WS009 A AND B, NEW ENDOTHELIN RECEPTOR ANTAGONISTS ISOLATED FROM *Streptomyces* sp. No. 89009

II. BIOLOGICAL CHARACTERIZATION AND PHARMACOLOGICAL CHARACTERIZATION OF WS009 A AND B

SUSUMU MIYATA, MICHIZANE HASHIMOTO, KEIKO FUJIE, MIWAKO SHOUHO, KEIZO SOGABE, SUMIO KIYOTO*, MASAKUNI OKUHARA and MASANOBU KOHSAKA

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

(Received for publication February 12, 1992)

WS009 A and B, produced by *Streptomyces* sp. No. 89009, were found to be competitive and specific antagonists against endothelin (ET)-1 receptors in *in vitro* studies and also active in *in vivo* studies. Furthermore, WS009 A and B were specific antagonists 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.

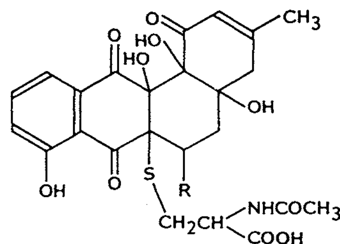
Endothelin (ET) belongs to a family of 21 amino-acid residue peptides with important cardiovascular actions. The existence of several ET receptors was first hypothesized from pharmacological analysis and then confirmed by the cloning and sequencing of two types of receptor molecules[1,2]. The first, designated ET_A, shows high selectivity for ET-1, with the order of affinity of the ET isoforms being ET-1>ET-2>ET-3[3]. The other ET_B is non-selective for the ET isoforms[4]. The ET_A receptor is responsible for the action of ETs on vascular smooth muscle cells and on atrial cells[5]. The nonselective ET_B receptor subtype is mainly expressed in neurons and astrocytes[5]. ET_A and ET_B receptors are coupled with phospholipase C.

The mechanism of action of ET-1 has been shown to involve activation of phospholipase C with the release of inositol phosphates and diacylglycerol and elevation of calcium from intracellular stores[6]. This would in turn activate protein kinase C and, subsequently, phosphorylation of myosin light chains to initiate contraction of smooth muscle cells[6].

Intravenously infused ET-1 induces a profound pressor effect[7], 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[8], myocardial infraction[9] and acute renal failure[10]. The precise pathophysiological roles of ET-1 are as yet unclear. The presence of ET-1 binding site in blood vessels and in several organ systems suggests ET-1 may have important regulatory functions.

In this paper, we describe the ET-1 antagonistic activities of WS009 A and B. The results indicated

Fig. 1. Structures of WS009 A and B.



WS009 A R=H
WS009 B R=OH

that WS009 A and B can displace the binding of ET-1 at all receptor sites in various vascular tissues. Moreover, WS009 A can attenuate the second messenger (IP_3) production induced by ET-1 in rat aorta tissues. The chemical structures of WS009 A and B are shown in Fig. 1.

Materials and Methods

Receptor Binding Assay

The ET receptor binding assay is described in detail in a preceding paper[11]. All other assays were performed using different kinds of tissue membranes in the receptor binding assays; porcine aorta for ET, guinea-pig lung for SP-P (substance P: NK-1), guinea-pig lung for SP-E (eledoisin: NK-2), rat lung for Ang II (angiotensin II), rat kidney for AVP (arginine vasopressin), guinea-pig ileum for BK (bradykinin) and baby hamster kidney cells for FGF (fibroblast growth factor) using the procedures as described previously[11]. In WS009 A profiling experiments various kind of tissue membranes were used as follows: porcine (aorta, brain, lung and kidney), rabbit (renal artery and kidney), canine (aorta, kidney and basilar artery) and human (aorta, kidney and liver). Binding assays were performed by incubating freshly prepared membranes with varying concentrations of ligands (SP-P 10^{-9} M, SP-E 10^{-9} M, AVP 10^{-9} M, BK 3×10^{-11} M, ET 10^{-11} M and FGF 10^{-10} M). The binding studies described above were conducted at 25°C except for SP-P receptor and SP-E receptor binding which were performed at 4°C.

