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BIOLOGICAL PROFILES OF HIGHLY POTENT NOVEL ENDOTHELIN ANTAGONISTS SELECTIVE FOR THE ET_A RECEPTOR

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Summary

We describe novel potent endothelin (ET) antagonists that are highly potent and selective for the ET_A receptor (selective to ET-1). Of the synthetic analogs based on ET_A antagonist BE-18257A isolated from *Streptomyces misakiensis* (IC_{50} value for ET_A receptor on porcine aortic smooth muscle cells (VSMCs); 1.4μM), the compounds BQ-123 and BQ-153 greatly improved the binding affinity of [^{125}I]ET-1 for ET_A receptors on VSMCs (IC_{50}; 7.3 and 8.6 nM, respectively), whereas they barely inhibited [^{125}I]ET-1 binding to ET_B receptors (nonselective with respect to isopeptides of ET family) in the cerebellar membranes (IC_{50}; 18 and 54 μM, respectively). Associated with the increased affinity for ET_A receptors, these peptides antagonized ET-1-induced constriction of isolated porcine coronary artery. However, there was a small amount of ET-1-induced vasoconstriction resistant to these antagonists, which paralleled the incomplete inhibition of [^{125}I]ET-1 binding in the membrane of the aortic smooth muscle layer. These data suggest that the artery has both ET_A and ET_B receptors responsible for ET-1-induced vasoconstriction. The antagonists shifted the concentration-response curve to the right for ET-1 in the coronary artery, and increased the apparent dissociation constant in the Scatchard analysis of [^{125}I]ET-1 binding on the VSMCs without affecting the binding capacity, indicative of the competitive antagonism for ET_A receptor. In conscious rats, pretreatment with the antagonists markedly antagonized ET-1-induced sustained pressor responses in dose-dependent fashion without affecting ET-1-induced transient depressor action, suggesting that the pressor action is mediated by ET_A receptors, while the depressor action is mediated by ET_B receptors. In addition, pretreatment with the potent antagonists prevented ET-1-induced sudden death in mice. Thus, these potent ET_A antagonists should provide a powerful tool for exploring the therapeutic uses of ET_A antagonists in putative ET-1-related disorders.

Endothelin (ET)-1, a potent vasoconstrictor peptide, consisting of 21 amino acids, was first isolated from cultured endothelial cells in 1988 (1). Subsequent studies have revealed the existence of two additional related peptides termed ET-2 and ET-3 (2,3), and two distinct ET-receptor subtypes termed ET_A (selective for ET-1) and ET_B (nonselective with respect to isopeptides of the ET family) (4-6). The existence of 'ET-3-selective' receptor has been also suggested (7). These peptides and receptors are widely distributed in many tissues and are involved in numerous biological responses (8). The distribution of these ET-receptor subtypes is clearly differentiated by the selective inhibition of [^{125}I]ET-1 binding by ET-1 and ET-3 (9) or by ET_A-selective receptor antagonists (10). ET_A receptors are abundant in cardiovascular tissues, while ET_B receptors are abundant in noncardiovascular tissues including kidney, adrenal gland and the central nervous system. Furthermore, the involvement of ET in the pathogenesis of certain diseases such as hypertension, heart failure, asthma, renal failure and vasospasm has been suggested (11). However, it is unclear

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whether ET biology is linked to such diseases and which ET-receptor subtype is involved in each disease, mainly because of the lack of potent and subtype-specific ET-receptor antagonists. Recently, we described an ET_A antagonist BE-18257B, which is a novel cyclic pentapeptide isolated from *Streptomyces misakiensis* (10). It is relatively weak but highly selective for ET_A receptors and antagonizes ET-1-induced vasoconstriction and pressor action. These initial findings led us to the design of more potent and selective ET_A antagonists. This report describes the *in vitro* and *in vivo* biological profiles of the synthesized cyclic pentapeptides with improved potency as ET_A antagonists.

