# RES-1214-1 and -2, Novel Non-peptidic Endothelin Type A Receptor Antagonists Produced by *Pestalotiopsis* sp.

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(Received for publication July 10, 1995)

RES-1214-1 and -2, novel and non-peptidic endothelin antagonists, were isolated from the culture broth of a fungus, *Pestalotiopsis* sp. RE-1214. RES-1214-1 and -2 selectively inhibited the ET-1 binding to endothelin type A receptor ($ET_A$ receptor) with $IC_{50}$ values of 1.5 $\mu$M and 20 $\mu$M, respectively. RES-1214-1 and -2 inhibited the increase in intracellular $Ca^{2+}$ concentration elicited by 1 nM ET-1 in A10 cells. Taxonomy of producing strains, fermentation, isolation, structural determination, and biochemical properties of RES-1214-1 and -2 are described.

Endothelin-1 (ET-1) was isolated from the culture medium of porcine aortic endothelial cells as a potent and long lasting vasoconstrictor peptide[1]. It became apparent that it was member of a family of structurally homologous peptides characterized by the disulfide bonds and six conserved amino acid residues. Other members are designated ET-2 and ET-3 differing by two and six amino acid residues, respectively[2]. The endothelin actions are mediated through at least two distinct receptor subtypes, $ET_A$ and $ET_B$. The $ET_A$ receptor has a high affinity for ET-1 but not for ET-3, while the $ET_B$ receptor is non-selective for ET isopeptides. $ET_A$ receptors are distributed predominantly in vascular smooth muscle to mediate vasoconstriction. $ET_B$ receptors are present on endothelial cells and mediate endothelium-dependent relaxation. Since the discovery of the ETs, many agonists and antagonists were developed to elucidate their pharmacological and physiological functions (reviewed in ref. 3).

In the course of screening of endothelin antagonists, we have found that a fungus strain, *Pestalotiopsis* sp. RE-1214 produced non-peptidic antagonists of $ET_A$ receptor, designated RES-1214-1 and -2. In this article, we report taxonomy of the producing strains, fermentation, isolation, structural elucidation and biochemical properties of RES-1214-1 and -2.

## 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. Bovine cerebellum and lung were obtained from a local slaughterhouse. Asterric acid was purified from the cultured broth of *Penicillium* sp. in our laboratories as was done for RES-1214-1. RES-701-1 was purified from the cultured broth of *Streptomyces* sp. RE-701 in our laboratories as reported previously[4]. All other chemicals were of analytical grade.

### Culture and Medium Conditions

A loopful spore of microorganism, grown on an agar slant, was inoculated into 10 ml of the seed medium composed of V8 vegetable juice (Campbell) 20% and dextrin 3% (pH 6.5 before sterilization) in a test tube (21 i.d. × 200 mm). The agar slant medium consisted of malt extract 2%, glucose 2%, peptone (Kyokuto) 0.1% and agar 2% (pH 6.5 before sterilization). The inoculated tube was incubated at 25°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 1 day on a rotary shaker (200 rpm) at 25°C, 50 ml of the second seed culture was transferred to a 2-liter Erlenmeyer flask containing 400 ml of the fermentation medium composed of glucose 2%, dried mashed potato (Yukijirushi) 2%, peptone (Kyokuto) 0.5%, $K_2HPO_4$ 0.05%, and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.5% (pH 6.0 before sterilization). The fermentation was carried out for 7 days on a rotary shaker (200 rpm) at 25°C. The production of RES-1214-1 and -2 was traced by $ET_A$ receptor binding assay.

For this measurement, 2 ml of the culture broth was sampled. The broth was adjusted pH to 3.0 with 1 N HCl and then extracted with 2 ml of ethyl acetate. The ethyl acetate extract was concentrated and then dissolved in 0.5 ml of methanol. The methanol solution (10 $\mu$l) was provided for receptor binding assay.

