Biological and Pharmacological Properties of Highly Selective New Endothelin Converting Enzyme Inhibitor WS79089B Isolated from *Streptosporangium roseum* No. 79089

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WS79089B† a highly specific endothelin converting enzyme (ECE) inhibitor has been isolated from the fermentation broth of *Streptosporangium roseum* No. 79089. WS79089B showed highly selective ECE inhibition activity with IC_{50} value of 0.14 μM and behaved as a competitive inhibitor of ECE, with *Ki* values of 8.9×10^{-8} M. The sodium salt of WS79089B (FR901533) inhibited big endothelin-1 (big ET-1) induced pressor effect in a dose dependent manner when administered to male Sprague-Dawley rats intravenously dosed 2 minutes prior to big ET-1 challenge.

The 21-amino acid peptide endothelin-1 (ET-1), initially isolated from endothelial cells in culture, is a potent vasoconstrictor *in vitro* and *in vivo*[1]. It is synthesized as a precursor peptide of 203 amino acids, which is proteolytically cleaved to produce either 38 (human) or 39 (porcine) amino acid intermediate big endothelin-1 (big ET-1). The big ET-1 is converted to ET-1, a mature form, through an unusual cleavage between Trp^{21} and Val^{22} by endothelin converting enzyme (ECE)[1]. The normal human plasma concentration of big ET-1 is about twice that of ET-1[2], and various endothelial cell lines secrete both big ET-1 and ET-1 into culture medium[3~5], indicating that ECE does not perfectly process the available big ET-1 intracellularly. Recent studies suggest that a phosphoramidon-sensitive neutral metalloprotease is a likely candidate for the physiological ECE functioning in vascular endothelial cells[5,6]. It has been suggested that the precursor of ET-1, big ET-1, induces various pharmacological effects *in vivo* and *in vitro* following an active conversion by phosphoramidon-sensitive ECE[7,8]. Phosphoramidon also blocks the *in vivo* effects of exogenously added big ET-1, suggesting that ECE may be involved in both intracellular and extracellular processing of big ET-1[9,10]. Phosphoramidon is not a selective inhibitor, rather it is more of a neutral endopeptidase than an ECE inhibitor. The development of more selective inhibitors will be required to elucidate further the specific nature of ECE.

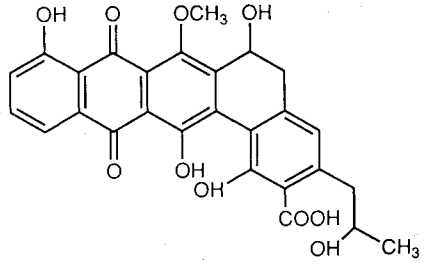
In the previous paper we described the discovery and characterization of three selective novel ECE inhibitors WS79089A, B and C from the culture broth of *Streptosporangium roseum* No. 79089[11]. In this paper, we report the pharmacological characteristics of WS79089B (Fig. 1). In the *in vivo* studies, the hypertensive effect of big ET-1 midon did not affect the ET-1 induced hypertensive effects. Therefore, the present study was carried out to provide the direct evidence that big ET-1 was converted by a specific ECE which is different from neutral endopeptidase.

Materials and Methods

Endothelin Converting Enzyme (ECE) Inhibition Assay

Endothelin converting enzyme (ECE) inhibition assay was performed as described previously[11]. Briefly, human big ET-1 (50 ng) was incubated with the bovine carotid endothelial cell homogenate preparation (20~30 μg protein) for 16 hours at 37°C in 250 μl of 50 mM Tris-HCl buffer (pH 7.0) containing 0.5 mM *p*-chloromercuriphenylsulfonic acid and 0.05 mM diisopropylfluorophosphate. Twenty microliters of the reaction mixture was diluted with 80 μl of RIA buffer (50 mM

Fig. 1. Proposed chemical structure of WS79089B.



† WS79089B (free form) is FR901532 and sodium salt of WS79089B is FR901533

Tris-HCl buffer containing 0.1% BSA, 0.1% Triton X-100 and 1 mM EDTA). Fifty microliters of anti-ET-1 serum (antisera against the C-terminal peptide of ET-1 (16~21), final dilution, 1 : 20,000) and 50 μl of ^{125}I-ET-1 (Amersham Japan, 37 KBq/10 ml) were added and incubated for 2 hours at 4°C. After 2 hours, 0.5 ml of Amerlex-M donkey anti-rabbit (Amersham Japan, 1/4 conc.) was added, the tubes were mixed and incubated for 10 minutes at room temperature. After magnetic separation (Amerlex-M system) the supernatant was removed and the immunocomplex was counted in a gamma counter (Packard Auto Gamma Model 5650).

