

# Identification of a domain of ET<sub>A</sub> receptor required for ligand binding

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Various chimeric ET<sub>A</sub> and ET<sub>B</sub> receptors were produced in CHO cells for the elucidation of a specific domain which influences the affinity of the receptor toward BQ-123, a selective ET<sub>A</sub> antagonist. Replacement of the first extracellular loop domain (B-loop) of the ET<sub>A</sub> receptor with the corresponding domain of the ET<sub>B</sub> receptor, reduced the inhibition by BQ-123 drastically, while the replacements of other extracellular domains of ET<sub>A</sub> did not. By contrast, the introduction of the B-loop of ET<sub>A</sub> in place of the corresponding domain of the ET<sub>B</sub> receptor endowed the ET<sub>B</sub>-based chimeric receptor with a sensitivity to BQ-123. These observations suggest that the B-loop domain of the ET<sub>A</sub> receptor is involved in ligand binding.

Endothelin (ET); Receptor; Binding site; Ca<sup>2+</sup> mobilization; Expression; Antagonist

## 1. INTRODUCTION

Endothelin (ET), a 21-amino acid peptide, induces strong and long acting vasoconstriction in a wide variety of vascular beds, and serves as an important modulator of vascular tone [1]. There are three isoforms of endothelin: ET-1, ET-2 and ET-3 [2]. Concerning its receptor, there are at least two types of endothelin receptors, ET<sub>A</sub> and ET<sub>B</sub> [3]. The ET<sub>A</sub> receptor is predominantly located in vascular smooth muscle cells [4,5], where it plays a major role in vasoconstriction; the ET<sub>B</sub> receptor is predominantly located in vascular endothelium cells [5,6] and is linked to vasodilation through the release of endothelium-derived relaxation factor and prostacycline [7,8]. We have cloned cDNAs coding for human ET<sub>A</sub> and ET<sub>B</sub> receptors [9,10]. The cloned cDNA sequences indicated that both ET<sub>A</sub> and ET<sub>B</sub> are members of the seven transmembrane-spanning and G-protein-coupled receptor family [4,9,11]. Both of the cloned cDNA have recently been expressed in the transfected COS cells [9,10]. The expressed ET<sub>A</sub> receptor represented different affinities to three endothelin isoforms in the order of ET-1 > ET-2 ≫ ET-3 [5,9], while the ET<sub>B</sub> binds to ET-1, 2 and 3 with an almost equal affinity [6,11].

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Abbreviations: ET, endothelin; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular calcium concentration; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMEM, Dulbecco Modified Eagle Medium; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; CHO cells, Chinese hamster ovary cells; Fura-2, Fura-2-penta-acetoxymethyl ester.

Several receptor antagonists have been reported, which compete with endothelins for binding to receptor and block the G-protein-mediated signal transduction and calcium channeling. The antagonist that inhibits the ET<sub>A</sub> or ET<sub>B</sub> receptor specifically or inhibits a certain step in signal transduction, provides a powerful tool for the elucidation of the functional domains of the receptor structure. BQ-123 is a novel cyclic pentapeptide, cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-), and binds to the ET-1 selective ET<sub>A</sub> receptor, but not to the ligand non-selective ET<sub>B</sub> receptor [12]. In this paper, the structure and function of two ET receptor species were investigated by construction and production in CHO cells of various chimeric receptors, and by inhibition of their activities by the ET<sub>A</sub>-specific antagonist BQ-123.

## 2. MATERIALS AND METHODS

### 2.1. Materials

ET-1 was purchased from Peptide Institute Inc. (Osaka, Japan) and the <sup>125</sup>I-labeled ET-1 (81.4 TBq/mmol) was from New England Nuclear. The site-directed mutagenesis system, Mutan-K, was from Takara Co. Ltd. (Osaka, Japan). 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and Fura-2 (Fura-2-penta-acetoxymethyl ester) was from Dojin Chemical Institute Co. (Kumamoto, Japan). BQ-123 was chemically synthesized and purified to homogeneity. Details of the procedure will be described elsewhere.