### Isolation and Purification

The cultured broths in the 2-liter Erlenmeyer flasks were combined. The pH of the cultured broth was adjusted to 3.0 with 2 N HCl, and then the broth was extracted with ethyl acetate (5 liters). The organic layer was extracted with 2% $NaHCO_3$ solution (5 liters), and then the aqueous layer was adjusted to pH 3.0 with 2 N HCl. The aqueous layer was extracted with ethyl acetate (5 liters) again.

The ethyl acetate extract was dried over anhydrous sodium sulfate and then concentrated *in vacuo*. The resultant material was dissolved with *n*-hexane-ethyl acetate (1 : 1) and then applied to a silica gel column (1000 ml). The column was eluted with *n*-hexane-ethyl acetate-acetic acid (50 : 50 : 1). The active fractions were combined and then concentrated *in vacuo* to give a crude material. The crude material was dissolved in a small volume of acetonitrile and applied to an octadecylated silica gel column (ODS AQ-S-50 20 i.d. × 500 mm, YMC). The column was developed with 43% acetonitrile solution containing 0.1% trifluoroacetic acid at a flow rate of 10 ml/minute. The active fractions were concentrated *in vacuo* to give white powder.

### Receptor Binding Assay

ET-1 binding assays were performed as described previously[5]. Briefly, bovine cerebellum membranes were used for a source of $ET_B$ receptor. Bovine lung membranes, which are expressed both $ET_A$ and $ET_B$ receptors were used for 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 the 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 described[5,6].

### Measurement of Intracellular $Ca^{2+}$ Concentration

A10 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 A10 cells that had been loaded with fura-2 was filled with 1 ml BSS containing ET-1 and/or RES-1214-1 or -2. 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.

### General Procedures

Melting point was determined with a Yanagimoto melting point apparatus. Optical rotation was measured on a JASCO DIP-370 digital polarimeter in MeOH solution. FAB-MS spectrum was obtained with a JEOL JMS HX/HX110A mass spectrometer. UV spectrum was taken on a Shimadzu UV-2200 spectrometer in MeOH solution. IR spectrometry was carried out on a JEOL JIR-RFX3001 spectrometer with KBr method. NMR spectra were recorded on a JEOL α 400 (400 MHz for $^1$H, 100 MHz for $^{13}$C) spectrometer using TMS as an internal standard.

## Results

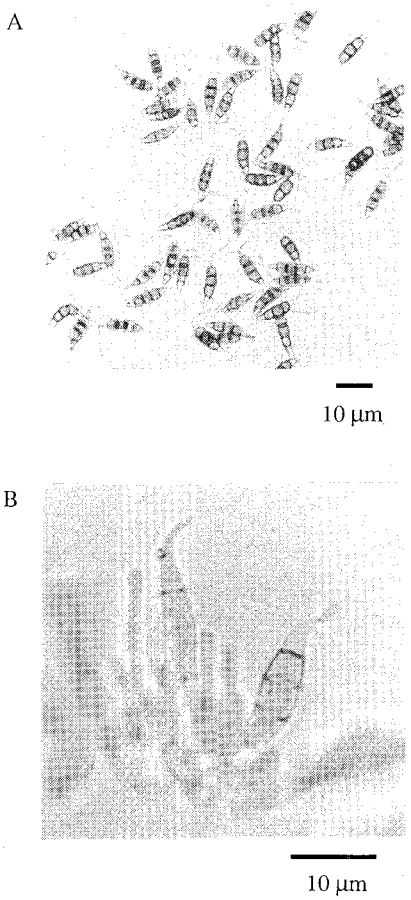
### Taxonomy

The fungal strain RE-1214 was isolated from a soil sample collected in Kanagawa Pref., Japan. Colonies on 2% malt extract agar are more than 80 mm in diameter after culturing at 25°C for 1 week. The surface of a colony is white to gray. The color of the reverse of the colony is brown at the center and cream at the marginal area. Colonies on potato-glucose agar are more than 80 mm in diameter after culturing at 25°C for 1 week. The surface of a colony is white. The color of the reverse of the colony is cream. The micrographs of strain RE-1214 grown on 2% malt extract agar are shown in Fig. 1. The hyphae are eparated and well-branched, but not synnematous on 2% malt extract agar medium. The conidia are produced on the medium either in enclosed acervuli or on exposed solitary to aggregated conidiophores which are smooth, colorless and cylindrical or lageniform, and measure 8～18 × 2～5 μm. Conidiogenous cells are annellidic, indeterminate, integrated, cylindrical, hyaline, smooth with several percurrent proliferations. Conidia are fusiform, straight or slightly curved, 4 euseptate (rarely 5 euseptate), 21～27 μm long and 6～8.5 μm wide. The basal cells are hyaline, truncate,