[^{125}I]Endothelin-1 (2,200 Ci/mmol), [^{125}I]endothelin-2 (2,200 Ci/mmol), [^{125}I]endothelin-3 (2,200 Ci/mmol), [2-*prolyl*-3,4-^{3}H]substance P (55 Ci/mmol), [*prolyl*-3,4-^{3}H]eledoisin (55 Ci/mmol), [*tyrosyl*-3,5-^{3}H(N)]angiotensin II (50 Ci/mmol), [*phenylalanyl*-3,4,5-^{3}H(N)]8-L-arginine vasopressin (70 Ci/mmol) and [2,3-*prolyl*-3,4-^{3}H(N)]bradykinin (80 Ci/mmol) were purchased from New England Nuclear Research Products (Daiichi Kagaku).

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 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 and 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. WS009 A and B were tested against the contractile response of ET-1 (3.2×10^{-9} M). Synthetic ET-1 was purchased from Peptide Institute Inc. (Osaka, Japan). WS009 A and B were added after the full contractile response induced by ET-1.

Vasoconstrictor Effect of ET-1 in Rats

Male spontaneously hypertensive (SHR) rats (200~250 g) were anesthetized with pentobarbital sodium (Nembutal, Abbott, 50 mg/kg of body weight). 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 was 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. Two groups of rats were injected with the following: ET-1 (1 nmol/kg) alone ($n=5$) and ET-1 (1 nmol/kg) with WS009 A (10 mg/kg) iv bolus injection ($n=5$).

Measurement of Inositol Phosphates

The tissue level of inositol monophosphate (IP) was determined by a slight modification of the procedure of BERRIDGE *et al.* (1983)[12]. Briefly, aorta rings of male Sprague-Dawley rats (200~250 g) were incubated at 37°C for 3 hours in Krebs solution containing *myo*-[2-^{3}H]inositol (4 μCi/0.5 ml/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 WS009 A (10^{-4} M and 10^{-3} 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).

Results and Discussion

We demonstrated specificity of WS009 A for antagonism of binding in the various kind of tissue membranes such as kidney, liver, lung, brain, aorta and other vascular tissues (renal artery and basilar artery) (Table 1). WS009 A inhibits [^{125}I]ET-1 and [^{125}I]ET-2 binding in aorta and other vascular receptors. WS009 A is a selective ET antagonist on vascular tissue binding sites. Moreover, WS009 A was shown to be active in the human aorta membranes binding assay, with an IC_{50} value of 1.3×10^{-5} M (Table 1).

Inhibition of ET-1 binding in the porcine aorta membranes by WS009 A was shown to be competitive in the Lineweaver-Burk plot with a *Ki* value of 5.8×10^{-6} M (Fig. 2).

The dose-response curve for WS009 A and B against ET-1-induced rabbit aorta tissues constriction were studied in drug pretreatment experiments (Fig. 3). WS009 A inhibited rabbit aorta constriction induced by ET-1 (3.2×10^{-9} M) in a dose-dependent manner.

The specificity of WS009 A and B for vascular tissues binding sites compared with other common peptides was demonstrated using several radioligand binding assays (Table 2) and they were shown to have no affinity for the other peptide binding sites such as substance P (SP-P), eledoisin (SP-E), angiotensin II (Ang II), arginine vasopressin (AVP), bradykinin (BK) and fibroblast growth factor (FGF). These results suggest that WS009 A and B are selective antagonists for ET-1, ET-2 at the vascular receptors.

Table 1. ET receptor-binding profiles of WS009 A.

