WS-7338, NEW ENDOTHELIN RECEPTOR ANTAGONISTS ISOLATED FROM *Streptomyces* sp. No. 7338

II. BIOLOGICAL CHARACTERIZATION AND PHARMACOLOGICAL CHARACTERIZATION OF WS-7338 B

SUSUMU MIYATA, MICHIZANE HASHIMOTO, KEIKO FUJIE, MOTOAKI NISHIKAWA, SUMIO KIYOTO*, MASAKUNI OKUHARA and MASANOBU KOHSAKA

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba City, Ibaraki 300-26, Japan

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WS-7338, produced by *Streptomyces* sp. No. 7338, was found to be a competitive and specific antagonist against ET-1 receptors in *in vitro* studies and WS-7338 B is also active *in vivo*. Furthermore, WS-7338 B was a specific antagonist for vascular ET-1 receptors (ET_A receptors) and significantly prevented the accumulation of intracellular inositol 1,4,5-triphosphate (IP_3) in endothelin treated rat aorta tissues.

Three distinct human ET related genes have been identified, coding for peptides, ET-1, ET-2 and ET-3[1]. Endothelial cells can only release ET-1. The expression of ET-2 has not been demonstrated to be physiological in any normal tissue yet, while ET-3-like immunoreactive material has been found in porcine brain homogenate[2]. Intravenously infused ET-1 induces a profound pressor effect[3], suggesting a potential role for ET-1 in the pathogenesis of systemic hypertension. Furthermore, ET-1 may have a possible role in development of cerebral vasospasm after subarachnoid hemorrhage[4], myocardial infarction[5] and acute renal failure[6]. The precise pathological mechanism of action of ET-1 is not known. The presence of ET-1 binding sites in blood vessels and in several organ systems suggests ET-1 may have important regulatory functions.

In this paper, we described the ET-1 antagonistic activity of WS-7338 B. The results indicated that WS-7338 B can displace the binding of ET-1 at all receptor sites in rat aorta and human aorta. Moreover, WS-7338 B can attenuate the second messenger (inositol 1,4,5-triphosphate (IP_3)) production induced by ET-1.

Materials and Methods

ET Receptor Assay

ET receptor binding assay was described in detail in a preceding paper[7]. Receptors of porcine brain membranes, kidney membranes, lung membranes and human aorta membranes were prepared in the same procedures mentioned in the same paper[7].

Rabbit Aorta Constriction *In Vitro*

Thoracic aorta were isolated from freshly sacrificed male albino rabbits (11 weeks old) and cut into 2 mm width and 25 mm length of arterial segments. The tissues were suspended in 25 ml organ chambers filled with Krebs-Ringer solution (113 mM NaCl, 4.8 mM KCl, 2.2 mM glucose) maintained at 37°C and gassed with 95% O_2/5% CO_2. A preload of 1 g was applied after the aorta had been conditioned by

application of increasing concentration of KCl. Contractions were measured as an increase in isometric tension. WS-7338 B was tested against the contractile response of ET-1 (3.2×10^{-9} M). Synthetic ET-1 was purchased from Peptide Institute Inc. (Osaka, Japan). WS-7338 B was added after the full contractile response induced by ET-1.