with an endogenous, cellular, simple or rarely branched appendage which measures 6~14 μm long. The apical cells are conic, hyaline, with 2~5 (mainly 3) apical and simple spathulate or espathulate appendages which measure 7.5~30.5 μm long. The median cells are brown, thicker-walled, smooth and 16~18 μm long. No teleomorph was observed in this strain.

Fig. 1. Micrographs of strain RE-1214.

Strain RE-1214 was grown on 2% malt extract agar for 1 week.
A: ×200, B: ×500.




From the characteristics mentioned above, the fungal strain RE-1214 was identified as *Pestalotiopsis* sp.[7]. The fungus has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-4464.

## Production and Purification of RES-1214-1 and -2

Producing organism, *Pestalotiopsis* RE-1214 was cultured according to the method described in materials and methods. The binding inhibitory activity was reached maximum on day 7 (data not shown). RES-1214-1 and -2 were purified from the cultured broth with the method described in materials and methods. The yields of pure RES-1214-1 and -2 from 5.5 liters of whole broth were 183 mg, 35 mg, respectively. Physico-chemical properties of RES-1214-1 and -2 are summarized in Table 1.

## Structural Determination of RES-1214-1 and -2

The molecular formula of RES-1214-1 was determined to be $C_{17}H_{16}O_8$ by HR-FABMS. The UV absorption maxima at 248 and 310 nm in MeOH resembled those of asterric acid which possesses the biphenyl ether structure[8]. In the IR spectrum, absorption band at 1720 $cm^{-1}$, and the combination of 1684 $cm^{-1}$ absorption and broad band from 3600 to 2600 $cm^{-1}$ suggested the presence of ester and carboxyl group, respectively. In the $^1H$ NMR spectrum, one methyl proton, two methoxy protons and four aromatic methine protons

Table 1. Physico-chemical properties of RES-1214-1 and -2.

| | RES-1214-1 | RES-1214-2 |
|---|---|---|
| Appearance | White powder | White powder |
| Molecular formula | $C_{17}H_{16}O_8$ | $C_{17}H_{15}ClO_8$ |
| High resolution FAB-MS | | |
| Observed | 348.0847 $(M)^+$ | 382.0449 $(M)^+$ |
| Calculated | 348.0845 (as $C_{17}H_{16}O_8$) | 382.0455 (as $C_{17}H_{15}O_8{}^{35}Cl$) |
| IR (KBr) $cm^{-1}$ | 3403, 3317, 1720, 1684, 1624, 1487, 1435, 1288, 1209, 1159, 1057, 829 | 3437, 3020, 2923, 1720, 1645, 1620, 1468, 1437 1248, 1213, 1097, 1061 |
| UV $\lambda_{max}$ nm (ε) (MeOH) | 310 (12,100), 248(18,400) | 310 (6,000), 253(12,900) |
| TLC (Rf) | | |
| n-hexane-EtOAc-AcOH (33:66:1) | 0.41 | 0.23 |
| Solubility | | |
| Soluble | MeOH, acetone, EtOAc, $CHCl_3$ | MeOH, acetone, EtOAc, $CHCl_3$ |
| Insoluble | $H_2O$ | $H_2O$ |

were observed and the $^{13}C$ NMR showed a total 17 resonances. After the assignment of the carbons bearing hydrogens by HSQC experiment, the structures of two aromatic ring moieties were determined by HMBC and NOESY experiments shown in Fig. 2. In conjugation with the molecular formula, these data suggested the presence of three exchangeable protons which were not observed in the $^1H$ NMR spectrum, even in DMSO-$d_6$ solution. Consequently it was not possible to determine which of the two oxygen-bearing carbons of each aromatic ring ($\delta$ 164.1, $\delta$ 160.2 and $\delta$ 153.2, $\delta$ 135.7) were hydroxyl bonded or ether bonded. It was finally determined by HMBC experiments for the permethylated derivative (data not shown).

