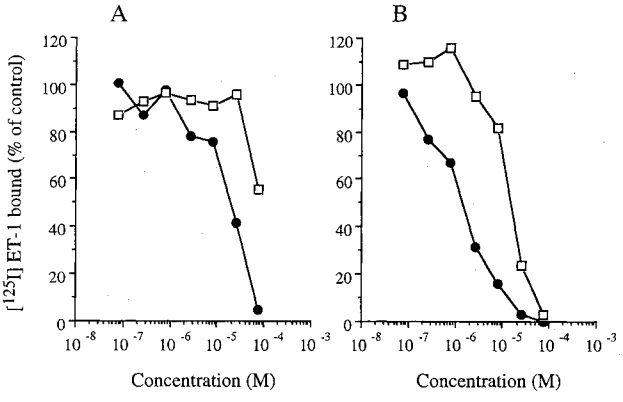
Scatchard analysis of ^{125}I-ET-1 binding to ET_B receptor of bovine cerebellum membranes was performed (Fig. 2). The plots in the absence or presence of 1 μM or 2 μM RES-1149-1 showed a reversible inhibition. *Ki* value of RES-1149-1 was calculated to be 0.8 μM.

Effect of RES-1149-1 on Intracellular Ca^{2+} Increase Induced by ET-1

In order to confirm the antagonistic property, effects of RES-1149-1 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 receptor were measured. The RES-1149-1 inhibited the ET-1-induced increase in $[Ca^{2+}]_i$ in ET_B receptor-expressing COS-7 cells, whereas it did not inhibit the $[Ca^{2+}]_i$ increase in

Fig. 1. Competition of ^{125}I-ET-1 binding to bovine ET_A (A) and ET_B (B) receptors by non-peptide antagonists, RES-1149-1 and RES-1149-2.

RES-1149-1 (●) and -2 (□) were dissolved in dimethylsulfoxide at various concentrations, and 10 μl were added to the reaction mixture.



All experiments were performed in duplicate.

Table 1. Receptor specificity of RES-1149-1 and -2 in binding assays with various tissues.

Receptor	Subtype	Radioligand	Receptor source	IC_{50} value (μM)	
				RES-1149-1	RES-1149-2
ET	ET_A	^{125}I-ET-1	Bovine lung	25.8	>80
	ET_B	^{125}I-ET-1	Bovine cerebellum	1.55	20.0
ANP	NPR-A or B	^{125}I-rANP	Rabbit kidney cortex	>80	>80
Angiotensin II	AT_1	^{125}I-Angiotensin II	Bovine adrenal cortex	23.3	25.8
Bradykinin	BK_2	[^{3}H]Bradykinin	Guinea pig ileum	>80	>80

Fig. 2. Scatchard analysis of spcific binding of ^{125}I-ET-1 to bovine cerebellum membrane in the absence of RES-1149-1 (○) or presence of 1 μM (□) or 2 μM (■) RES-1149-1.

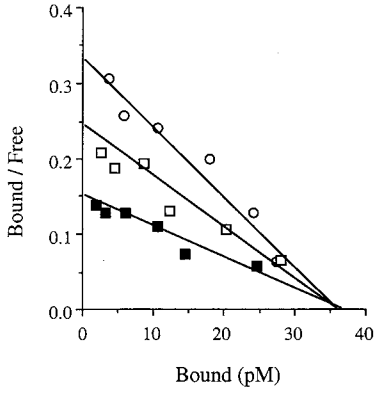
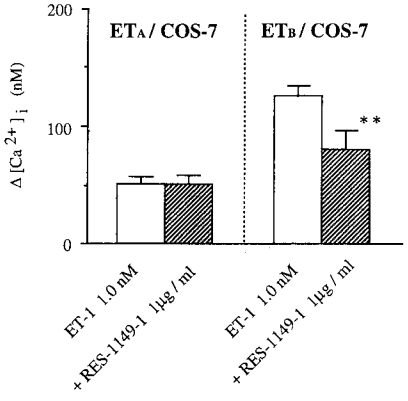


Fig. 3. Effect of RES-1149-1 and -2 on 1 nM ET-1- induced $[Ca^{2+}]_i$ increase in human ET_A or ET_B receptor expressing COS-7 cells.



** : $0.001 < p \leq 0.01$

The values are means ± standard errors of determinations on 23 ~ 30 cells.

ET_A receptor-expressing COS-7 cells (Fig. 3). RES-1149-1 alone did not influence $[Ca^{2+}]_i$ in ET_A or ET_B receptor-expressing COS-7 cells (data not shown). These data show that the RES-1149-1 was functional antagonist for ET_B receptor.

Species Difference

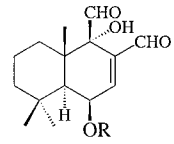
To evaluate the species difference of inhibitory effect of RES-1149-1, ET_B receptor binding experiments were performed using lung membranes prepared from rat, rabbit, pig and cow. The BQ-123, ET_A receptor selective antagonist, was added to the reaction mixture at the concentration of 10 μM to block the ^{125}I-ET-1 binding to the ET_A receptor. The IC_{50} values of RES-1149-1 for ^{125}I-ET-1 binding to ET_B receptor of lung membrane from various animals ranged from 1.0 μM to 3.2 μM, whereas those of RES-701-1 ranged from 0.0042 μM to 1.3 μM (Table 2). Therefore, significant species differences of RES-1149-1 were not observed.