Materials and Methods

Chemicals: ET-1 and ET-3 were purchased from Peptide Institute Inc. (Osaka, Japan). [^{125}I]ET-1 was obtained from Amersham Japan, Tokyo. BE-18257A and BE-18257B were isolated from the cultured broth of *Streptomyces misakiensis* BA18257 as previously described (12,13). BQ-123, BQ-153 and BQ-162 were synthesized in our laboratories (see Table I for the chemical structures), and the chemical syntheses will be described elsewhere. BE-18257A and B sodium salts are barely soluble in water (0.14 and .0.21 mg/ml in saline), whereas BQ-123 and BQ-153 sodium salts have greatly improved water solubility (>1 g/ml in saline).

[^{125}I]ET-1 binding experiments: Cultured porcine aortic smooth muscle cells (VSMCs) were obtained according to a previously described method (14), with minor modifications. The confluent VSMCs in 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), the VSMCs were incubated at 37°C with 10pM [^{125}I]ET-1 in the presence and absence of test compounds in 95% air - 5% CO_2 humidified atmosphere for 2 hours. The VSMCs were then washed three times with ice-cold buffer A and the 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. The membranes of the endothelium-denuded porcine aortic smooth muscle layer and cerebellum were prepared as previously described (9), and incubated at 25°C with 10pM [^{125}I]ET-1 in the presence and absence of test compounds in 50mM Tris/HCl buffer pH 7.4 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, cold 5mM Hepes/Tris buffer pH 7.4 containing 0.3% BSA (Buffer B) was added to the mixture, followed by a rapid filtration through GF/C glass fiber filters (Whatman, England). After the filters were washed with buffer B, the radioactivity remaining on the filter was determined by the gamma counter. Nonspecific binding was defined by adding 200nM ET-1 to the assay mixture. The Hill slopes were obtained by analysis of Hill plots on the binding inhibition of [^{125}I]ET-1 (15).

Other binding experiments: For [^{125}I]angiotensin II binding experiments, calf cerebellar membranes were prepared, and the effect of test compounds on [^{125}I]angiotensin II binding to the membranes was assessed as previously described (16). For [^{3}H]oxytocin binding experiments, rat uterus membranes were prepared from uteri taken from diethylstilbestrol dipropionate-treated (0.3 mg/kg i.p.; 18-24hours) rats, and the effect of test compounds on [^{3}H]oxytocin binding to the membranes was assessed as previously described (17).

Vasocontraction experiments: Contraction studies were performed as previously described (18). Briefly, left anterior descending coronary arteries were isolated from fresh porcine hearts. Connective tissues and adherent fats were removed. For removal of the vascular endothelium, the intimal surface of spiral strips was rubbed gently with wet filter paper. The endothelium-denuded arteries were cut into spiral strips about 10 mm long and 1mm wide. Each strip was suspended in an organ bath containing Krebs-Henseleit solution bubbled with 95% O_2-5% CO_2 at 37°C. After equilibration, reference contraction was isometrically obtained with 50mM KCl. Concentration-response curves for ET-1 were obtained by cumulative additions of ET-1. The test compounds were added 20 minutes before the cumulative addition of ET-1. The pA_2 values and slopes were obtained by analysis of Schild plots according to Arunlakshana and Schild (19).

Blood pressure experiments: Blood pressure studies were performed as previously described (18).

Briefly, male Wistar Kyoto rats of 15-16 weeks of age (331 ± 9 g, mean ± S.E.M.) were anesthetized with pentobarbital-Na (50 mg/kg s.c.), and catheterized into the femoral artery for measurement of blood pressure and into the femoral vein for administration of ET-1. One day after being catheterized, each conscious, unrestrained rat was placed in an individual cage for continuous measurement of blood pressure, and given ET-1 intravenously (i.v.) at a dose of 1 nmol/kg. Test compounds were given intravenously 5 minutes before the bolus injection of ET-1. Control animals received vehicle on the same time schedule.

Sudden death experiments: Male ddY mice of 5 weeks of age (30 ± 2 g) were used. Test compounds or saline (control) were given intravenously (0.1 ml/kg) through the tail vein 5 minutes before the i.v. injection of ET-1 (5 nmol/kg). Death was defined as the cessation of respiration. Survival rate was recorded for 24 hours after the injection.

TABLE I
Chemical Structures of Cyclic Peptides in This Study
Cyclo(- A - B - C -L-Leu-D-Trp-)

compounds	A	B	C
BE-18257A	D-Glu	L-Ala	D-Val
BE-18257B	D-Glu	L-Ala	D-*allo*-Ile
BQ-162	D-Glu	L-Pro	D-Val
BQ-123	D-Asp	L-Pro	D-Val
BQ-153	D-Sal*	L-Pro	D-Val

* D-Sulfoalanine.