### 2.2. Cells and transfection

CHO cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Laboratories, New York, USA) supplemented with heat-inactivated 10% fetal bovine serum. The cells were grown at ca. 3 to 4 × 10<sup>6</sup> cells per 90 mm-diameter dish 1 day before transfection and the medium was replaced with 6 ml of DMEM supplemented with heat-inactivated 10% Nu-serum. About 8 μg of pCDM8-based chimeric plasmids in 100 μl of distilled water and 60 μl of 40 mg/ml of DEAE-dextran in phosphate-buffered saline were mixed with vortexing and added to the cell monolayer. After the further addition of 60 μl of 10 mM Chloroquine (Sigma Co., St. Louis, USA), the mixture

was incubated for 3 h. Cells were treated with 3 ml of 10% dimethylsulfoxide for 3 min, and were fed with fresh medium again and incubated for 72 h prior to ligand binding or  $Ca^{2+}$  induction assays.

### 2.3. Binding assay

CHO cells were removed from the monolayer plates and collected by centrifugation. After sonication of CHO cells, the crude membrane was prepared from the cell homogenate by centrifugation at 50,000 rpm for 20 min, and the pellet was suspended in 200  $\mu$ l of assay buffer (50 mM sodium phosphate buffer, pH 7.4, and 0.1% CHAPS). The assay mixture (50  $\mu$ l) consists of 30  $\mu$ l of assay buffer, 15  $\mu$ l of crude membrane from transfected CHO cells, 2.5 fmol of  $^{125}I$ -labeled ET-1 and  $10^{-10}$ – $10^{-5}$  M BQ-123. It was incubated for 2 h at room temperature. The receptor- $^{125}I$ ET-1 complex was separated from free  $^{125}I$ ET-1 and determined the radioactivity of the bound ET-1 as described [13]. Non-specific binding was determined in the presence of 1  $\mu$ M nonradioactive ET-1.

### 2.4. Measurement of intracellular calcium concentration ( $[Ca^{2+}]_i$ )

Three days after transfection, the CHO cells were treated with trypsin, washed twice with solution A (140 mM NaCl, 4 mM KCl, 1 mM  $Na_2HPO_4$ , 1 mM  $MgCl_2$ , 1.25 mM  $CaCl_2$ , 11 mM glucose, 5 mM HEPES buffer, pH 7.4, 0.2% bovine serum albumin) and incubated in solution A containing 10  $\mu$ M Fura-2 for 1 h at 20°C. The Fura-2-loaded cells were washed twice and resuspended in 4.5 ml of fresh solution A without the dye. After transfer of one ml aliquot of the cell suspension to the chamber of the intracellular ion analyzer (Japan Spectroscopic Co., Tokyo, Japan). ET-1 was added at a final concentration of 1 nM and the fluorescence of the cells was measured with excitation at 340 nm and 380 nm, and emission at 500 nm. Next, BQ-123 was added to another one ml aliquot of the Fura-2-loaded cell suspension in the chamber at the final concentration of 1  $\mu$ M, 1 min and 20 s before the addition of  $10^{-9}$  M ET-1, and ET-1 induced  $[Ca^{2+}]_i$  was measured. The total intracellular calcium concentrations of transfected CHO cells before and after the addition of ET-1 were determined as described [14,15].

## 3. RESULTS

### 3.1. Expression of chimeric ET receptors

We have constructed pCDM8-based expression plasmids to synthesize various chimeric ET receptors, in which each extracellular domain, A, B, C and D of  $ET_A$  receptor, was replaced with the corresponding domain of the  $ET_B$  receptor. List of these chimeric receptors is shown in Fig. 1. After transfection of CHO cells with these plasmids, the binding of  $^{125}I$ -labeled ET-1 to the receptor was determined as described in section 2. The parental and chimeric ET receptors bound 0.6 to 2.2 fmol range of  $^{125}I$ -labeled ET-1 in the assay condition described in section 2.

