WS9326A, A NOVEL TACHYKININ ANTAGONIST ISOLATED FROM *Streptomyces violaceusniger* No. 9326

II. BIOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF WS9326A AND TETRAHYDRO-WS9326A (FK224)

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(Received for publication January 16, 1992)

WS9326A binds competitively to [^{3}H]substance P (NK-1 receptor) binding sites on guinea-pig lung membranes ($IC_{50}=3.6\times10^{-6}$ M), and acts as a tachykinin antagonist in various functional assays.

WS9326A inhibited tracheal constrictions produced by exogenously added substance P and neurokinin A, with IC_{50} values of 9.7×10^{-6} M and 3.5×10^{-6} M, respectively. WS9326A inhibited neurokinin A-induced bronchoconstriction in a dose dependent manner when administered to guinea-pigs intravenously together with neurokinin A, and was also effective in preventing capsaicin-induced bronchoconstriction, which is known to be caused by release of endogenous tachykinins (substance P and neurokinin A).

FK224 (tetrahydro-WS9326A; catalytic hydrogenation of WS9326A gave FK224) was more potent than WS9326A in the [^{3}H]substance P receptor binding assay using guinea-pig lung membrane ($IC_{50}=1.0\times10^{-7}$ M).

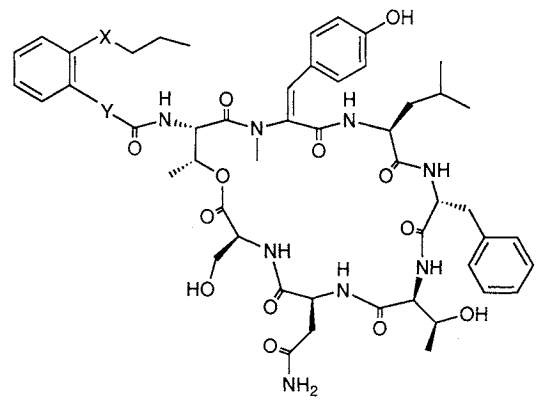
Substance P (SP) and neurokinin A (NKA) have been found in capsaicin sensitive C-fibre afferent to the lower airways of the guinea-pig respiratory system[1]. SP immunoreactive nerves in the airway are found beneath and within the airway epithelium, around blood vessels, and to a lesser extent, within airway smooth muscle[2]. In the guinea-pig tracheal preparation, SP and NKA exhibit the most potent contractile activity of all mediators tested, and were found to act directly on airway smooth muscle[3,4]. These studies have also confirmed that tachykinins are important in the control of bronchial smooth muscle tone. Since the effects induced by tachykinins in airways resemble the typical pathological features of asthma, there has been considerable interest in the possible involvement on tachykinins in airway pathophysiology[5].

Tachykinins play many roles in airway function of various mammals including human as demonstrated in both *in vitro* and *in vivo* experiments. Non-specific stimulation of airway tissue causes the release of SP and NKA followed by marked bronchoconstriction, airway edema and hypersecretion of mucus. Although SP and NKA are released from the same neuron (C-fibre) simultaneously, these neuropeptides have different effects on airways. For airway constriction, NKA is more potent than SP, whereas SP is more potent at inducing airway edema than NKA. SP constricts human airways *in vitro* but has no effect when given by infusion or inhalation *in vivo*[6,7]. By contrast, NKA is more potent as a constrictor of human bronchi *in vitro* and also causes bronchoconstriction *in vivo* by inhalation[8,9]. Recently many tachykinin analogs have been synthesized that competitively inhibit tachykinin-mediated responses. When administered in sufficient

doses some of these analogs block the tachykinin-mediated effects on airway smooth muscle and vascular endothelium[10~12].

In the previous paper we described the discovery and characterization of WS9326A. We here report on the pharmacological characteristics of WS9326A and tetrahydro-WS9326A (FK224). WS9326A and FK224 are novel tachykinin receptor antagonists which competitively and selectively interfere with responses mediated *via* NK-1 and NK-2 receptors *in vitro*. We have examined their effects on airway constriction and airway edema in *in vivo* experiments. The chemical structures of WS9326A and FK224 are shown in Fig. 1. Determination of the chemical structure of WS9326A and chemical induction of FK224 will be published elsewhere[13].