In the FAB-MS spectrum of RES-1214-2, $MH^+$ ions were observed at *m/z* 383, 385 with a peak intensity ratio of approximately 3 : 1, which indicated the presence of one Cl atom. This was confirmed by the molecular formula ($C_{17}H_{15}O_8Cl$) determined by HR-FABMS. The structure was determined by the similar procedure used to elucidate RES-1214-1. RES-1214-2 has the same structure as RES-1214-1 except a Cl substitution for the 4′ aromatic proton.

The structures are shown in Fig. 3 and the $^1H$ and $^{13}C$ NMR signal assignments are summarized in Table 2. In contrast to asterric acid and its derivatives synthesized with methoxy functions at 5′ positions, RES-1214-1 and RES-1214-2 differ in having hydroxyl functions in these positions.

Fig. 2. Results of NMR analysis of RES-1214-1.

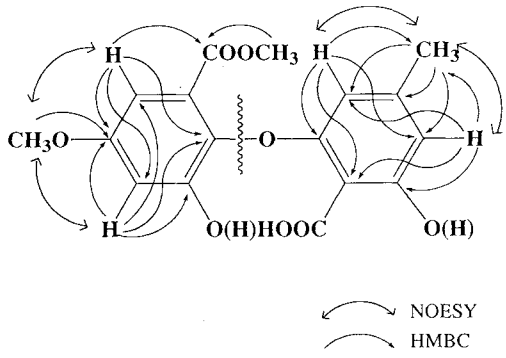
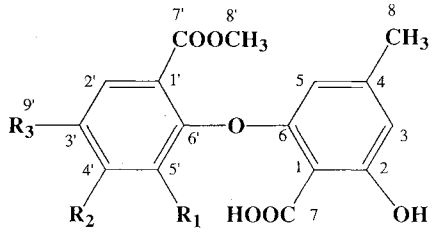



Fig. 3. Structures of RES-1214-1, RES-1214-2, and asterric acid.




RES-1214-1 $R_1$=OH, $R_2$=H, $R_3$=$OCH_3$
RES-1214-2 $R_1$=OH, $R_2$=Cl, $R_3$=$OCH_3$
Asterric acid $R_1$=$OCH_3$, $R_2$=H, $R_3$=OH

Table 2. $^1H$ and $^{13}C$ NMR of RES-1214-1 and RES-1214-2.

| Position | RES-1214-1 (in $CD_3OD$) $\delta_C$ (m) | $\delta_H$ (m, J in Hz) | RES-1214-2 (in DMSO-$d_6$) $\delta_C$ (m) | $\delta_H$ (m, J in Hz) |
|---|---|---|---|---|
| 1 | 102.3(s) | | 105.0(s) | |
| 2 | 164.1(s) | | 159.8(s) | |
| 3 | 112.6(d) | 6.45(d,0.8) | 110.7(d) | 6.39(d, 0.7) |
| 4 | 147.7(s) | | 143.4(s) | |
| 5 | 106.3(d) | 5.82(d,0.8) | 105.2(d) | 5.73(d, 0.7) |
| 6 | 160.2(s) | | 157.8(s) | |
| 7 | 172.7(s) | | 170.4(s) | |
| 8 | 22.1(q) | 2.13(s) | 21.3(q) | 2.09(s) |
| 1' | 126.2(s) | | 123.0(s) | |
| 2' | 108.0(d) | 6.99(d, 3.0) | 103.1(d) | 7.01(s) |
| 3' | 159.2(s) | | 152.4(s) | |
| 4' | 108.4(d) | 6.78(d, 3.0) | 114.1(s) | |
| 5' | 153.2(s) | | 148.2(s) | |
| 6' | 135.7(s) | | 136.0(s) | |
| 7' | 167.0(s) | | 164.6(s) | |
| 8' | 52.9(q) | 3.71(s) | 52.2(q) | 3.65(s) |
| 9' | 56.3(q) | 3.82(s) | 56.4(q) | 3.89(s) |