TABLE II
Inhibitory effects on [^{125}I]ET-1 binding and ET-1-induced vasoconstriction*

compounds	The IC_{50}(nM) values for [^{125}I]ET-1 binding VSMC#	VSMM$	cerebellum	Anti-vasoconstriction pA_2	slope
BE-18257A	1,400	3,000§	>100,000	n.d.¶	
BE-18257B	470	1,400§	>100,000	n.d.¶	
BQ-162	230	410§	>100,000	n.d.¶	
BQ-123	7.3	22§	18,000	7.4	0.96
BQ-153	8.6	21§	54,000	7.4	0.96
ET-1	0.1	0.16	0.11	------	
ET-3	70	5.7	0.07	------	

* The values in this table represent the averages of more than three experiments.
# Porcine aortic smooth muscle cell.
$ Porcine aortic smooth muscle membrane.
§ The half-maximum inhibition concentration (IC_{max50}) for the high affinity sites.
¶ These compounds showed an inhibitory effect on ET-1-induced vasoconstriction, but the pA_2 values could not be calculated because of their low potency and solubility.

Results

The specific binding of [^{125}I]ET-1 to the cultured aortic smooth muscle cells (VSMCs) and to the endothelium-denuded aortic smooth muscle membranes (VSMMs) was inhibited by ET-1 700 and 30 times more selectively than by ET-3, respectively, indicating that the 'ET-1 selective' ET_A receptor predominates there. In cerebellar membranes, [^{125}I]ET-1 specific binding was inhibited by ET-1 and ET-3 equipotently, indicating that the 'ET-isopeptides nonselective' ET_B receptor predominates there (Table II, Fig.1).