Ligand	(tissue)	IC_{50} (M)	Ligand	(tissue)	IC_{50} (M)
ET-1	(porcine aorta)	5.8×10^{-6}		(porcine kidney)	$>8.0 \times 10^{-4}$
	(porcine brain)	$>8.0 \times 10^{-4}$		(rabbit kidney)	$>8.0 \times 10^{-4}$
	(porcine lung)	$>8.0 \times 10^{-4}$		(canine aorta)	6.9×10^{-5}
	(porcine kidney)	$>8.0 \times 10^{-4}$		(canine kidney)	$>8.0 \times 10^{-4}$
	(rabbit renal artery)	2.1×10^{-5}	ET-3	(porcine aorta)	—*
	(rabbit kidney)	$>8.0 \times 10^{-4}$		(porcine brain)	$>8.0 \times 10^{-4}$
	(canine aorta)	1.2×10^{-5}		(porcine lung)	$>8.0 \times 10^{-4}$
	(canine basilar artery)	3.0×10^{-6}		(porcine kidney)	$>8.0 \times 10^{-4}$
	(human aorta)	1.3×10^{-5}		(rabbit kidney)	$>8.0 \times 10^{-4}$
	(human kidney)	$>8.0 \times 10^{-4}$		(canine kidney)	$>8.0 \times 10^{-4}$
	(human liver)	$>8.0 \times 10^{-4}$		(human aorta)	—*
ET-2	(porcine aorta)	6.9×10^{-6}		(human kidney)	$>8.0 \times 10^{-4}$
	(porcine brain)	$>8.0 \times 10^{-4}$		(human liver)	$>8.0 \times 10^{-4}$
	(porcine lung)	$>8.0 \times 10^{-4}$			

* No existence of specific binding for [^{125}I]ET-3.

Fig. 2. Lineweaver-Burk plot for ET-1 antagonism on porcine aorta receptors with WS009 A.

△ WS009 A 1.5×10^{-6} M, ▲ WS009 A 3.0×10^{-6} M, ● control. $Ki = 5.8 \times 10^{-6}$ M.

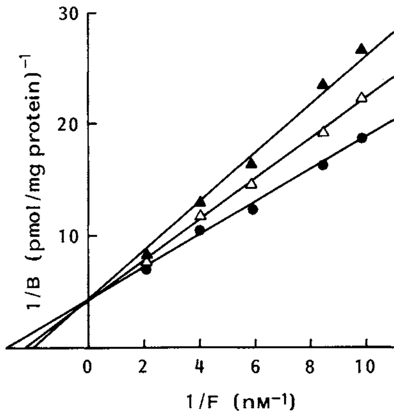


Fig. 3. Effect of WS009 A and B on the contractile response of rabbit aorta induced by ET-1.

△ Nilvadipine, ○ WS009 A, ● WS009 B.

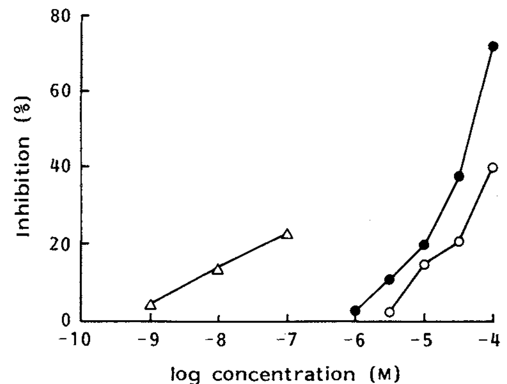


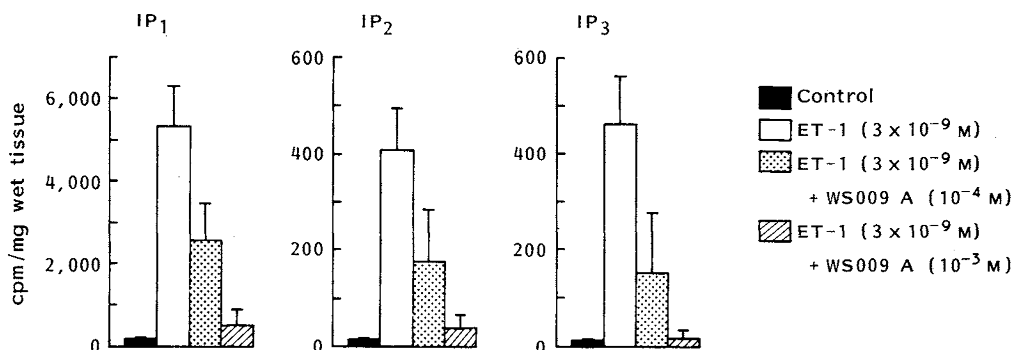
Table 2. Comparison of the receptor on specificity of WS009 A and B binding assays using different tissues.

