

# SSR240600 [(R)-2-(1-{2-[4-{2-[3,5-Bis(trifluoromethyl)-phenyl]acetyl}-2-(3,4-dichlorophenyl)-2-morpholinyl]ethyl}-4-piperidinyl)-2-methylpropanamide], a Centrally Active Nonpeptide Antagonist of the Tachykinin Neurokinin-1 Receptor: I. Biochemical and Pharmacological Characterization

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## ABSTRACT

The biochemical and pharmacological properties of a novel antagonist of the tachykinin neurokinin 1 (NK<sub>1</sub>) receptor, SSR240600 [(R)-2-(1-{2-[4-{2-[3,5-bis(trifluoromethyl)phenyl]acetyl}-2-(3,4-dichlorophenyl)-2-morpholinyl]ethyl}-4-piperidinyl)-2-methylpropanamide], were evaluated. SSR240600 inhibited the binding of radioactive substance P to tachykinin NK<sub>1</sub> receptors in human lymphoblastic IM9 cells ( $K_i = 0.0061$  nM), human astrocytoma U373MG cells ( $K_i = 0.10$  nM), and human brain cortex ( $IC_{50} = 0.017$  nM). It also showed subnanomolar affinity for guinea pig NK<sub>1</sub> receptors but was less potent on rat and gerbil NK<sub>1</sub> receptors. SSR240600 inhibited [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-induced inositol monophosphate formation in human astrocytoma U373MG cells with an  $IC_{50}$  value of 0.66 nM (agonist concentration of 100 nM). It also antagonized substance P-induced contractions of isolated human small bronchi with a  $pIC_{50}$  value of 8.6

(agonist concentration of 100 nM). The compound was >100- to 1000-fold more selective for tachykinin NK<sub>1</sub> receptors versus tachykinin NK<sub>2</sub> or NK<sub>3</sub> receptors as evaluated in binding and in vitro functional assays. In vivo antagonistic activity of SSR240600 was demonstrated on tachykinin NK<sub>1</sub> receptor-mediated hypotension in dogs (3 and 10  $\mu$ g/kg i.v.), microvascular leakage (1 and 3 mg/kg i.p.), and bronchoconstriction (50 and 100  $\mu$ g/kg i.v.) in guinea pigs. It also prevented citric acid-induced cough in guinea pigs (1–10 mg/kg i.p.), an animal model in which central endogenous tachykinins are suspected to play a major role. In conclusion, SSR240600 is a new, potent, and centrally active antagonist of the tachykinin NK<sub>1</sub> receptor, able to antagonize various NK<sub>1</sub> receptor-mediated pharmacological effects in the periphery and in the central nervous system.

Substance P belongs to a group of related neuropeptides named tachykinins, which includes neurokinin A and neurokinin B. These peptides are widely distributed in the peripheral and central nervous systems where they exert various biological actions as neuromodulators or neurotransmitters.

Biological activities of tachykinins are mediated by three different, but related, G-protein-coupled receptors with seven  $\alpha$ -helical transmembrane segments, denoted NK<sub>1</sub>, NK<sub>2</sub>, and NK<sub>3</sub>. Substance P is the natural endogenous ligand of tachykinin NK<sub>1</sub> receptors, whereas neurokinin A and neurokinin B are the preferential ligands of tachykinin NK<sub>2</sub> and NK<sub>3</sub> receptors, respectively (Regoli et al., 1994; Maggi, 1995; Quartara and Maggi, 1997).

