Vol. 178, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1991 Pages 132-137

AN ENDOTHELIN RECEPTOR (ET_A) ANTAGONIST ISOLATED FROM *STREPTOMYCES MISAKIENSIS*

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Received May 25, 1991

SUMMARY: A competitive endothelin (ET) antagonist, BE-18257B, was isolated from the fermentation products of *Streptomyces misakiensis*. It is a novel cyclic pentapeptide, cyclo(-D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp-), and binds to ET_A receptors (ET-1 selective) in cardiovascular tissues, but not to ET_B receptors (equally sensitive to isopeptides of ET family) in kidney, adrenal gland and cerebellum tissues. BE-18257B also antagonizes ET-1-induced vasoconstriction in rabbit iliac artery and pressor action in rats. Thus it is a selective ET_A antagonist and should provide a valuable tool for elucidation of the pharmacological and pathophysiological roles of ET-1.

Endothelin (ET)-1, a potent vasoconstrictor peptide, consisting of 21 amino acids, was first isolated from cultured endothelial cells in 1988 (1). Further studies revealed the existence of two additional related peptides, ET-2 and ET-3 (2,3), and of at least two distinct ET receptor subtypes termed ET_A (ET-1 selective) and ET_B (equally sensitive to isopeptides of ET family) (4-6). These peptides and their receptors are widely distributed in the peripheral tissues and the central nervous system (3,7) and are involved in numerous biological responses, such as constriction of various smooth muscles (1,8,9), positive inotropic effect of the heart (10), mitogenic action (11-13), and stimulation of endothelium-derived relaxing factor, prostaglandins, aldosterone and substance P release (14-17). Although possible relevance to hypertension (18), renal failure (19), vasospasm (20) and endotoxin shock (21) has been suggested, the pathophysiological roles of ET are still unclear. For the purpose of clarifying the biological roles, the specific receptor antagonists would be quite useful. Thus we have screened natural products to discover a selective ET antagonist.

MATERIALS AND METHODS

Materials: ET-1 and ET-3 were purchased from Peptide Institute Inc. (Osaka). [^{125}I]ET-1 and [^{125}I]ET-3 were obtained from Amersham Japan, Tokyo.

Binding experiments on cultured cells: Cultured porcine aortic smooth muscle cells (SMCs) were obtained according to a previously described method, with minor modifications (22). Cultured cells from the 3rd to 14th passages were used in these experiments. After washing with Hank's balanced salt solution containing 0.1% glucose and 0.3% bovine serum albumin (BSA) (Buffer A), confluent SMCs were incubated at 37°C with 10pM [^{125}I]ET-1 in

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the presence and absence of BE-18257B in 95% air - 5% CO_2 humidified atmosphere for 2 hours. The SMCs were then washed three times with ice-cold buffer A and cell-bound radioactivity was determined by a gamma counter (PACKARD: COBRA 5002). Nonspecific binding was defined by adding 200nM unlabeled ET-1 to the assay.

Binding experiments in membranes: Porcine tissues were homogenized in 10 volumes of 10mM 3-[N-morpholino]propane sulfonic acid (MOPS) pH 7.4 containing 20% sucrose with a Polytron (setting 7 for 30 second x 2) at 4°C. The homogenates were centrifuged at 1,000 x g for 15 minutes, and the resulting supernatants were centrifuged at 10,000 x g for 15 minutes. The supernatants were then centrifuged at 90,000 x g for 40 minutes. The pellets were homogenized in 5mM Hepes/Tris pH 7.4. The resulting homogenates were incubated at 25°C with 10pM [^{125}I]ET-1 or [^{125}I]ET-3 in the presence and absence of BE-18257B in 50mM Tris-HCl buffer pH7.4 containing 0.1mM phenylmethylsulfonyl fluoride, 1μM pepstatin, 2μM leupeptin, 1mM 1,10-phenanthroline, 1mM EDTA, 10μM $CaCl_2$, 10μM $MgCl_2$ and 0.1% BSA. After 4 hours of incubation, 2ml of cold 5mM Hepes/Tris pH 7.4 containing 0.3% BSA (Buffer B) was added to the mixture, then rapidly filtered through Whatman GF/C glass fiber filters. After washing the filters with buffer B, the radioactivity on the filter was determined by the gamma counter. Nonspecific binding was defined by adding 200nM ET-1 or ET-3 to the assay mixture.