Measurement of Inositol Phosphates

The tissue level of inositol monophosphate (IP) was determined by a slight modification of the procedure of MICHAEL J. BERRIDGE (1983)[8]. Briefly, rings of rats aorta were incubated at 37°C for 3 hours in Krebs solution containing *myo*-[2-^{3}H]inositol(4 μCi/0.5 ml for 1 ring). *myo*-[2-^{3}H]Inositol was purchased from New England Nuclear (15 Ci/mmol). The tissues were transferred into Krebs solution containing 10 mM LiCl and placed on ice for 30 minutes. ET-1(3×10^{-9} M) and WS-7338 B (10^{-4} M) were added to the solution and the mixture was incubated at 37°C for 60 minutes. Each tissue was then homogenized in 1.0 ml of 10% trichloroacetic acid with a Pyrex-glass homogenizer. The homogenates were centrifuged at $300 \times g$ for 15 minutes and the supernatants were extracted 3 times with 2.0 ml of ether. Four ml of 5 mM $NaHCO_3$ was added to 1 ml of the aqueous phase and the mixture was transferred to a column containing anion exchange resin (Bio-Rad AGI-X8, 100~200 mesh, formate form). The column was washed with 10 ml of water to remove free *myo*-[2-^{3}H]inositol. Labeled *myo*-[2-^{3}H]inositol phosphates (IP_1, IP_2 and IP_3) were eluted by the stepwise addition of 6 ml of solutions containing increasing levels of ammonium formate, IP_1 was eluted with 0.1 M formic acid plus 0.2 M ammonium formate, for IP_2 0.1 M formic acid plus 0.4 M ammonium formate was used and for IP_3 0.1 M formic acid plus 1.0 M ammonium formate. Scintillation cocktail (Aquasol-2, New England Nuclear) was added to the eluate and the samples were counted in a liquid scintillation counter (Packard TRI-CARB 4530).

Fig. 1. Scatchard plot for specific [^{125}I]-ET-1 binding in porcine aorta membranes.

$K_d = 0.38 \times 10^{-9}$.

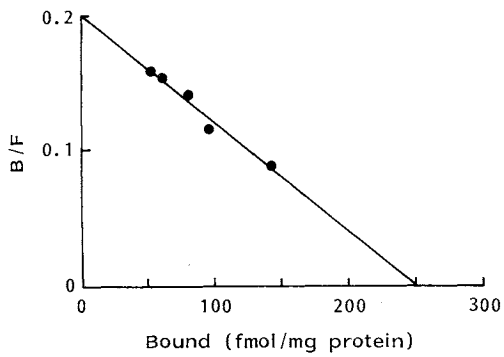


Table 1. Comparison of IC_{50} values of WS-7338 B for various tissue receptors of ET-1 and ET-3.

	IC_{50} (μM)				
	Porcine				Human aorta
	Lung	Aorta	Kidney	Brain	
ET-1	>130	0.27	50	>130	0.49
ET-3	>130	—	>130	119	—

—: No specific binding.

Table 2. Comparison of binding affinity to various ligand receptors of WS-7338 B (IC_{50} M).

Ligand	(receptor)	WS-7338 B
ET-1	(porcine aorta)	2.7×10^{-7}
FGF	(BHK cells)	$>6.6 \times 10^{-5}$
Ang II	(bovine adrenal)	$>6.6 \times 10^{-5}$
BK	(guinea-pig ileum)	$>6.6 \times 10^{-5}$
SP	(guinea-pig lung)	$>6.6 \times 10^{-5}$

Fig. 2. Lineweaver-Burk plot for ET-1 antagonism on porcine aorta receptor with WS-7338 B.

□ WS-7338 B 1.6×10^{-7} M, ● WS-7338 B 3.2×10^{-7} M, ○ control.