### 3.2. Differential effect of BQ-123 on the ligand binding activity of various chimeric ET receptors

BQ-123 was reported to be a  $ET_A$ -specific antagonist [12]. We have synthesized BQ-123 to investigate its inhibitory effect on the ligand binding activity of chimeric ET receptors. The synthesized BQ-123 indeed inhibited the  $^{125}I$ ET-1 binding in an  $ET_A$ -specific manner: the binding  $IC_{50}$  was 6 nM for the  $ET_A$  and more than 10  $\mu$ M for the  $ET_B$  receptor (Fig. 2(1)). When the N-terminal half of  $ET_A$  receptor was substituted with the corresponding half of  $ET_B$  receptor (Fig. 1K), the resulting

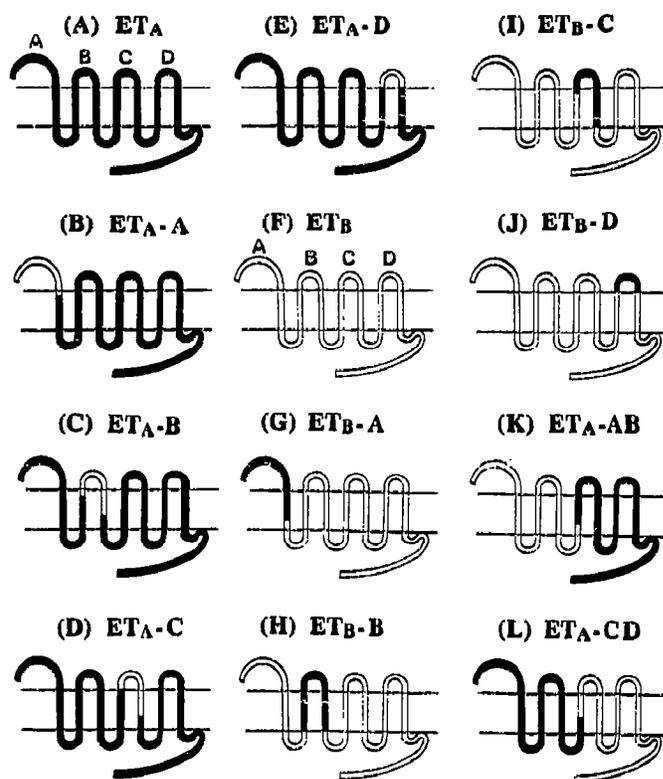


Fig. 1. Structure of chimeric ET receptors. (A)  $ET_A$  receptor. (F)  $ET_B$  receptor. (K and L) Half of  $ET_A$  receptor was replaced by that of  $ET_B$  receptor and vice versa. Numbers on the chimeric receptors represent the regions of changed amino acid sequences of the parental ET receptor:  $ET_A$ -CD (212–427 of C-terminus),  $ET_A$ -AB (1–210), (B–D and G–I) Each of the extracellular A, B and C domains of  $ET_A$  receptor was replaced by the same domain of  $ET_B$  receptor and vice versa. To construct the expression plasmids for various chimeric ET another ET receptor through several ligation steps. The following amino acid sequences of  $ET_A$  receptor were substituted with the corresponding sequences of the  $ET_B$  receptor and vice versa. Numbers on the chimeric receptors represent the regions of changed amino acid sequences of the parental ET receptor:  $ET_A$ -A (1–79),  $ET_A$ -B (139–175),  $ET_A$ -C (229–270),  $ET_B$ -A (1–120),  $ET_B$ -B (138–197),  $ET_B$ -C (241–291). (E and J) D domains of the  $ET_A$  and  $ET_B$  receptors were mutually exchanged.  $ET_A$  and  $ET_B$  cDNAs were subcloned in the *Xba*I site of M13-mp19 plasmid vector and were subjected to site-directed mutagenesis using the Kunkel procedure [16]. The nucleotide sequence encoding the D-loop domain of  $ET_A$  receptor was changed to that of the  $ET_B$  receptor. The same approach was done to form the chimeric  $ET_B$  receptor that contains the D-loop of the  $ET_A$ . The mutated cDNAs were inserted into *Xba*I site of pCDM8 vector at correct orientation. The resultant plasmids were designated as  $ET_A$ -D and  $ET_B$ -D. The closed bar and open bar represent the sequences of human  $ET_A$  and  $ET_B$  receptor, respectively. The extracellular A, B, C and D domains are indicated.