Vasoconstriction experiments: Constriction studies were done as previously described (23). Briefly, iliac arteries were isolated from rabbits. The preparations were cut into spirals and were suspended in an organ bath containing Krebs-Henseleit solution. After equilibration, reference contraction was isometrically obtained with 50mM KCl. Concentration-response curves for ET-1 were obtained by cumulative additions of the compound.

***In vivo* experiments:** *In vivo* studies were done as previously described (23). Briefly, male Wistar Kyoto rats 15-16 weeks of age were anesthetized with pentobarbital-Na (50mg/kg s.c.), and catheterized into the femoral artery for measurement of blood pressure and the femoral vein for the administration of ET-1. One day after being catheterized, each conscious, restrained rat was placed in an individual cage for continuous measurement of blood pressure. ET-1 at a dose of 1nmol/kg was given intravenously to rats. BE-18257B (10 or 50mg/kg) suspended in 0.5% carboxy-methyl cellulose Na solution was administered intraperitoneally (i.p.) 1 hour before the bolus injection of ET-1. Control animals received vehicle on the same time schedule.

RESULTS AND DISCUSSION

From the screening of natural products for ET binding inhibitors, we discovered a novel ET receptor antagonist, BE-18257B. It was isolated from the fermentation products of *Streptomyces misakiensis* BA18257, which was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-10751. BE-18257B is a novel cyclic pentapeptide, cyclo(-D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp-), as determined by amino acid analysis of the acid hydrolysate, mass spectrometry and nuclear magnetic resonance (manuscript in preparation).

[^{125}I]ET-1 binding in cardiovascular tissues, such as vascular smooth muscle and ventricle, was inhibited by ET-1 (IC_{50}: 0.1-0.19nM) 40-700 times more potently than by ET-3, while the binding in non-cardiovascular tissues, such as kidney, adrenal gland and cerebellum, was inhibited to the same extent by ET-1 and ET-3 (IC_{50}: 0.07-0.12nM) (Table 1). Therefore, the cardiovascular tissues are rich in ET-1-selective ET_A receptors, while the non-cardiovascular tissues are rich in ETs-nonselective ET_B receptors. In ET_A receptor-rich tissues, namely aortic smooth muscle and ventricle membranes and cultured aortic smooth muscle cells, BE-18257B inhibited [^{125}I]ET-1 binding in a concentration-dependent manner with IC_{50} values of 1.4, 0.8 and 0.5μM, respectively. However, in the ET_B receptor-rich tissues, BE-18257B failed to inhibit [^{125}I]ET-1 binding even at 100μM (Table 1, Fig.1A).

Table 1. The IC_{50} values of ET-1, ET-3 and BE-18257B on [^{125}I]ETs binding

Tissues	[^{125}I]ET-1 binding			[^{125}I]ET-3 binding		
	ET-1 (nM)	ET-3 (nM)	BE-18257B (μM)	ET-1 (nM)	ET-3 (nM)	BE-18257B (μM)
Cells:						
Aorta SM*	0.10§	70	0.47	n.t.¶	n.t.	n.t.
Membranes:						
Aorta SM	0.19	5.6	1.4	0.23	0.19	>100
Ventricle	0.10	3.9	0.80	0.10	0.07	>100
Kidney cortex	0.08	0.07	>100	0.07	0.05	>100
Adrenal cortex	0.12	0.07	>100	0.10	0.06	>100
Cerebellum	0.11	0.09	>100	0.11	0.08	>100

* SM: smooth muscle.
§ The values in this table represent mean values determined in at least three different preparations.
¶ Not tested due to the absence of specific [^{125}I]ET-3 binding.