Bovine, Human and Rat Endothelial Cell Cultures

Endothelial cells from bovine carotid artery were cultured as previously described[11]. Human and rat endothelial cells were isolated by gentle scraping of the intimal surface of the human umbilical cord veins and rat abdominal aortas with scalpel blade under stelile conditions. The cells were grown in DULBECCO's modified EAGLE's minimum essential medium supplemented with 20% (v/v) fetal bovine serum and 50 U/ml penicillin, 50 μg/ml streptomycin in 25 cm^2 culture flasks at 37°C in a humid atmosphere (5% CO_2-95% air). Endothelial cells were identified by the typical phase contrast cobblestone morphology and immunofluorescence to Factor VIII related antigen. Cells were subcultured by trypsinization and used between passages 6 to 8.

Rat Mesangial Cell Cultures

The isolation of glomeruli and culture of mesangial cells methods (explant growth methods) were essentially the same as described by J. I. KREISBERG[12,13].

The glomeruli were isolated from the kidney of male Sprague-Dawley rats weighing 50~70 g (3~4 weeks). Under ether anesthesia, rats were exsanguinated, and the kidneys were immediately removed and the renal capsule was removed. The cortex was excised and minced into small pieces (< 1 mm) under sterile conditions. The mince was mashed through a 200 mesh sterile stainless sieve with an angulated spatula and suspended in cold RPMI1640 medium and centrifuged at 800 rpm for 5 minutes after which supernatant was removed. The pellet was gently resuspended RPMI1640 medium supplemented with 20% fetal calf serum and 50 U/ml penicillin, 50 μg/ml streptomycin and grown at 37°C in a humid atmosphere (5% CO_2-95% air). The mesangial cells were obtained by culturing isolated glomeruli at 37°C for 3~5 weeks in RPMI1640 medium supplemented with 20% fetal calf serum and 50 U/ml penicillin in a humid atmosphere (5% CO_2-95% air). The obtained mesangial cells were used for the experiments for ECE activity between passages 5 and 10.

The Cellular Conversions of Big ET-1 to Mature ET-1

Assay of big ET-1 converting activity was performed as described previously[11]. For the measurement of cellular big ET-1 converting activity, a synthetic human big ET-1 (Peptide Institute) was exogenously supplied (50 ng/well) and incubated with confluent human endothelial cells, bovine endothelial cells, rat endothelial cells and rat mesangial cells in 96 well plates at 37°C for 16 hours, and the formed ET-1 was measured by a specific radio immunoassay (RIA) for the carboxyl terminal tail of ET-1 using the antiserum[11].

Measurement of Plasma Concentration of Drugs

Male Sprague-Dawley rats weighing 200~250 g were administered intravenously 0.5 ml of saline solution of Na salt of WS79089B (FR901533) or phosphoramidon (10 mg/kg, respectively). Blood was withdrawn through orbital vein in heparinized capillary tubes (Drummond Scientific Co. Ltd.) at 0, 5, 15, 30, 60, 120 and 240 minutes after the i.v. injection and after sealing one side of the capillary tubes by tube sealer (seal-ease; Becton Dickinson Co. Ltd.) centrifuged at 4°C, 500 $\times g$ for 10 minutes. Plasma portion of the capillary tubes were cut off by the ampoule cutter and 20 μl of plasma was extracted with 80 μl of methanol, centrifuged at 4°C, 500 $\times g$ for 10 minutes and the supernatant was stored at −20°C until analyzed. Plasma levels of WS79089B and phosphoramidon were measured by HPLC analyses using a Hitachi Model 655 pump. A steel column (4.00 mm diameter, 250 mm length) packed with LiChrospher 100 RP-18 (E. Merck) was used at a flow rate of 1.0 ml/minute. Ten μl of the supernatant was injected into HPLC. The mobile phase for measurement of WS79089B was an aqueous solution of 75% methanol with 0.1% trifluoroacetic acid (TFA) and the detector wavelength was set at 245 nm (retention time: 6.2 minutes). The mobile phase for measurement of phosphoramidon was aqueous solution of 10% acetonitrile with 10 mM Tris-HCl buffer (pH 7.0) and the detector wavelength was set at 215 nm (retention time: 8.1 minutes).