chimeric receptor, surprisingly, showed a strong resistance to inhibition by BQ-123. The concentration of BQ-123 required for 50% inhibition ( $IC_{50}$ ) went up from 6 nM (to  $ET_A$  receptor) to over 10  $\mu$ M for this chimeric receptor (Fig. 2(1)). By contrast, the substitution of the C-terminal half of  $ET_A$  with that of  $ET_B$  receptor (Fig. 1L) showed only a subtle change in the  $IC_{50}$  of BQ-123

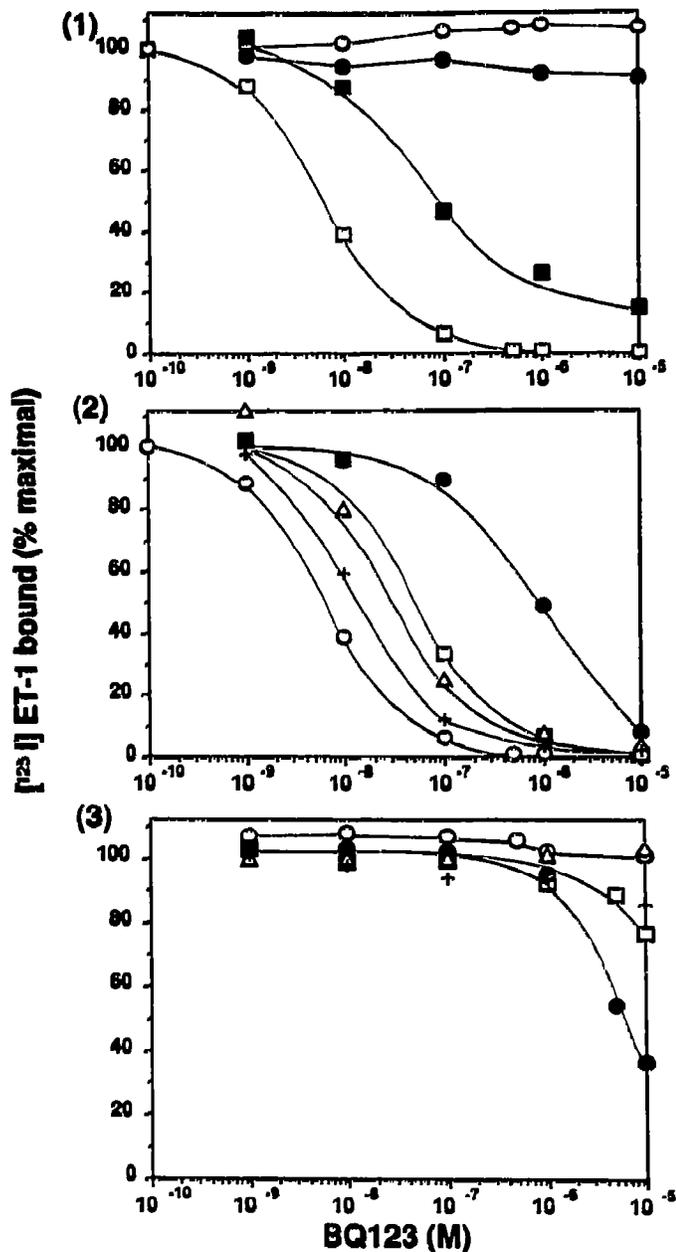


Fig. 2. Inhibitory effects of BQ-123 on the ligand binding of parental and chimeric ET receptors transiently expressed in CHO cells. (1) human  $ET_A$  ( $\square$ ) and  $ET_B$  ( $\circ$ ) receptors,  $ET_A$ -CD ( $\blacksquare$ ) and  $ET_A$ -AB ( $\bullet$ ) chimeric receptors; (2) human  $ET_A$  receptor ( $\circ$ ) and the chimeric  $ET_A$ -A ( $\square$ ),  $ET_A$ -B ( $\bullet$ ),  $ET_A$ -C ( $\Delta$ ) and  $ET_A$ -D ( $+$ ) receptors; (3) human  $ET_B$  ( $\circ$ ),  $ET_B$ -A ( $\square$ ),  $ET_B$ -B ( $\bullet$ ),  $ET_B$ -C ( $\Delta$ ) and  $ET_B$ -D ( $+$ ) receptors.

(Fig. 2(1)). These results suggest that the N-terminal half which includes A and B-domains of  $ET_A$  is responsible for the specific inhibition by BQ-123.