These results indicate that BE-18257B selectively binds to ET_A receptors, but not to ET_B receptors. Furthermore, [^{125}I]ET-3 binding to all the assayed tissues was inhibited equally by both ET-1 and ET-3 (IC_{50}: 0.05-0.23nM), but not by BE-18257B even at 100μM (Table 1). These data indicate that [^{125}I]ET-3 binds to ETs-nonselective ET_B receptors and that BE-18257B does not. Inhibition of [^{125}I]ET-1 binding to aorta and ventricle membranes by BE-18257B was incomplete even at 100μM, although complete inhibition was achieved for

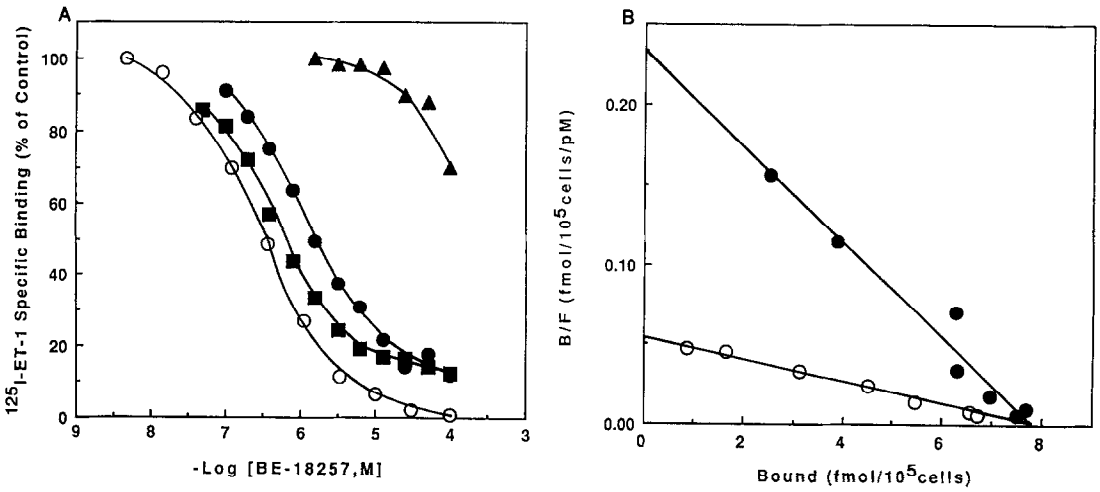
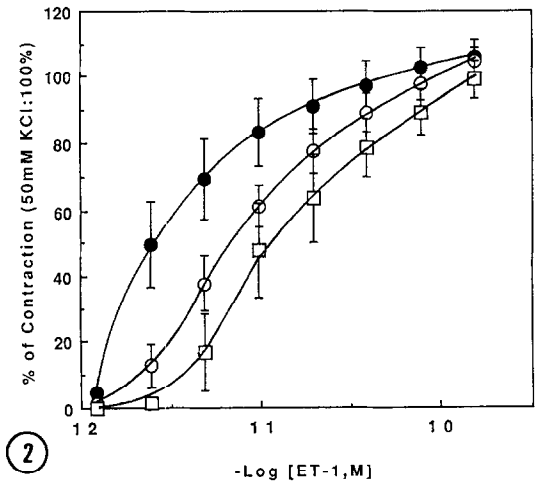


Fig.1. (A)Effect of BE-18257B on [^{125}I]ET-1 binding to porcine aortic smooth muscle (●), ventricle (■) and cerebellum (▲) membranes and to cultured aortic smooth muscle cells (○). (B)Scatchard analysis of [^{125}I]ET-1 binding to smooth muscle cells in the absence (●) and presence (○) of BE-18257B (1.1μM). The mean ± SD values of K_D were 46±11 and 214±66pM, respectively. The B_{max} values in the presence of BE-18257B were 109±11% (mean ± SD) of control values. These experiments were repeated more than three times.

binding on aortic smooth muscle cells in culture (Fig.1A). This finding indicates that aorta and ventricle tissues may contain a small population of ET_B receptors, which are insensitive to BE-18257B. The mechanism of BE-18257B inhibition of [^{125}I]ET-1 binding was investigated using cultured aortic smooth muscle cells in the presence and absence of BE-18257B (1.1μM). The linearity of the resultant Scatchard plots (24) indicates a single population of binding sites (K_D: 46pM, B_{max}: 45,600 sites/cell). In addition, the reduced slope (K_D: 214pM) and unchanged X-intercept (B_{max}) in the presence of BE-18257B indicate a competitive interaction of BE-18257B with the ET_A receptors on these cells (Fig.1B).