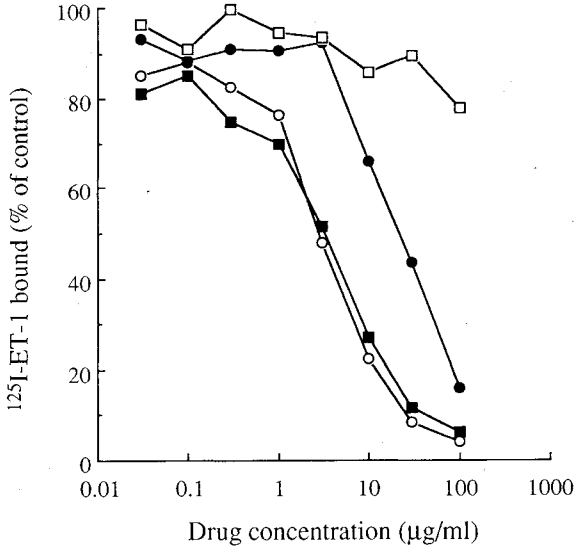
m: multiplicity, $\delta$: ppm from TMS.

Table 3. Receptor specificity of RES-1214s and asterric acid in binding assays with various tissues.

| Receptor | Subtype | Radioligand | Receptor source | $IC_{50}$ value (μg/ml) | | | |
|---|---|---|---|---|---|---|---|
| | | | | RES-1214-1 | RES-1214-2 | RES-1214-Me | Asterric acid |
| ET | $ET_A$ | $^{125}$I-ET-1 | Bovine lung | 5.2 | 19.0 | >100 | 5.2 |
| | $ET_B$ | $^{125}$I-ET-1 | Bovine cerebellum | 100 | >100 | >100 | 100 |
| ANP | NPR-A or B | $^{125}$I-rANP | Rabbit kidney cortex | >100 | >100 | >100 | NT |
| Angiotensin II | $AT_1$ | $^{125}$I-Angiotensin II | Bovine adrenal cortex | >100 | >100 | >100 | NT |
| Bradykinin | $BK_2$ | [$^3$H]Bradykinin | Guinea pig ileum | >100 | >100 | >100 | NT |

Fig. 4. Inhibition of $^{125}$I-ET-1 binding to $ET_A$ receptor by RES-1214-1, RES-1214-2 RES-1214-M, and asterric acid.

RES-1214-1 (■), RES-1149-2 (●), RES-1214-M (□), asterric acid (○).




Compounds were dissolved in dimethylsulfoxide at various concentrations, and 10 μl were added to the reaction mixture. All experiments were performed in duplicate.

Fig. 5. Scatchard analysis of specific binding of $^{125}$I-ET-1 in the absence of RES-1214-1 and presence of 10 μM RES-1214-1.

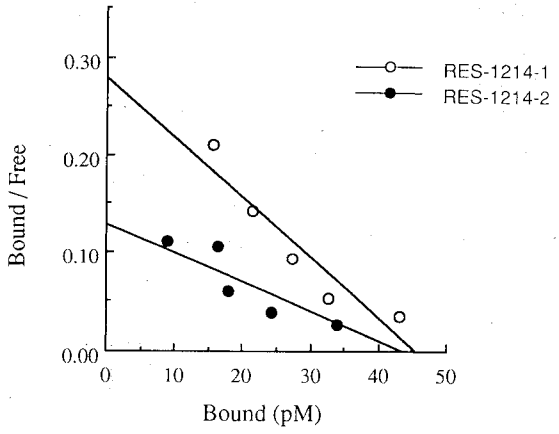
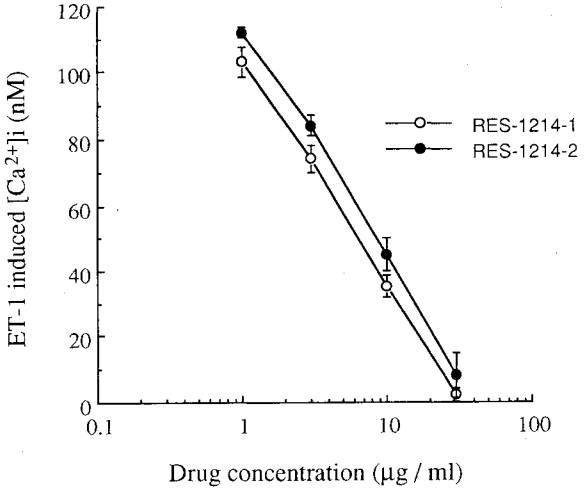