Vasoconstrictor Effect of Big ET-1 in Rats

Male Sprague-Dawley rats (200~250 g) were anesthetized with pentobarbital sodium (Nembutal-Abbott, 50 mg/kg of body weight, i.p.). A femoral artery catheter was implanted for monitoring arterial pressure and heart rate, and a femoral vein catheter was used for injection of saline or drugs. Blood pressure and heart rate (data not shown) were recorded from the femoral artery through a cannula (PE-50) connected to a pressure transducer (Nihon Koden, PT-200T) coupled to a Biophysiograph RM 6000 system (Nihon Koden). Two groups of rats were injected with the following: big ET-1 (1 nmol/kg) alone (n = 11) and sodium salt of WS79089B (FR901533; 1, 3.2 and 10 mg/kg, n = 5~6) or phosphoramidon (1, 3.2 and 10 mg/kg, n = 3~5) i.v. bolus injection 2 minutes prior to big ET-1 (1 nmol/kg) challenge.

Statistical Analysis

Results are expressed as means ± S.E. of number of experiments as indicated. Statistical analysis was done

by means of Student's *t*-test.

Results

In Vitro Kinetic Study

We studied the potency of WS79089B to inhibit ECE by comparing it with that of phosphoramidon. In the previous report we described that WS79089B selectively inhibited ECE activity with an IC_{50} value of 1.4×10^{-7} M, while it did not inhibit collagenase and NEP activities below 4.9×10^{-5} M[11]. Our results showed that WS-79089B is at least 3 times more potent than phosphoramidon in ECE inhibition of bovine carotid endothelial cell homogenate membrane fractions[11].

The dependence of inhibition on the substrate concentration was examined for ECE activities. Fig. 2 shows double-reciprocal plots of kinetic data for conversion of big ET-1 to ET-1 by ECE obtained from bovine carotid endothelial cells. The *Km* value of ECE is approximately 1×10^{-6} M in our assay system. Lineweaver-Burk analysis revealed that WS79089B behaved as a competitive inhibitor of ECE, with *Ki* values of 8.9×10^{-8} M.

Duration of Plasma Concentration

In the first series of experiments, plasma concentration of WS79089B was compared with phosphoramidon when administered intravenously to Sprague-Dawley rats. WS79089B was administered in solution as sodium salt (FR901533). The plasma concentration values of WS79089B were slightly higher than phosphoramidon, obtained from 0 time to 2 hours after dosing (Fig. 3). Phosphoramidon blocks the *in vivo* hypertensive effect of exogenously added big ET-1[8~10]. Serum concentrations of WS79089B declined to 4.5 μg/ml 2 hours after dosing, a concentration which is in excess of 60 times the concentration required for effective inhibition of ECE. Thus WS79089B is expected to be more effective than phosphoramidon in the *in vivo* evaluation.

In Vivo ECE Inhibition

Intravenous administration of big ET-1 to anesthetized rats caused a dose-dependent and long-lasting hypertensive effect the magnitude of which was similar to that evoked by ET-1. On the contrary big ET-1 did not cause the transient hypotensive effect observed with ET-1[1,9] (Fig. 4).

In the present study, a bolus of 1 nmol/kg of big ET-1 was used. When the sodium salt of WS79089B (FR901533) and phosphoramidon were injected in the dose of 1.0, 3.2 and 10.0 mg/kg, the hypertensive effects of big ET-1 were markedly suppressed in a dose-dependent manner (Fig. 4). It was reported that phsphoramidon did not affect the ET-1 induced hypertensive effect[9]. We also confirmed that Na salt of WS79089B (FR901533) did not affect the ET-1 induced hypertensive effect at the dose of 10 mg/kg (Fig. 5). The mean ED_{50} values of WS79089B and phosphoramidon at 2, 4, 6, 10, 20 and 30 minutes post big ET-1 administration are summarized in Table 1. The fall in mean blood pressure produced by phosphoramidon (10 mg/kg) was more gradual, with the maximum reduction achieved 10 minutes after medication. However, the efficacy of WS79089B was relatively weak in comparison with phosphoramidon, especially when

Fig. 2. Lineweaver-Burk plot for inhibition of endothelin converting enzyme of bovine endothelial cells.