Next, we have constructed a series of recombinant chimeric receptors to investigate in detail the regions that influence the inhibition profile of BQ-123. Each

individual domain of  $ET_A$ , the A, B, C and D domains, was replaced with the corresponding region of  $ET_B$  receptor. Furthermore, the construction of various chimeric receptors with the same replacements were carried out for  $ET_B$  receptor. The substitution of each of A, C and D domain of the  $ET_A$  receptor with each of the corresponding region of the  $ET_B$  receptor (Fig. 1B,D,E) caused only a marginal decrease in the inhibitory activity of BQ-123, to less than one-tenth-fold of the original inhibitory activity (Fig. 2(2)). However, when the B-loop domain of the human  $ET_A$  receptor was substituted with the same domain of the  $ET_B$  receptor (Fig. 1C), a drastic decrease in antagonistic activity (more than 1/100-fold) was observed (Fig. 2(2)). Conversely, when the B-loop domain of  $ET_B$  receptor was substituted with that of the  $ET_A$  receptor (Fig. 1H), the relative antagonistic activity of BQ-123 was increased by more than one order of magnitude (Fig. 2(3)). Thus, the B-loop domain of  $ET_A$  receptor must be involved in the interaction with BQ-123.

### 3.3. Effect of BQ-123 on the ET induced increase of the $[Ca^{2+}]_i$

The  $[Ca^{2+}]_i$  increased from the 27–77 nM range to the 73–290 nM range, by the addition of  $10^{-9}$  M ET-1 to CHO cells which were transfected with expression plasmids pCDM8- $ET_{(A,B)}$  and chimeric plasmids. As expected, BQ-123 inhibited the ET-induced transient increase of  $[Ca^{2+}]_i$  in CHO cells transfected with pCDM8- $ET_A$ , but it did not inhibit the increase in CHO cells transfected with pCDM8- $ET_B$  (Table I). Therefore, BQ-123 inhibited ET-1 binding without any effect on the downstream pathway of signal transduction by which the transient increase of  $[Ca^{2+}]_i$  is induced.

The effect of BQ-123 on transient increase of  $[Ca^{2+}]_i$  in the transfected CHO cells was examined in detail. When the B-loop region of the human  $ET_A$  receptor was substituted with the corresponding region of the  $ET_B$  receptor, the inhibition percentage of the ET-1-induced transient increase of  $[Ca^{2+}]_i$  by BQ-123 decreased from 98% to 70% (Table I). When the crude membranes were prepared from both recombinant CHO cells, BQ-123 inhibited the binding of  $^{125}I$ -labeled ET-1 to the  $ET_A$  and  $ET_A$ -B receptors by 99% and 51% at the concentration of  $1 \mu\text{M}$ , respectively (Fig. 2). On the contrary, when the B-loop region of  $ET_B$  receptor was substituted with the corresponding region of  $ET_A$  receptor, the inhibition percentage by BQ-123 increased from 24% to 89% under the same experimental conditions (Table I). The exchange of A, C and D domains between  $ET_A$  and  $ET_B$  receptors, however, did not significantly affect the extent of inhibition by BQ-123 for the transient increase of  $[Ca^{2+}]_i$  (Table I). Again, these results indicate that the B-loop domain of the  $ET_A$  receptor is a site which interacts with BQ-123. The same domain of  $ET_B$  receptor, for yet unknown reasons, does not interact with BQ-123.

Table 1  
Inhibition by BQ-123 of ET-1 induced transient increase of  $[Ca^{2+}]_i$  in transfected CHO cells