Fig. 6. Effects of RES-1214-1 and -2 on 1 nM ET-1-induced $[Ca^{2+}]i$ increase in A10 cells.




The values are means ± standard errors of determinations on 42 cells.

## Biochemical Properties

The various receptor binding assays of RES-1214s and asterric acid were carried out. RES-1214-1, -2 and asterric acid inhibited the binding of $^{125}$I-ET-1 to $ET_A$ receptor in a dose dependent manner, whereas the permethylated derivative of RES-1214-1 (RES-1214-M) did not affect the binding of $^{125}$I-ET-1 to $ET_A$ receptor at the concentration of 100 μg/ml (Fig. 4). The potency of RES-1214-1 was same as that of asterric acid. The $IC_{50}$ values of these compounds in receptor binding assays are summarized in Table 3. The RES-1214s did not inhibit the binding of $^{125}$I-rANP, $^{125}$I-angiotensin II and [$^3$H]bradykinin at concentrations up to 100 μg/ml. The inhibition of $^{125}$I-ET-1 binding in the bovine lung membranes by RES-1214-1 was shown to be competitive in the scatchard plot (Fig. 5).

The antagonistic properties of RES-1214-1 and -2 were assessed by measuring the effects on the increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]i$) induced by 1 nM ET-1 in A10 cells (Fig. 6). The RES-1214-1 and -2 blocked the ET-1-induced increase in $[Ca^{2+}]i$ in A10 cells in a dose dependent manner. RES-1214-1 and -2 alone did not influence $[Ca^{2+}]i$ in A10 cells (data not shown).

## Discussion

We have isolated RES-1214-1 and -2 from the cultured broth of *Pestalotiopsis* sp. RE-1214 and demonstrated that these compounds selectively inhibited the binding of ET-1 to $ET_A$ receptor in a competitive manner and blocked the ET-1-induced increase in $[Ca^{2+}]i$ in A 10 cells. These data show RES-1214-1 and -2 are selective antagonists that block the function of ETA receptor.

RES-1214-1 and -2 are novel compounds, the structures of which are close to that of asterric acid. The asterric acid was first isolated from the cultured broth of *Aspergillus terreus* as a metabolite of a fungus[9]. Recently, it has been reported that asterric acid inhibits the binding of ET-1 to $ET_A$ receptor[8]. The structure of RES-1214-1 is different from that of asterric acid in the position of hydroxyl group and methoxyl group. But the RES-1214-1 inhibited the ET-1 binding to $ET_A$ receptor as potent as asterric acid. Halogenated derivative, RES-1214-2, inhibited the binding but was four times less potent than RES-1214-1. The methylated derivative RES-1214-M had no inhibitory activity. These data suggest that hydroxyl group and methoxyl group are essential for binding inhibitory activities, but positions of hydroxyl group and methoxyl group are not so important.

We have recently isolated non-peptidic antagonist, RES-1149-1, from the culture broth of fungus[10]. RES-1149-1 was the first non-peptidic antagonist selective for $ET_B$ receptor. RES-1214-1 and RES-1149-1, subtype-specific non-peptidic antagonists, can be useful tools to elucidate the physiological and pathological roles of ET receptors and might be useful in the treatment of disease involving ET-1.

Acknowledgement

We would like to thank for the expert assistance of Mrs. CHIEKO IWAHASHI and Miss TOSHIKO NAKANO.

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