WS79089B (○: 9.5×10^{-8} M, ●: 3.8×10^{-7} M), absence of inhibitor (▲).

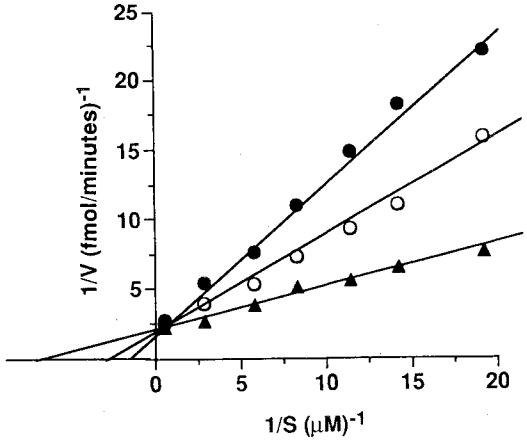


Fig. 3. Comparison of the plasma concentrations by intravenous administration of WS79089B and phosphoramidon in SD rats.

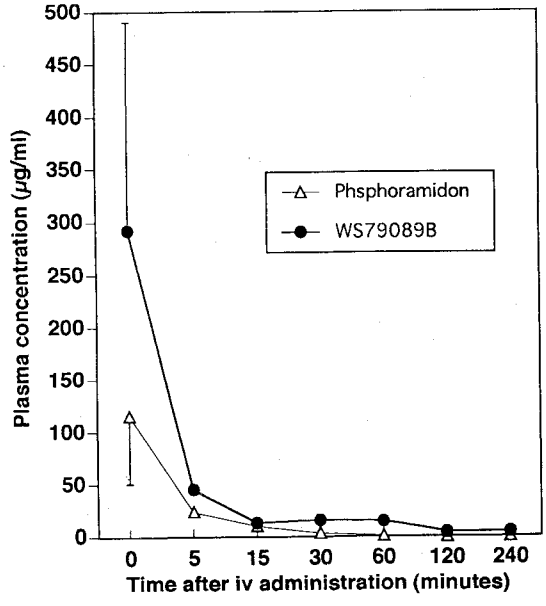
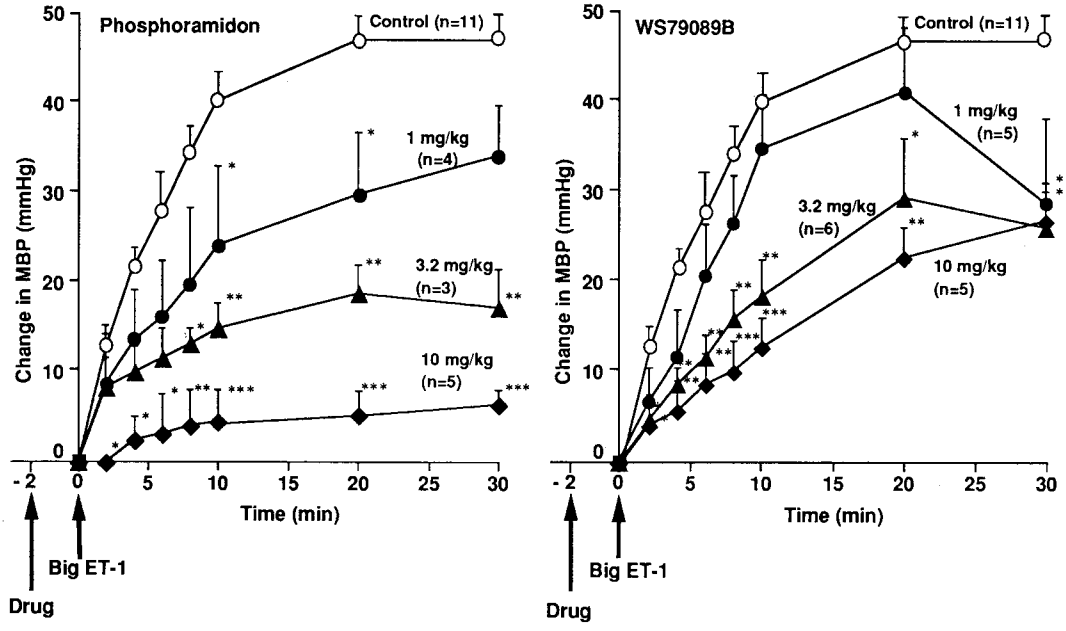


Fig. 4. Effects of WS79089B and phosphoramidon on the pressor response induced by big ET-1 in SD rats.
At the time indicated by the arrows, drugs and big ET-1 were injected iv, respectively.