Ligand	Ligand conc (M)	(tissue)	IC_{50} (M)	
			WS009 A	WS009 B
ET-1	10^{-11}	(porcine aorta)	5.8×10^{-6}	6.7×10^{-7}
SP-P (NK-1)	10^{-9}	(guinea-pig lung)	$>8.0 \times 10^{-4}$	$>8.0 \times 10^{-4}$
SP-E (NK-2)	10^{-9}	(guinea-pig lung)	$>8.0 \times 10^{-4}$	$>8.0 \times 10^{-4}$
Ang II	10^{-9}	(rat lung)	$>8.0 \times 10^{-4}$	$>8.0 \times 10^{-4}$
AVP	10^{-9}	(rat kidney)	$>8.0 \times 10^{-4}$	$>8.0 \times 10^{-4}$
BK	3×10^{-11}	(guinea-pig ileum)	$>8.0 \times 10^{-4}$	$>8.0 \times 10^{-4}$
FGF	10^{-10}	(BHK cells)	$>8.0 \times 10^{-4}$	$>8.0 \times 10^{-4}$

The current view is that ET-1 binds to specific receptors, leading to intracellular 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 WS009 A to a similar intensity (Fig. 4). The concentrations of ET-1 and WS009 A used in rabbit aorta contractile response experiments (Fig. 3) were almost the same that used in the inositol phosphate formation experiments (Fig. 4). Thus blocking of IP_3 formation may be an essential element for ET-1 receptor antagonism.

Most recently, two reports that describe distinct cDNA clones of ET receptor have been published[3,4]. The ET-1 selective receptor found in vascular smooth muscle receptors, is named the ET_A receptor, and the non-selective receptor is named the ET_B receptor. A large amount of the ET_B receptor is expressed in lung, kidney and brain, on the other hand, vascular aorta tissues express the ET_A receptor[3,4]. As shown in Tables 1 and 2, WS009 A and B are active against various vascular tissues such as aorta, renal artery and basilar artery. On the other hand WS009 A and B are not active against non-vascular tissues such as lung, kidney, liver and brain. These findings suggest that WS009 A and B are selective ET_A receptor antagonists.

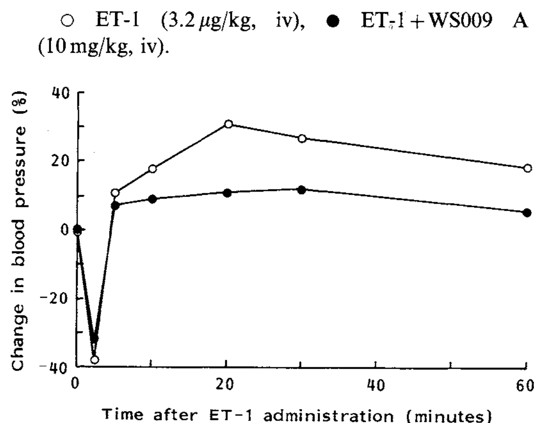
Fig. 4. Inhibitory effect of WS009 A on IP_1, IP_2 and IP_3 formation induced by ET-1.