Fig. 1. Chemical structures of WS9326A and FK224.



	WS9826A	FK224
X:	-C=C- (H H)	$-CH_2-CH_2-$
Y:	H -C=C- H	$-CH_2-CH_2-$

Materials and Methods

Receptor Binding Assay

The SP receptor binding assay is described in detail in a preceding paper[14]. All other assays were performed using different kinds of tissue membranes in the receptor binding assay; cynomolgus monkey lung for SP, porcine lung for endothelin (ET), rat lung for angiotensin II (Ang II), rat kidney for arginine vasopressin (AVP), guinea-pig ileum for bradykinin (BK) and guinea-pig lung for quinuclidinyl benzilate ((QNB): muscarinic acetylcholine antagonist) were used with the procedures described previously[14]. ET receptor binding assays using porcine aorta membranes were performed as described before[15], and rat lung membrane and porcine lung membrane preparations are described in the same paper[15]. Binding assays were performed by incubating freshly prepared membranes with varying concentration of ligands (SP 10^{-9} M, AVP 10^{-9} M, BK 3×10^{-11} M, ET 10^{-11} M and QNB 10^{-10} M). The binding studies described above were conducted at 25°C except for SP receptor binding which was perfomed at 4°C.

Guinea-pig Tracheal Constriction

Male albino guinea-pigs weighing 300~400 g were stunned and bled. The trachea were rapidly removed and a zigzag strip preparation of trachea was placed in oxygenated (95% O_2 and 5% CO_2) standard Tyrode solution under a resting tension of 0.5 g. After 60-minute equilibration period, the response to each agonist was recorded 2~3 times to obtain a stable and reproducible contraction response. When the tissue was washed thoroughly, the tension returned rapidly to the initial basal level. Furthermore, the test drug was added and 10 minutes later, contraction was induced with the same agonist. The contractile response obtained in the presence of drug was compared with the control response. The agonist used and their final concentrations were as follows: NKA (10^{-9} M), SP (10^{-7} M), histamine (3.2×10^{-7} M) and acetylcholine (10^{-6} M).

Guinea-pig Airway Constriction *In Vivo*

Male albino guinea-pig weighing 340~440 g were anesthetized by ip injection of pentobarbital (10 mg/animal). The jugular vein and trachea were cannulated and the animals were artificially ventilated (5 ml air/60 strokes per minute). The airway resistance was recorded by using the method of KONZETT and ROSSLER with minor modification. The pressure in the respirator system, *i.e.* the insufflation pressure, was

measured constantly with a transducer (TP 200T, Nihon Kohden) connected to a polygraph (AP-601G, Nihon Kohden), NKA (1 nmol/kg) or capsaicin (10 nmol/kg) was injected iv every 15 minutes through the jugular vein cannula to induce airway constriction. In other experiments NKA was injected intratracheally (it) every 15 minutes through the tracheal cannula. NKA was administered repeatedly until a reproducible constriction was obtained (control). A test drug was then administered iv or it 2 minutes prior to a further challenge with the same agonist. The resulting constriction was compared with the control constriction. In some experiments, systemic arterial blood pressure was monitored at the same time *via* a catheter in a carotid artery connected to a pressure transducer and polygraph.