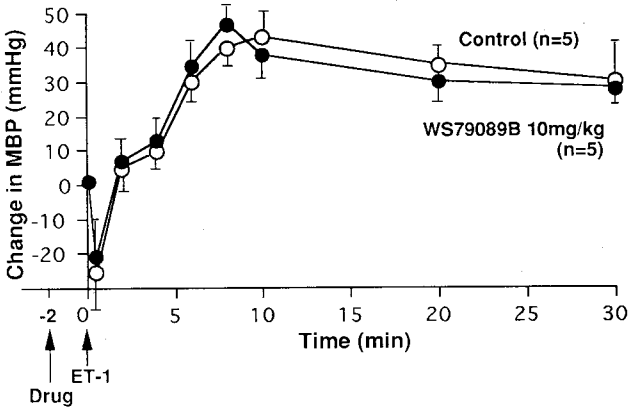


*p < 0.05, **p < 0.01, *** p < 0.001 vs control

Table 1. The mean ED_{50} values of WS79089B and phosphoramidon of hypotensive effects for big ET-1 challenge.

Time after big ET-1 challenge (minutes)		2	4	6	8	10	20	30
ED_{50} (mg/kg)	Na salt of WS79089B (FR901533)	1.31	1.69	2.42	2.90	2.89	>10	>10
	Phosphoramidon	4.67	2.79	2.22	1.82	1.81	2.22	2.07

Fig. 5. Effect of WS79089B on the pressor response induced by ET-1 in SD rats.



the efficacy of 10 mg/kg of WS79089B was compared with the same dose of phosphoramidon. These findings were not in agreement with the data on plasma concentrations and *in vitro* studies data. The above mentioned hemodynamic *in vivo* experiments were carried out using rats and the *in vitro* ECE inhibition studies employed bovine ECE.

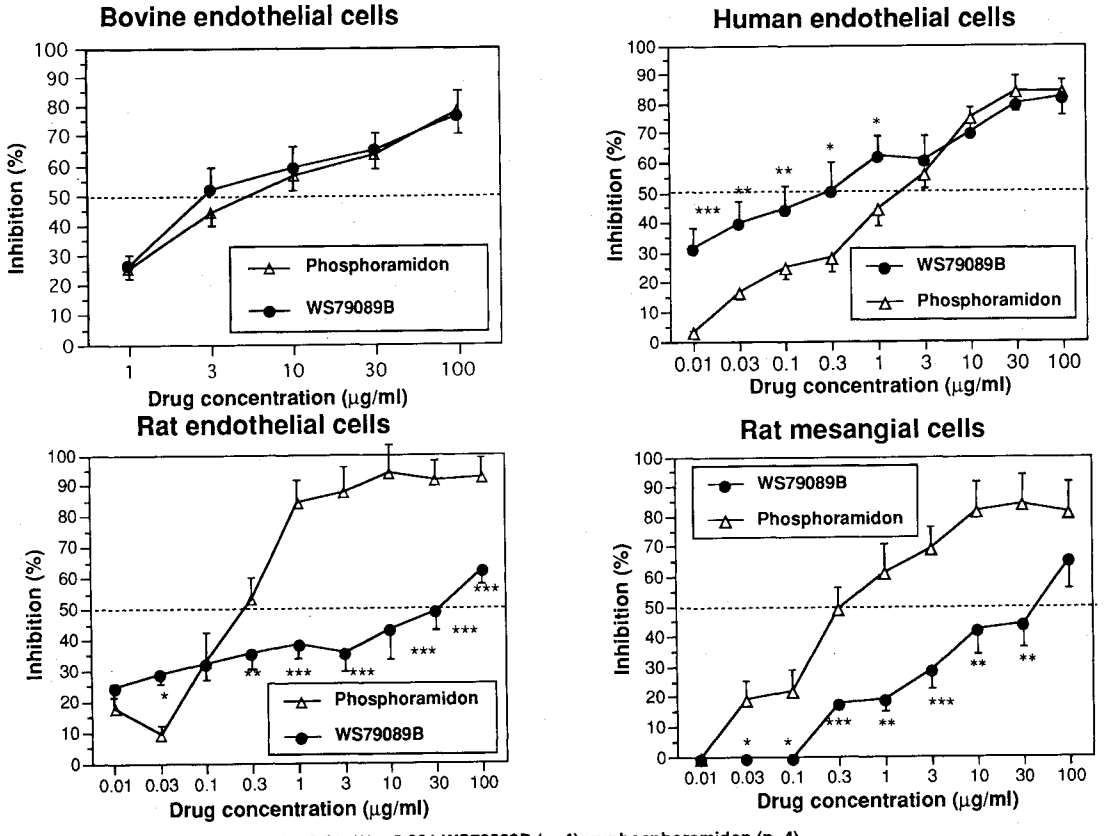
Effect of WS79089B on ECE Activities in Various Different Species Cells