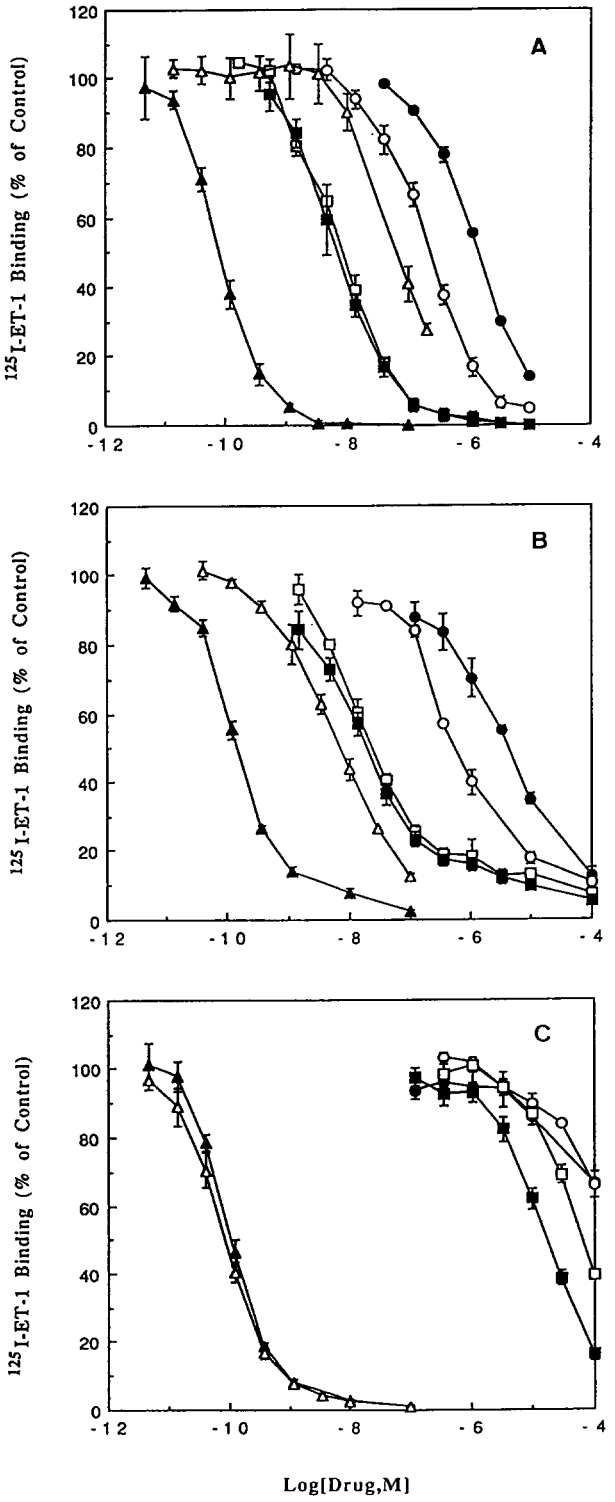

FIG.1
Inhibitory effects of BE-18257A (●), BQ-162 (○), BQ-123 (■), BQ-153 (□), ET-1 (▲) and ET-3 (△) on the specific binding of [^{125}I]ET-1 to (A) porcine aortic smooth muscle cells, (B) the smooth muscle membranes and (C) cerebellum membranes. The values represent the mean ± S.E.M. of more than three experiments.

The affinity (IC_{50} value) of the cyclic peptide analogs for ET_A receptors on VSMCs is listed in Table II. BE-18257A showed 34% of the potency of BE-18257B. Substitution of L-Ala for L-Pro in position B of BE-18257A (BQ-162) increased the potency 6-fold, and further substitution of D-Glu for D-Asp in position A (BQ-123) increased the potency 32-fold, for an overall increase of 190-fold compared to BE-18257A. Furthermore, BQ-153, in which the γ-carboxyl group of D-Asp in BQ-123 was replaced by sulfonic acid, was almost equal in potency to BQ-123. The displacement curves for BE-18257A, BQ-162, BQ-123 and BQ-153 are shown in Fig.1A. These cyclic pentapeptides displaced the bound [^{125}I]ET-1 completely in a dose-dependent manner and exhibited Hill slopes not significantly different from unity. Furthermore, we carried out a saturation kinetic study of the binding of [^{125}I]ET-1 to VSMCs in the presence and absence of 14nM BQ-123. Scatchard analysis (20) of the binding data revealed that the VSMCs have a single population of high affinity binding sites (B_{max}=21,000 ± 4,200 sites/cell, mean ± S.E.M.(n=3)) for [^{125}I]ET-1 with an apparent dissociation constant (K_D) of 25.1 ± 2.0 pM, and that in the presence of BQ-123, the K_D value of [^{125}I]ET-1 was increased to 187 ± 16 pM, but the total number of binding sites (B_{max}) was unchanged (Fig.2).

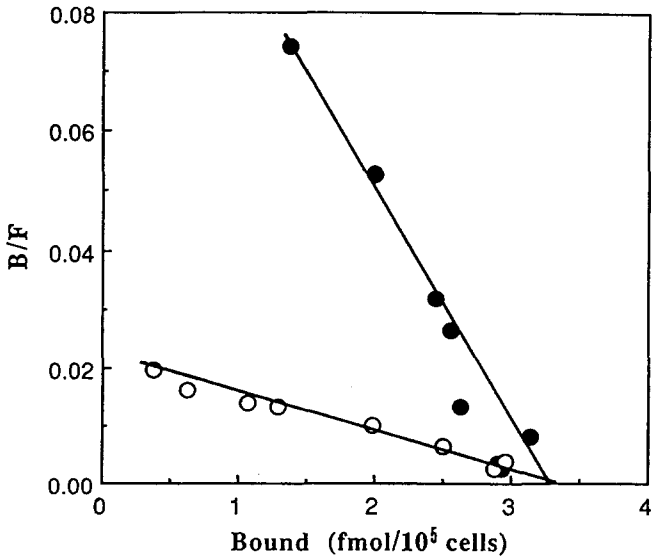

FIG.2
Scatchard analysis of BQ-123 on the binding characteristics of [^{125}I]ET-1 on porcine aortic smooth muscle cells. Specific binding of [^{125}I]ET-1 in the absence (●) (K_D 25.1 ± 3.4 pM) and in the presence (○) of 14nM of BQ-123 (K_D 187 ± 28 pM). The K_D values represent the mean ± S.E.M. of three experiments.