Guinea-pig Airway Edema *In Vivo*

SP (1 nmol/kg) or histamine (320 nmol/kg) solutions containing Evans Blue dye (20 mg/kg) and heparin (200 IU/kg) were administered to male albino guinea-pigs (260~420 g) by iv injection. Ten minutes later animals were stunned, bled and perfused through the pulmonary artery with 50 ml saline. The trachea and main bronchi were removed, blotted dry and weighed. After they were incubated overnight at 37°C in 1 N KOH (0.5 ml), 4.5 ml of 0.6 N H_3PO_4 - acetone (5 : 13) was added, stirred and centrifuged at 3,000 rpm for 15 minutes. The concentration of extractable Evans Blue dye in the supplement was quantified from light absorbance at 620 nm (UV-160, Shimadzu) by interpolation on a standard curve of dye concentrations in the 0~4.0 μg/ml range. Evans Blue contents per 1 μg wet weight tissue were calculated. The test drugs or control vehicles were administered iv 2 minutes prior to a further agonist challenge. The Evans Blue content extracted from the tissues of animal groups which were injected Evans Blue dye and heparin solution without agonist were subtracted from similar values obtained from each agonist treated animal group. The test drug treated group was compared with the vehicle group.

Materials

The drugs used were as follows: SP, NKA, ET-1, Ang II, AVP and BK (Peptide Institute, Inc.), capsaicin (Sigma), acetylcholine (Daiichi Seiyaku), histamine and QNB (Nakarai). All radioligands were purchased from NEN Research Products (Daiichi Kagaku) as follows: [2-*prolyl*-3,4-^{3}H]substance P (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), [2,3-*prolyl*-3,4-^{3}H(N)]bradykinin (80 Ci/mmol), [^{125}I]endothelin-1 (2,200 Ci/mmol) and L-[*benzilic*-4,4'-^{3}H(N)]quinuclidinyl benzilate (50 Ci/mmol).

Statistical Analysis

Results are expressed as means $\pm$ SE of number of experiments as indicated. Statistical analysis was done by means of Student's t-test for paired data.

Results

Receptor Binding Studies

The specificity of WS9326A and FK224 (tetrahydro-WS9326A) for lung SP binding sites compared with other common peptides and quinuclidinyl benzilate binding sites was demonstrated using several radioligand binding assays (Table 1). WS9326A and FK224 are specific antagonists for the guinea-pig lung membranes receptor. Moreover, these compounds were shown to be active in the monkey lung membrane binding assay. The data in Table 1 demonstrate that WS9326A and FK224 are selective antagonists for SP binding sites. To determine whether FK224 interacts competitively or noncompetitively with [^{3}H]SP binding sites on guinea-pig lung membranes, binding in the presence and absence of FK224 (3.2×10^{-8} M and 1.0×10^{-7} M) was analyzed using Scatchard and Lineweaver-Burk plots. Inhibition of SP binding in the porcine aorta membranes receptor by FK224 was shown to be competitive in both Scatchard analysis (Fig. 2) and Lineweaver-Burk plots (Fig. 3). As shown in Fig. 2, FK224 alters the dissociation constant (*Kd*) of SP binding without a change in the maximum number of binding sites (Bmax). These data suggest that FK224 interacts competitively with SP binding sites on lung membranes with an inhibition constant

Table 1. Comparison of the receptor of specificity of WS9326A and FK224 binding assays using different tissues.

Ligand	Ligand conc (M)	(tissue)	IC_{50} (M)	
			WS9326A	FK224
SP	10^{-9}	(guinea-pig lung)	3.6×10^{-6}	1.0×10^{-7}
SP	10^{-9}	(cynomolgus monkey lung)	3.7×10^{-6}	2.2×10^{-7}
Ang II	10^{-9}	(rat lung)	$>1.0\times10^{-5}$	$>1.0\times10^{-5}$
AVP	10^{-9}	(rat kidney)	$>1.0\times10^{-5}$	$>1.0\times10^{-5}$
BK	3×10^{-11}	(guinea-pig ileum)	$>1.0\times10^{-5}$	$>1.0\times10^{-5}$
ET	10^{-11}	(porcine aorta)	$>1.0\times10^{-5}$	$>1.0\times10^{-5}$
ET	10^{-11}	(porcine lung)	$>1.0\times10^{-5}$	$>1.0\times10^{-5}$
QNB	10^{-10}	(guinea-pig lung)	$>1.0\times10^{-5}$	$>1.0\times10^{-5}$

Fig. 2. Scatchard analysis of SP binding to guinea-pig lung membranes in the absence and presence of FK224.