Intravenous bolus injection of ET-1 causes a transient depressor response (due to the ET_B receptor) followed by a sustained pressor response (due to the ET_A receptor). In the *in vivo* evaluation experiment, ET-1 (3.2 µg/kg) is administered intravenously to SHR rats 10 minutes after administration of WS009 A (10 mg/kg iv). Pretreatment with 10 mg/kg of WS009 A reduces the pressor effect of ET-1, in contrast WS009 A has no effect on the depressor response of ET-1 (Fig. 5). The results of the *in vivo* study are in agreement with the data given by the *in vitro* receptor binding studies mentioned above.

Fig. 5. Effect of WS009 A on ET-1 induced hypotensive and hypertensive response in SHR rats.

○ ET-1 (3.2 µg/kg, iv), ● ET-1 + WS009 A (10 mg/kg, iv).



In conclusion, it may be worthwhile investigating the structure-activity relationships of these unique lead compounds. WS009 A and B are useful tools for investigating and clarifying the nature of endothelin pathophysiology.

References

1) WATANABE, H.; H. MIYAZAKI, M. KONDOH, M. MASUDA, S. KIMURA, M. YANAGISAWA, T. MASAKI & K. MURAKAMI: Two distinct types of endothelin receptors are present on chick cardiac membranes. Biochem. Biophys. Res. Commun. 161: 1252~1259, 1989
2) YANAGISAWA, M. & T. MASAKI: Molecular biology and biochemistry of the endothelin. Trends Pharmacol. Sci. 10: 374~378, 1989
3) ARAI, H.; S. HORI, I. ARAMORI, H. OHKUBO & S. NAKANISHI: Cloning and expression of a cDNA encoding an endothelin receptor. Nature 348: 730~732, 1990
4) SAKURAI, T.; M. YANAGISAWA, Y. TAKUWA, H. MIYAZAKI, S. KIMURA, K. GOTO & T. MASAKI: Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 348: 732~735, 1990
5) WEBB, D. J.: Endothelin receptors cloned, endothelin converting enzyme characterized and pathophysiological roles for endothelin proposed. Trends Pharmacol. Sci. 12: 43~46, 1991
6) TAKUWA, N.; Y. TAKUWA, M. YANAGISAWA, K. YAMASHITA & T. MASAKI: A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblast. J. Biol. Chem. 265: 7856~7861, 1989
7) YANAGISAWA, M.; H. KURIHARA, S. KIMURA, Y. TOMOBE, M. KOBAYASHI, Y. MITSUI, T. YAZAKI, K. GOTO & T. MASAKI: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:

411~415, 1988

8) SUZUKI, H.; S. SATO, Y. SUZUKI, K. TAKEKOSHI, N. ISHIHARA & S. SHIMODA: Increased endothelin concentration in CSF from patients with subarachnoid hemorrhage. Acta Neuro. Scand. 81: 553~554, 1990

9) MIYAUCHI, T.; M. YANAGISAWA, T. TOMIZAWA, Y. SUGISHITA, N. SUZUKI, M. FUJINO, R. AJISAKA, K. GOTO & T. MASAKI: Increased plasma concentrations of endothelin-1 and big-endothelin-1 in acute myocardial infarction. Lancet 1989-II: 53~54, 1989

10) TOMITA, K.; K. UJIE, T. NAKANISHI, S. TOMURA, O. MATSUDA, K. ANDO, M. SHICHIRI, Y. HIRATA & F. MARUMO: Plasma endothelin levels in patients with acute renal failure. New Engl. J. Med. 321: 1127, 1989

11) MIYATA, S.; N. OHHATA, H. MURAI, Y. MASUI, M. EZAKI, S. TAKASE, M. NISHIKAWA, S. KIYOTO, M. OKUHARA & M. KOHSAKA: WS009 A and B, new endothelin receptor antagonists isolated from *Streptomyces* sp. No. 89009. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. J. Antibiotics 45: 1029~1040, 1992

12) BERRIDGE, M. J.; R. M. C. DAWSON, C. P. DAWSON, J. P. HESLOP & R. F. IRVINE: Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochemical. J. 212: 473~482, 1983