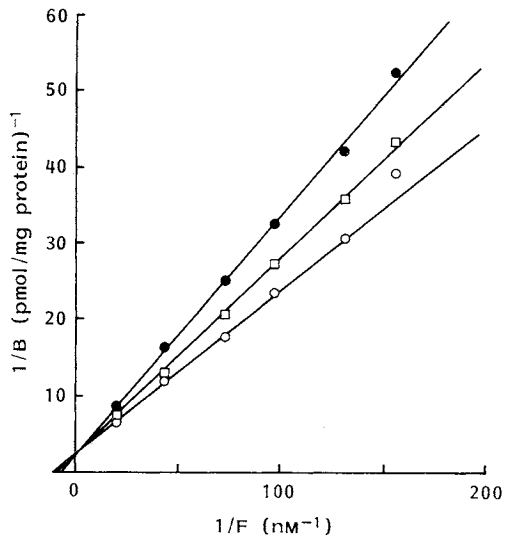
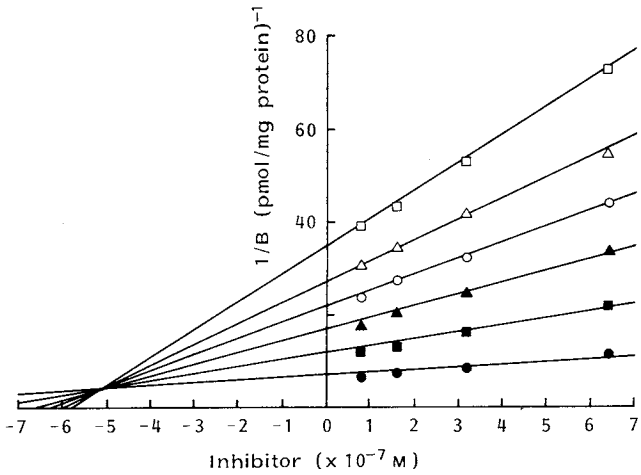


Fig. 3. Dixon plot for ET-1 antagonism on porcine aorta receptors with WS-7338 B.

The abscissa represents the concentration of WS-7338 B. ET-1 concentrations: ● 200 pM, ■ 100 pM, ▲ 67 pM, ○ 50 pM, △ 40 pM, □ 33 pM.



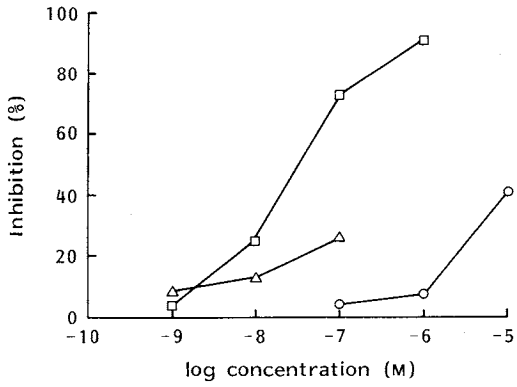
Results and Discussion

High-affinity binding site for ET-1 were present in porcine aorta membrane receptors. Scatchard analysis of the binding data in the porcine aorta revealed a straight line, indicating a single class of binding site and the equilibrium dissociation constant was 0.38×10^{-9} M, the maximal specific binding was 250 fmol/mg protein (Fig. 1).

We demonstrated specificity of WS-7338 B for antagonism of binding in the receptor membranes of lung, aorta, kidney and brain (Table 1). WS-7338 B is a specific aorta ET-1 antagonist. Moreover, WS-7338 B was shown to be active in the human aorta receptor membranes binding assay, with an IC_{50} value of 4.9×10^{-7} M (Table 1). However, WS-7338 B had no affinity for the FGF receptors, angiotensin II (Ang II) receptors, bradykinin (BK) receptors and substance P (SP) receptors (Table 2). These results suggest that WS-7338 B is a selective antagonist for ET-1 at the vascular aorta membrane receptors.

Fig. 4. Effect of WS-7338 B on the contractile response of rabbit aorta induced by ET-1.

□ Nitroglycerin, △ nilvadipine, ○ WS-7338 B.