○ Control, ● FK224 3.2×10^{-6} M, ▲ FK224 1.0×10^{-7} M.

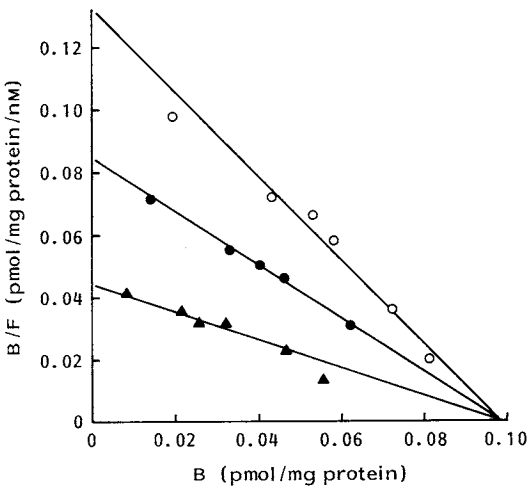
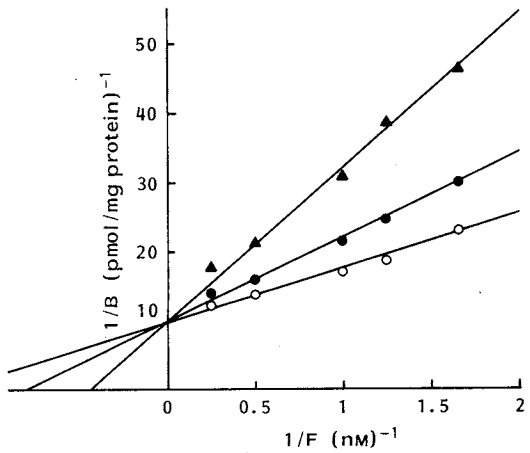


Fig. 3. Lineweaver-Burk plot (double-reciprocal plot).

○ Control, ● FK224 3.2×10^{-8} M, ▲ FK224 1.0×10^{-7} M.



(*Ki*) of 4.57×10^{-8} M.

Guinea-pig Tracheal Constriction *In Vitro*

The dose-response curve for WS9326A and FK224 against SP- or NKA-induced tracheal constriction was studied in test drug pretreatment experiments. WS9326A and FK224 inhibited tracheal constriction induced by NKA (10^{-9} M) and SP (10^{-8} M) in a dose-dependent manner. The IC_{50} values for WS9326A against NKA- and SP-induced contractions were 3.5×10^{-6} M and 9.7×10^{-6} M, respectively. The IC_{50} values for FK224 against NKA- and SP-induced contractions were 1.6×10^{-6} M, and 2.6×10^{-6} M respectively. The inhibitory effects of WS9326A and FK224 were specific for NKA- and SP-induced contraction because histamine- and acetylcholine-induced contractions were unaffected by WS9326A and FK224 at a dose of 10^{-5} M.

Guinea-pig Airway Constriction *In Vivo*

WS9326A or FK224 administered by intravenous (iv) or intratracheal (it) injection 2 minutes prior to challenge with NKA clearly inhibited the NKA (1.1 μg/kg iv)-induced increase in airway resistance in

Fig. 4. Effect of WS9326A and FK224 on airway constriction induced by iv injection of neurokinin A (1 nmol/kg) in guinea-pigs.

WS9326A and FK224 were given iv 2 minutes before the neurokinin A. WS9326A: ▲ 1 mg/kg, ■ 10 mg/kg. ($n=5$). FK224: △ 0.1 mg/kg, ○ 1 mg/kg, □ 10 mg/kg. ($n=5$).