In the VSMM, the cyclic peptides inhibited specific ET-1 binding in the same order of potency as in the VSMCs, but the displacement curve for these cyclic peptides was different from that in the VSMCs (Fig.1). It should be noted in the VSMM that approximately 15% of the specific binding of [^{125}I]ET-1 was resistant to displacement by the cyclic peptides (Fig.1B). In addition, the affinity of the cyclic peptides in the VSMM was represented by the half-maximum inhibition concentration (IC_{max50} values) for 85% of the [^{125}I]ET-1 binding to these cyclic peptides, and the IC_{max50} values in the VSMM were 2-3 times lower than those in the VSMCs (Table II).

In cerebellar membranes, where ET_B receptors predominate, these cyclic peptides inhibited the specific binding of [^{125}I]ET-1 very slightly. The IC_{50} values of BQ-162, BE-18257A and BE-18257B were higher than 100μM, and those of BQ-123 and BQ-153 were 18 and 54μM, respectively. In addition, the cyclic peptides barely inhibited the specific binding of [^{125}I]angiotensin II to bovine cerebellum and the binding of [^{3}H]oxytocin to rat uterus membranes, even at a concentration of 10μM (data not shown).

In isolated porcine coronary arteries, the cyclic peptides caused no contraction even at 10μM, indicating the lack of the agonistic activity. As illustrated in Fig.3, ET-1 caused a dose-dependent increase in tension and produced a maximal response at 1nM. BQ-153 (Fig.3), BQ-162, BQ-123,

BE-18257A and BE-18257B (data not shown) caused a parallel, dose-dependent shift to the right in the concentration-response curve for ET-1 without affecting the maximal response. Also noted is that a small portion of the ET-1-induced constriction was resistant to these antagonists. The pA_2 values are listed in Table II. The Schild plot for each antagonist was not significantly different from unity. BQ-123 and BQ-153 are potent analogs with pA_2 values of 7.4, and these pA_2 values are similar in extent to the IC_{50} values with respect to displacement of [^{125}I]ET-1 binding to the VSMM. On the other hand, BQ-123 and BQ-153 even at a high concentration (10μM) did not significantly affect the concentration-response curves or maximal constrictive responses to potassium chloride or norepinephrine. In addition, BQ-153 (10μM) did not significantly affect them to prostaglandin F2α, acethylcholine or histamine (data not shown).

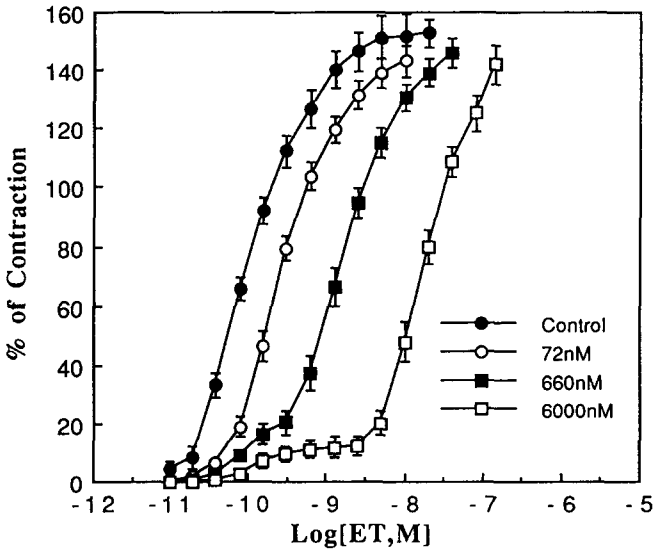

FIG.3
Effect of BQ-153 (72-6000nM) on the concentration-response curve for ET-1 in isolated porcine coronary artery. Values represent the mean ± S.E.M. (n=6).