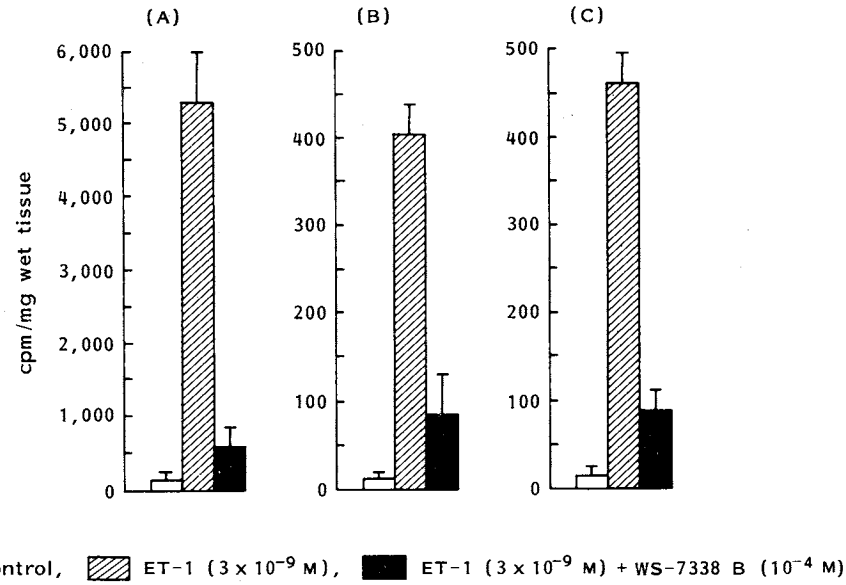


Inhibition of ET-1 binding in the porcine aorta membane receptors by WS-7338 B was shown to be competitive in the Lineweaver-Burk plot (Fig. 2). The *Ki* value was calculated as 5.1×10^{-7} M from the Dixon plot (Fig. 3).

The dose-response curve for WS-7338 B against 3.2×10^{-9} M ET-1-induced rabbit aorta constriction was studied in drug pretreatment experiments (Fig. 4). WS-7338 A inhibited ET-1-induced rabbit aorta constriction in a dose-dependent manner.

The current view is that ET-1 binds to a specific membrane receptors, leading to intracellular

Fig. 5. Inhibitory effect of WS-7338 B on IP_1 (A), IP_2 (B) and IP_3 (C) formation induced by ET-1.



biochemical signals involving the activation of inositol phosphates and diacylglycerol and the elevation of calcium levels from intracellular calcium stores[9]. In this respect, we demonstrated the ET-1-induced increase of inositol phosphates (IP_1, IP_2 and IP_3) in rat aorta rings, and the inhibition of this increase by the ET-1 antagonist WS-7338 B. Inositol phosphates (IP_1, IP_2 and IP_3) formation was blocked by WS-7338 B in a similar intensity (Fig. 5). The concentrations of ET-1 and WS-7338 B used in rabbit aorta contractile response experiments (Fig. 4) were almost same that used in the inositol phosphate formation experiments (Fig. 5). Thus IP_3 formation blocking activity may be an essential element for ET-1 receptor antagonism.

Most recently ET receptors were classified into three distinct subclasses (ET_A, ET_B and ET_C) on the basis of agonist affinities (ET-1 > ET-3; ET-1 = ET-3; ET-3 > ET-1). It was recommended that an ET-1 specific type be called ET_A receptors, nonselective receptors be named ET_B and ET-3 specific receptors be designated ET_C[10,11]. A large amount of ET_B receptors are expressed in lung, kidney and brain, on the other hand, vascular aorta tissues express ET_A receptors[12,13]. As shown in Table 1, WS-7338 B is active against aorta receptors but not against lung, kidney and brain receptors. These findings suggest that WS-7338 B is an ET_A specific receptor antagonist.

Intravenous bolus injection of ET-1 cause a transient depressor response (based on ET_B receptor) followed by a sustained pressor response (based on ET_A receptor). In the *in vivo* evaluation experiment, ET-1 (3.2 μg/kg) is given intravenously to SHR rats 10 minutes after administration of WS-7338 B (10 mg/kg iv). Pretreatment with 10 mg/kg of WS-7338 B reduces the pressor effect of ET-1 (55% inhibition), in contrast WS-7338 B has no effect on the depressor response of ET-1. The results of the *in vivo* study are in agreement with the data given by the *in vitro* receptor binding studies (Table 1) mentioned above.

In conclusion, it may be worthwhile investigating the chemical modification of WS-7338 B in order to increase the ET_A receptor specific antagonism activity or to study the structure-activity relationships of derivatives of this unique lead compound. Our final goal is to create a more potent ET antagonist in

order to clarify the natures of endothelin pathophysiology.

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