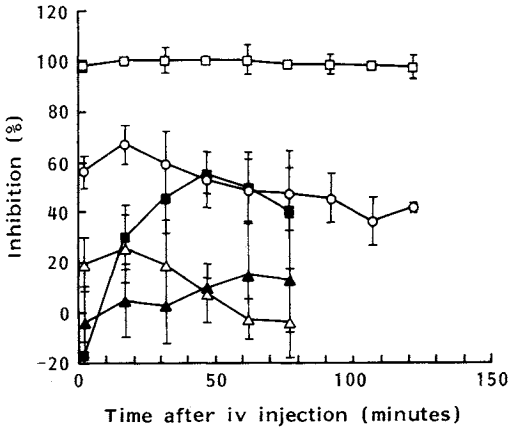
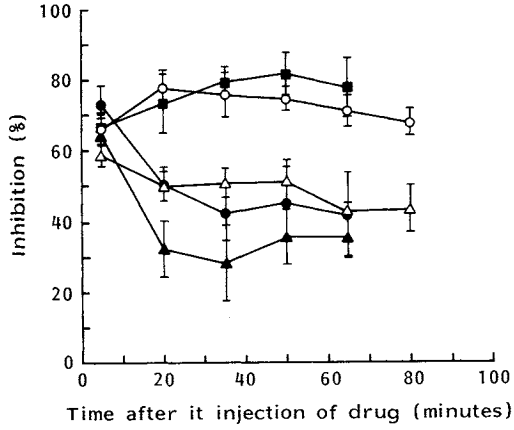


Fig. 5. Effect of WS9326A and FK224 on airway constriction induced by iv injection of neurokinin A (1 nmol/kg) in guinea-pigs.

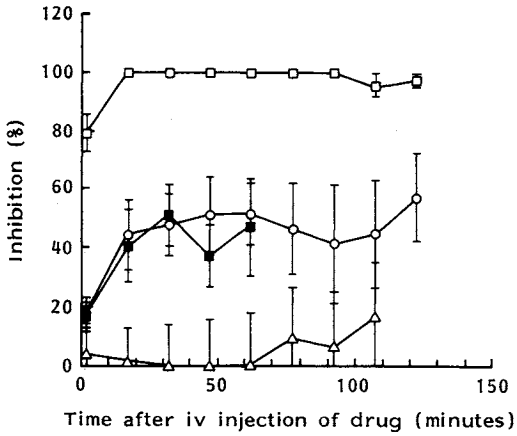
WS9326A and FK224 were given intratracheally (it) 2 minutes before the neurokinin A. WS9326A: ▲ 0.03 mg/kg, ● 0.3 mg/kg, ■ 3.0 mg/kg. ($n=4$). FK224: △ 0.03 mg/kg, ○ 0.3 mg/kg. ($n=4$).



a dose-dependent manner (Figs. 4 and 5). The increase in airway resistance induced by histamine (43 nmol/kg it) was not affected by treatment with either WS9326A or FK224, even at a dose of 10 mg/kg (iv). These results show that WS9326A and FK224 are also effective *in vivo*, and that the inhibitory actions of WS9326A and FK224 are selective for neurokinin responses. In some experiments, blood pressure was monitored simultaneously. NKA injected iv caused not only airway constriction but also a transient systemic hypotension. WS9326A and FK224 significantly inhibited airway constriction and also affected NKA-induced hypotension. The degree of NKA-induced hypotension was 57% (WS9326A 10 mg/kg iv), 65% (FK224 1 mg/kg iv) and 100% (FK224 10 mg/kg).

Fig. 6. Effect of WS9326A and FK224 on the airway constriction induced by iv injection of capsaicin in guinea-pigs.

WS9326A and FK224 were given iv 2 minutes before the capsaicin. WS9326A: ■ 10 mg/kg ($n=5$). FK224: △ 0.1 mg/kg, ○ 1 mg/kg, □ 10 mg/kg ($n=5$).



Capsaicin, the pungent ingredient of red peppers of the *Capsicum* family, has been widely used as a tool in the study of primary afferent sensory neurons[16]. Capsaicin is known to stimulate sensory neuron C-fibres and to induce the release of neuropeptides such as SP and NKA from nerve endings[1,5,16]. Capsaicin injected iv causes a marked increase in airway constriction that is considered to be due to neurokinin release[6]. WS9326A and FK224 injected iv 2 minutes before capsaicin significantly inhibited airway constriction (Fig. 6).