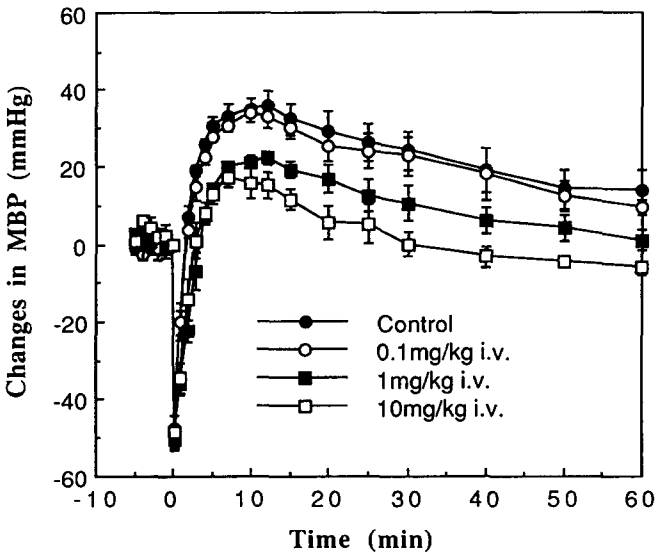

FIG.4
Effect of BQ-153 (0.1-10mg/kg i.v.) pretreatment (five minutes before ET-1 injection) on the changes in mean arterial blood pressure induced by ET-1 (1nmol/kg i.v.) in conscious, unrestrained Wistar Kyoto rats. Values represent the mean ± S.E.M. (n=4-7).

An intravenous dose of ET-1 (1 nmol/kg) in rats produced a rapid, transient decrease followed by a sustained increase in blood pressure (Fig.4). A bolus injection (0.1-10 mg/kg) of BQ-153 five minutes before the ET-1 bolus injection (1 nmol/kg) caused dose-dependent inhibition of the pressor effect of ET-1, but did not affect the ET-1-induced depressor effect. In addition, a bolus injection of BQ-153 or BQ-123 (10 mg/kg) alone produced no change in the blood pressure. A similar selective antagonism to ET-1-induced pressor effect was also obtained with BQ-123 (data not shown).

ET-1 had a potent lethal effect in mice. Mean survival time of the ET-1-treated mice (5 nmol/kg i.v.) was 3.2 ± 0.6 minutes (n=10). Pretreatment with BQ-123 or BQ-153 protected the mice from ET-1-induced sudden death, with ID_{50} values of about 0.5 mg/kg i.v., although BQ-162 could not prevent death or prolong the survival time even at a dose of 5 mg/kg i.v. (Table III).

TABLE III
Protective effect against ET-1-induced sudden death in mice

Dose (mg/kg i.v.)	Survival time* (min)	Survival Total	Survival rate (%)
Control	3.2	0/10	0
BQ-123			
0.1	4.3	0/10	0
0.25	9.2	1/10	10
0.5	9.8	7/10	70
1.0	----	10/10	100
BQ-153			
0.1	5.0	0/10	0
0.25	6.9	2/10	20
0.5	6.5	3/10	30
1.0	----	10/10	100
BQ-162			
1.0	3.8	0/10	0
5.0	3.4	0/10	0

* Mean survival time of mice after lethal ET-1 injection.

Discussion

As previously reported (10), the cyclic pentapeptide BE-18257B, isolated from the cultured broth of *Streptomyces misakiensis*, was identified as a weak but highly selective antagonist of the ET_A receptor. The same *Streptomyces* isolate also produced an analogous cyclic pentapeptide BE-18257A, in which the D-*allo*-Ile residue of BE-18257B is replaced by D-Val (12,13). BE-18257A was used as a structural template for synthesizing analogs with improved affinity for ET_A receptors due to the difficulty in synthesizing BE-18257B analogs containing D-*allo*-Ile even though BE-18257A had approximately half of the ET_A antagonistic activity of BE-18257B. The compounds presented in this paper are representative of a large series of analogs. Accordingly, it was observed that the replacement of L-Ala of BE-18257A with L-Pro (BQ-162) and the further replacement of D-Glu with D-Asp and D-Sal (BQ-123 and BQ-153) remarkably increased the binding affinity (IC_{50} values) for ET_A receptors on the VSMCs. Increases in the binding affinity correspond to increases in the antagonistic activity (pA_2 values) against ET-1-induced contraction of porcine coronary artery, indicating antagonism to common receptors shared by binding sites and contraction. Hill slopes of inhibition of [^{125}I]ET-1 binding on the VSMCs by these cyclic peptides are not significantly different from unity, and Scatchard analysis of the saturation binding data obtained in the presence and absence of BQ-123 showed that BQ-123 increased the apparent dissociation constant of ET-1 without changing the binding capacity, indicating that these cyclic peptides compete with ET-1 binding to the common sites on the ET_A receptors. Furthermore, in the isolated artery these compounds resulted in a parallel rightward shift of the concentration-response curve of ET-1, and the