To clarify the reason why the efficacy of WS79089B was relatively weak in comparison with phosphoramidon, we checked the species difference of WS79089B in ECE inhibition. We examined the inhibition ability of WS79089B for rat, bovine and human ECE-positive cells to convert exogenously supplied big ET-1. The results are shown in Fig. 6. Both WS79089B and phosphoramidon efficiently inhibited the production of ET-1 with similar potency in bovine and human endothelial cells. On the contrary, WS79089B was less potent when compared with phosphoramidon in rat endothelial cells and rat mesangial cells. Approximately 100-fold higher concentration of WS79089B was required for 50% inhibition of rat ECE activities compared with phosphoramidon when big ET-1 was exogenously supplied to the cells.

Discussion

In the present study we have demonstrated that an

Fig. 6. Effects of WS79089B and phosphoramidon on the ECE activities in various species different cells.



intravenously bolus injection of WS79089B had similar protective effect as the same dose of phosphoramidon in antagonizing the pressor effect induced by big ET-1 without affecting the pressor effects produced by ET-1. We previously repoted that phosphoramidon is a general neutral endopeptidase inhibitor and WS79089B is the only highly selective ECE inhibitor in *in vitro* studies[11]. It has been suggested that the marked pressor effects of big ET-1 are due to its conversion to ET-1[7,8]. It seemed that exogenously administrated big ET-1 is mainly converted to bioactive ET-1 by WS79089B and phosphoramidon-sensitive ECE in vascular tissues, and gradually increased ET-1 causes the pressor effects in cardiovascular systems. Our observations support and confirm that big ET-1 is cleaved *in vitro* and *in vivo* by specific ECE which appears to be distinct from general neutral endopeptidase. In this context it was reported that the selective neutral endopeptidase thiorphan did not show the ECE inhibitory effect[15].

It is reported that renal epithelial cell lines of MDCK and LLC-PK1 cells possess ECE activities[14]. We also demonstrated that rat renal mesangial cells possess remarkable ECE activity. In the present study, we investigated the effect of WS79089B on ECE activity by using the cultured endothelial cells and mesangial cells. We found that phosphoramidon efficiently suppressed the ET-1 release from cultured human, bovine, rat endothelial cells and rat mesangial cells. On the contrary, ECE inhibition activities of WS79089B was relatively weak in rat endothelial cells and rat mesangial cells. Our results showed that is probably a species difference in ECE activities or there is the possibility of ECE subtypes. The precise mechanisms of the above difference is unclear.

Recently ECE was purified from rat lung microsomes to homogeneity of 130 KDa single band by SDS-PAGE analysis[16]. More recently the cloning and functional expression of cDNA encoding a rat ECE[17] also have been repoted YANAGISAWA's group cloned and functional expressed a cDNA encoding a bovine adrenal cortex[18]. The precise mechanism of ECE activation has not be elucidated and the presence of ECE subtypes still remaines largely unexplored.

In our study, we demonstrated that WS79089B is a selective ECE inhibitor both *in vitro* and *in vivo*. Therefore, WS79089B will be a useful tool for clarifying the physiological and pathophysiological roles of ET-1 in vascular as well as nonvascular tissues.

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