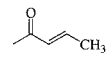
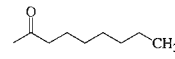
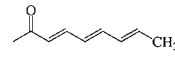
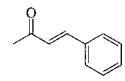
Table 2. Effect of RES-1149-1 and RES-701-1 on ^{125}I-ET-1 binding to ET_B receptor from various animal lung tissues.

Species	IC_{50} value	
	RES-1149-1	RES-701-1
Porcine	3.2	0.004
Bovine	2.2	0.017
Guinea pig	nt	0.013
Canine	nt	0.06
Rabbit	2.5	0.020
Rat	1.0	1.2

nt: Not tested.

Table 3. ^{125}I-ET-1 binding inhibitory activities of RES-1149-1 and their side chain derivatives.



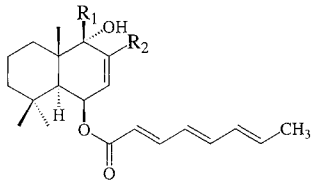
Compound	R	IC_{50} value (μM)	
		ET_A	ET_B
KT-7766	H	NT	11.6
Cinnamodial		NT	19.5
KT-7932		7.0	3.5
KT-7915		10.2	0.86
RES-1149-1		28.0	1.6
KT-7914		10.4	1.6
KT-7913		26	3.6

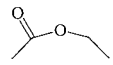
NT: Not tested.

Structure-activity Relationships

To examine structure-activity relationships, we synthesized some derivatives of RES-1149-1[9]. First, we investigated the relationship between the side chain structure of RES-1149-1 and the inhibitory activity. The

Table 4. ^{125}I-ET-1 binding inhibitory activites of RES-1149-1 and their aldehyde derivatives.



Compound	R_1	R_2	IC_{50} value (μM)	
			ET_A	ET_B
RES-1149-1			25.8	1.55
KT-7624			NT	23.3
KT-7619			NT	50.6
RES-1149-2			>80	20.0

NT: Not tested.

IC_{50} values of 6-*O*-acyl derivatives of RES-1149-1 for ET receptors are summarized in Table 3. Saturation of the acyl group (KT-7915) increased the affinity for both ET_A and ET_B receptor, but selectivity between ET_A and ET_B receptor was decreased. Comparison of three compounds, RES-1149-1, KT-7932, and cinnamodial, shows that RES-1149-1 (which contains the longest unsaturated acyl group) had the highest affinity for the ET_B receptor. Replacement of the octa-2,4,6-trienoyl residue with aromatic acyl groups did not affect the binding affinity for either the ET_A or the ET_B receptors. These results show that the structure of 6-*O*-acyl group is important for the binding to the ET_B receptor and selectivity between the ET_A and ET_B receptors.

Second, we assessed the necessity of aldehyde groups for binding inhibitory activity. The conversion of aldehyde groups to acetal significantly reduced affinity for the ET_B receptor (Table 4). RES-1149-2, which has no aldehyde group, had a lower affinity for ET_B receptor than RES-1149-1 which has two aldehyde groups. Thus, two aldehyde groups are essential for binding inhibitory activity.

Discussion

Endothelin may act through at least two distinct receptor subtypes, ET_A and ET_B. ET_A receptor is distributed in vascular tissues and mediates vasoconstriction, whereas ET_B receptor is widely expressed in vascular and non-vascular tissues and mediates vasodilatation as well as vasoconstriction. The development of subtype-selective non-peptidic antagonists is important for the elucidation of the function of ET receptors. Recently, several non-peptidic antagonists were reported. One type is selective for the ET_A receptor (97～139[10], BMS-182874[11]), and the other is non-selective for the ET_A and ET_B receptor (bosentan[12], SB 209670[13]). But a ET_B receptor-selective, non-peptidic antagonist has not yet been reported.

In a preceding paper, we reported a novel sesquiterpenoid, RES-1149-1, that was isolated from the cultured broth of a fungus and demonstrated that RES-1149-1 inhibited the binding of ET-1 to ET_B receptor[4]. The structure of RES-1149-1 is different from that of other nonpeptidic antagonists. In this paper, we demonstrate that RES-1149-1 selectively inhibits the binding of ET-1 to the ET_B receptor in a competitive manner and blocks the ET-1-induced increase in $[Ca^{2+}]_i$ in ET_B receptor-expressing COS-7 cells. Therefore, the RES-1149-1 is the first example of a nonpeptidic antagonist which blocks the function of ET_B receptor. However, RES-1149-1 was not so as potent as a peptidic antagonist RES-701-1 in blocking ET_B receptors. We modified the RES-1149-1 to study the structure-activity relationships of this compound. The results of this study revealed that 6-*O*-acyl and 8, 9 dialdehyde groups are important for the ET_B receptor blocking activities. Further investigation is now being undertaken to obtain a more potent compound.

RES-701-1 selectively blocks function of the ET_B receptor, but a species difference was observed in various animal tissues[7]. RES-701-1 inhibited the binding of ET-1 to ET_B receptor from canine, rabbit, porcine, and guinea pig lung tissues in the nM concentration range, but IC_{50} value for those from rat lung tissue was calculated to be 1.2 μM. However, a significant difference in the binding characteristics of RES-1149-1 was not observed between various species examined. In rat lung membrane, RES-1149-1 had a higher affinity for ET_B receptor than RES-701-1.

Finally, RES-1149-1together with its derivatives and RES-701-1 could be useful tools to elucidate the physiological and pathological roles of ET_B receptor, and might be useful in the treatment of disease involving ET-1.

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