Schild plots were not significantly different from unity. Thus, these compounds consistently exhibited a competitive type of antagonism against ET-1. BQ-153 and BQ-123 had no effect on the blood pressure of rats *in vivo* even at a high dose, and reduced the ET-1-induced pressor responses in a dose-dependent manner but not the depressor responses. Therefore, the ET_A-selective antagonistic effect observed *in vitro* is reproducible *in vivo* and these ET_A antagonists may be effective in ET-1-related hypertension. In addition, the potent antagonists BQ-123 and BQ-153 protected mice from acute death due to ET-1 (ID_{50}; about 0.5 mg/kg), although the weaker BQ-162 did not protect even at 5 mg/kg. These data indicate that potent ET_A antagonists protect against ET-1-induced sudden death in mice. The main mechanism involved in ET-1-induced sudden death is purported to be coronary vasoconstriction followed by cardiac ischemia (21); if so, ET_A antagonists would be effective in ischemic heart diseases.

One of the most attractive features of these analogs is the high specificity as pure antagonists for ET_A receptors. These cyclic peptides at high concentrations of up to 10μM showed no contractile response in coronary arteries, no effect on the contractile responses induced by various agonists, and no affinity for closely-related ET_B receptors and various other receptors in radioligand binding assay. In the VSMM, these peptides inhibited about 85% of the specific binding of [^{125}I]ET-1 with potency 2-3 times lower than that seen with the ET_A receptors on the VSMCs, indicating the predominant presence of ET_A receptors in the smooth muscle layer. However, 15% of the specific [^{125}I]ET-1 binding sites showed low affinity for the cyclic peptides although the binding sites on the VSMCs were completely displaced by those with high affinity, and a small portion of ET-1-induced contraction was resistant to the cyclic peptides. These data indicate that the smooth muscle layer contains small amounts of ET_B receptors which mediate vasoconstriction and which are resistant to the cyclic peptides. These observations were in good agreement with our previous study, in which we reported that porcine coronary arteries constrict *via* both ET_A (previously termed ET receptor site 1) and ET_B (termed ET receptor site 2) receptors by the analysis of two different types of constriction associated with ET-1 and ET-3 (9). The existence of both ET_A and ET_B receptors in the vasculature was also confirmed by the detection of their mRNAs in human aorta (22,23). Furthermore, in the *in vivo* study, BQ-153 did not block the ET-1-induced early transient depressor phase although BQ-153 antagonized the long-lasting pressor phase. It has been reported that in rat isolated perfused mesentery ET-1 was a more potent vasoconstrictor than ET-3, but the two peptides were equipotent vasodilators (24). Therefore, it is thought that the ET-1-induced transient depressor response may be dependent on vasodilation mediated by ET_B receptors on the endothelium (24,25,26). The existence of 'ET-3-selective' receptor has been also suggested (7). However, this putative ET receptor is insensitive to ET-1 so that ET-1-induced responses in the present study can not be mediated by the 'ET-3-selective' receptor. Thus the resistant phases observed in these *in vitro* and *in vivo* studies are supposed to be ET_B-mediated responses.

The present studies demonstrate that cyclic pentapeptides, BQ-123 and BQ-153, which were synthesized by amino acid substitutions of natural ET_A antagonist BE-18257, are extremely potent, competitive and specific ET_A receptor antagonists in radioligand binding assays, *in vitro* functional assays and *in vivo* study. Furthermore, BQ-123 and BQ-153 sodium salts improved water solubility to more than 1 g/ml in saline compared to the natural lead compounds BE-18257A and B sodium salts (0.14 and 0.21 mg/ml in saline, respectively). ET isopeptides, the related peptides from Israeli snake venom (sarafotoxins) and their synthetic analogs showed high affinity for ET_B receptors or to both ET_A and ET_B receptors (27). However, there are no reports on ET_A-selective compounds except for the weak ET_A antagonist (BE-18257B) we recently reported (10). These potent and water-soluble ET_A receptor antagonists should provide powerful tools for characterizing the function of ET_A receptors and for exploring the therapeutic uses of ET antagonists in ET-related disorders.

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