Guinea-pig Airway Edema *In Vivo*

Table 2 shows the effect of iv injection of WS9326A and FK224 on SP- and histamine-induced airway edema. WS9326A and FK224 inhibited SP-induced airway edema in a dose dependent manner and the ED_{50} values were 0.87 mg/kg and 0.14 mg/kg, respectively. These results demonstrate that WS9326A and FK224 are potent antagonists of SP-induced airway edema. WS9326A or FK224 did not induce any microvascular permeability changes in airway tissue at a dose of 10 mg/kg and 1 mg/kg, respectively.

Discussion

A novel non-peptide antagonist of NK-1 receptors (CP-96,345) has recently been reported to bind to bovine caudate membranes with an IC_{50} value of 3.4 nM. CP-96,345 has no activity for NK-2 and NK-3 receptors[17,18]. Selective NK-1 receptor antagonist appear to be promising anti-inflammation and analgesic agents. Recent interest in the peptide, selective NK-2 antagonist, MEN 10,376 has demonstrated a major role for NKA rather than for SP as an endogenous bronchoconstrictor in the guinea-pig isolated bronchi[19]. Since tracheal constriction in guinea-pig is very sensitive to NKA, this reaction is considered to be mediated *via* the NK-2 receptors. Cigarette smoke-induced or vagally-induced tracheal edema is most likely due to SP (which acts *via* NK-1 receptors) release from local capsaicin-sensitive afferent neurons in the airway mucosa[20]. SP induces mucus secretion *via* the NK-1 receptors which have been located overlying human bronchial glands[21].

Our aim is to find therapeutically useful anti-asthmatic agents. The involvement of tachykinins, especially SP and NKA, in the pathophysiology of asthmatic disease has been suggested because of their potent induction of airway constriction. Both NK-1 and NK-2 receptors are present in guinea-pig and human airways and a dual NK-1/NK-2 receptor antagonist may therefore be a useful treatment. In this paper we demonstrate that WS9326A and FK224 (tetrahydro-WS9326A) are specific dual NK-1 and NK-2 antagonists both *in vitro* and *in vivo* (Table 2). Moreover, FK224 is a competitive antagonist of SP-induced guinea-pig ileum contraction and NKA-induced contraction of rat portal vein, whereas FK224 has no activity at NK-3 receptors[22]. FK224 interacts with NK-1 and NK-2 receptors with a similar potency, and the pharmacological profile of FK224 is quite different from that of other tachykinin antagonists[23]. These findings suggest that FK224 could be a promising agent for development as an anti-asthmatic drug.

Table 2. The ED_{50} values (mg/kg) or IC_{50} values (M) of WS9326A and FK224 on various experimental models.

Expt	Agonist	(dose)	Drug	WS9326A	FK224	
			Route	it	iv	it
			Vehicle	DMSO	0.1% MC* in saline	DMSO
In vivo						
Bronchoconstriction	SP	(10 nmol/kg)		—	0.39	0.35
	NKA	(1 nmol/kg)		0.29	0.36	0.03
	Capsaicin	(10 nmol/kg)		—	1.1	—
	Histamine	(43 nmol/kg)		No effect	No effect	No effect
Tracheal edema	SP	(1 nmol/kg)		0.87	0.14	1.3
	NKA	(10 nmol/kg)		—	0.29	2.5
	Capsaicin	(320 nmol/kg)		—	0.3	0.35
	Histamine	(320 nmol/kg)		No effect	No effect	No effect
In vitro						
Binding assay	SP	(1×10^{-9} M)		3.6×10^{-6} M	1.0×10^{-7} M	
Tracheal contraction	SP	(1×10^{-8} M)		9.7×10^{-6} M	2.6×10^{-6} M	
	NKA	(1×10^{-9} M)		3.5×10^{-6} M	1.3×10^{-6} M	

* Methylcellulose. it: intratracheal.

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