ET receptors	Intracellular $Ca^{2+}$ concentration				Inhibition (%) by BQ123
	Basal	$10^{-9}$ M ET-1	Basal	$10^{-6}$ M BQ123 and $10^{-9}$ M ET-1	
ET <sub>A</sub>	76 nM	220 nM	26 nM	29 nM	98%
ET <sub>A</sub> -A	48 nM	120 nM	47 nM	47 nM	100%
ET <sub>A</sub> -B	34 nM	95 nM	39 nM	57 nM	70%
ET <sub>A</sub> -C	63 nM	160 nM	63 nM	63 nM	100%
ET <sub>A</sub> -D	44 nM	290 nM	45 nM	45 nM	100%
ET <sub>B</sub>	56 nM	140 nM	36 nM	100 nM	24%
ET <sub>B</sub> -A	69 nM	250 nM	72 nM	250 nM	2%
ET <sub>B</sub> -B	64 nM	240 nM	67 nM	87 nM	89%
ET <sub>B</sub> -C	55 nM	220 nM	41 nM	200 nM	4%
ET <sub>B</sub> -D	77 nM	250 nM	84 nM	250 nM	4%
ET <sub>A</sub> -CD	27 nM	86 nM	39 nM	42 nM	95%
ET <sub>A</sub> -AB	29 nM	73 nM	39 nM	91 nM	0%

Increase of intracellular calcium concentration by the addition of  $10^{-9}$  M ET-1, and the inhibition by  $1 \mu$ M BQ-123 was determined as described in section 2. Inhibitory effect of BQ-123 on the ET-induced increase of  $[Ca^{2+}]_i$  was determined and represented by percentages.

#### 4. DISCUSSION

It has been reported that the C-terminal domain of ETs, especially the tryptophan residue at the C-terminus, plays an important role in the binding of ETs to the receptor [17,18]. Supporting this hypothesis, the peptidic endothelin antagonists, BQ-123 and FR-139317 (1-hexahydroazepino-CO-Leu-D-Trp(CH<sub>3</sub>)-D-(2-pyridyl)alanine), and the agonist, IRL-1620, Suc-[Glu<sup>9</sup>,Ala<sup>11,15</sup>]-ET-1(8-21) [19], that contain C-terminal tryptophan residue, have been found to be specific ET antagonists or agonists. The two former antagonists are ET<sub>A</sub>-specific and the latter agonist is ET<sub>B</sub>-specific. All these peptidic compounds apparently bind to the endothelin receptor through the structural resemblance of the -Asp(Leu)-Pro(Asp)-Val(Ile)-Leu(Ile)-Trp-sequence to the C-terminal sequence (-Leu-Asp-Ile-Ile-Trp) of endothelins. Although the processing of preprobigET and the second messenger pathways of ET have been extensively investigated, little is known about the function of each ET receptor domain. First of all, it is not clear why the two distinct receptor species that bind ET-1 with an almost identical affinity exist in the different cells and tissues. The two types of receptor differ in binding to ET-3, while the overall structure of receptor molecule and the biological activity, as measured in vitro by ligand binding and induction of  $[Ca^{2+}]_i$  increase, are quite similar. The significant similarity of the two ET receptors makes it difficult to define the regions required for ligand selection and binding. BQ-123, a strong ET<sub>A</sub> specific inhibitor, is thus one of few valuable reagents that can distinguish the binding site of the ET<sub>A</sub> receptor from ET<sub>B</sub> receptor.

To assess the structural alterations of chimeric receptors that result in the changes in the profile of ligand binding, we used BQ-123 as a diagnostic reagent. The substitution of the B-loop of the ET<sub>A</sub> receptor with the same region of ET<sub>B</sub> receptor remarkably reduced the affinity by BQ-123, indicating that BQ-123 perhaps binds to the B-loop of the ET<sub>A</sub> receptor, but not, or weakly, to the B-loop of ET<sub>B</sub> receptor. As shown in the experiments (Table 1) in which the ET-dependent  $[Ca^{2+}]_i$  was monitored in CHO cells that express chimeric receptors, BQ-123 inhibited the ET-1-induced increase of  $[Ca^{2+}]_i$  significantly when the B-loop of ET<sub>A</sub> was present in the chimeric receptor, in accordance with the increased inhibition in ligand binding. The results indicated that BQ-123 is not an agonist and is a useful reagent that competes with ET-1 at the ligand binding step only.

Recently, we have found that part of the N-terminal extracellular domain (amino acid residues 50-76 of the predicted human ET<sub>A</sub> sequence), in the vicinity of the first transmembrane, is required for ET-1 binding [20]. The data indicates that part of the N-terminal domain is involved in the tertiary structure formation of ligand binding site, together with the B-loop domain of the ET<sub>A</sub> receptor.

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