In isolated rabbit iliac arteries, BE-18257B antagonized ET-1-induced vasoconstriction in a dose-dependent manner, although it had no direct vasoactivity even at 100μM. This antagonism was characterized by a parallel shift to the right of the vasoconstriction-concentration curve without a reduction in maximum response (Fig.2), indicating a competitive antagonism. The pA_2 value of BE-18257B analyzed by Shild plots was 5.9, which was in agreement with the IC_{50} value (-log IC_{50}= 5.9) for [^{125}I]ET-1 binding inhibition in aortic smooth muscle membrane. BE-18257B is also an ET receptor-specific antagonist because it (10μM) had no effect on vasoconstriction induced by acethylcholine, histamine or potassium chloride (data not shown). The effect of BE-18257B (10 or 50mg/kg) as an ET antagonist was then estimated by the intraperitoneal administration (i.p.) in conscious, unrestrained Wistar Kyoto rats. An intravenous bolus injection of ET-1 (1nmol/kg) produced early transient



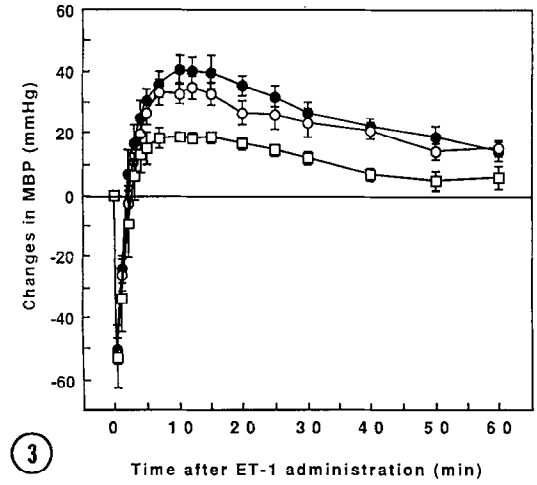


Fig.2. Antagonistic effect of BE-18257B on ET-1-induced vasoconstriction (control: ●) of rabbit iliac arteries. BE-18257B 1.6μM (○) and 4.9μM (□) were given 20 minutes before the addition of ET-1. The maximum response of 50mM KCl-induced contractions was taken as 100%. Data represent the means ± S.E. of three tests.

Fig.3. Effect of BE-18257B on ET-1-induced depressor and pressor responses in Wistar Kyoto rats. ET-1 at a dose of 1nmol/kg was given intravenously to conscious and unrestrained rats. BE-18257B 10mg/kg(○) or 50mg/kg(□) suspended in 0.5% carboxy-methyl cellulose Na solution was administered intra-peritoneally (i.p.) 1 hour before the ET-1 injection. Control animals (●) received vehicle on the same time schedule. Data represent the means ± S.E. of four tests.

depressor responses followed by sustained pressor responses. When BE-18257B was adnimistered intraperitoneally 1 hour before the intravenous injection of ET-1 (1nmol/kg), pretreatment with BE-18257B (50mg/kg i.p.) attenuated only the sustained pressor phase (Fig.3). However, the intraperitoneal administration of BE-18257B (50mg/kg i.p.) alone did not change the blood pressor. This result suggests that the two-phase response of ET-1 on the blood pressure is mediated by at least two ET receptor subtypes and that the response to the ET_A receptor is blocked by BE-18257B, resulting in suppression of the pressor action, while the response to the ET_B receptor is not blocked by BE-18257B, resulting in no effect on the transient depressor response. Since both ET-1 and ET-3 produce equivalent endothelium-dependent vasodilation in isolated tissues (14), ET-induced transient depressor response may be partially dependent on vasodilation mediated by ET_B receptors on the endothelium. Thus, the *in vitro* ET_A-selective antagonistic effect of BE-18257B was reproducible in the *in vivo* study.

Discovery of an ET_A-selective antagonist will aid elucidation of the pharmacological and pathophysiological roles of ET-1. Furthermore, in cardiovascular disorders such as hypertension and vasospasm, ET_A-selective antagonists, such as BE-18257B or potent synthetic analogs, may have more therapeutic potential than ET_B-selective or ET receptor-nonselective antagonists because the ET_A antagonist does not inhibit ET_B-mediated vasodilation.

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