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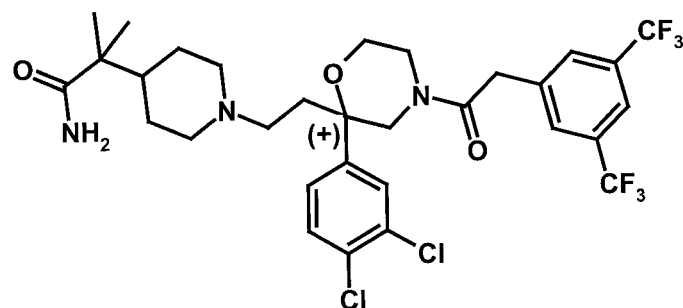
**ABBREVIATIONS:** NK, neurokinin; SSR240600, (R)-2-(1-{2-[4-{2-[3,5-bis(trifluoromethyl)phenyl]acetyl}-2-(3,4-dichlorophenyl)-2-morpholinyl]ethyl}-4-piperidinyl)-2-methylpropanamide; CHO, Chinese hamster ovary; SR140333, (S)-1-[2-[3-(3,4-dichlorophenyl)-1-[1-[3-(1-methylethoxy)phenyl]acetyl]-3-piperidinyl]ethyl]-4-phenyl-1-azabicyclo[2.2.2]octane chloride; SR48968, (S)-N-methyl-N-[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl)butyl]benzamide; FK888, N<sup>2</sup>-[[4(R)-4-hydroxy-1-[(1-methyl-1H-indole-3-yl)carbonyl]-L-propyl]-N-methyl-N-(phenylmethyl)-3-(2-naphthyl)-L-alaninamide; CP-99,994, (2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine; ANOVA, analysis of variance. SR144190, (R)-3-[1-[2-(4-benzoyl)-2-(3,4-difluorophenyl)-morpholin-2-yl]-ethyl]-4-phenylpiperidin-4-yl]-1-dimethylurea; SR142801, (R)-(N)-[1-[3-[1-benzoyl]-3-(3,4-dichlorophenyl)piperidin-3-yl]propyl]-4-phenylpiperidin-4-yl]-N-methylacetamide; SSR146977, (R)-(N)-[1-[3-[1-benzoyl]-3-(3,4-dichlorophenyl)piperidin-3-yl]propyl]-4-phenylpiperidin-4-yl]-N-dimethylurea; GR205171, (2S,3S)-2-methoxy-5-[(5-trifluoromethyl-tetrazol-1-yl)-benzyl-(2-phenyl-piperidin-3-yl)-amine; GR73632, D-Ala-[L-Pro<sup>9</sup>,Me-Leu<sup>8</sup>]substance P(7-11).

Over the past several years, potent nonpeptide antagonists, selective for the different tachykinin receptors, have been described and have provided tools to investigate the physiopathological role of tachykinins and their receptors both in the periphery and in the central nervous system (Regoli et al., 1994; Quartara and Maggi, 1997, 1998; Lagente and Advenier, 1998; Stout et al., 2001). Based on the more recent pharmacological data, confirmed by preliminary clinical trials, it has emerged that blockade of tachykinin NK<sub>1</sub> receptors may provide a novel treatment of major depression (Kramer et al., 1998; Rupniak and Kramer, 1999) and emesis (Rupniak and Kramer, 1999; Diemunsch and Grélot, 2000). These activities of tachykinin NK<sub>1</sub> receptor antagonists are essentially dependent on their ability to penetrate the brain (Rupniak et al., 1997; Kramer et al., 1998; Diemunsch and Grélot, 2000). We now describe some general biochemical and pharmacological activities of a novel nonpeptide tachykinin NK<sub>1</sub> receptor antagonist, SSR240600 [(*R*)-2-(1-[2-[4-(2-[3,5-bis(trifluoromethyl)phenyl]acetyl)-2-(3,4-dichlorophenyl)-2-morpholinyl]ethyl]-4-piperidinyl)-2-methylpropanamide] (Fig. 1). Its activity in various tests predictive of an antidepressant activity is described in the accompanying paper (Steinberg et al., 2002).

## Materials and Methods

**Binding Assays.** The affinity of SSR240600 for tachykinin receptors was evaluated in several receptor-radioligand binding assays: 1) binding of [<sup>125</sup>I]Bolton-Hunter region-labeled substance P to tachykinin NK<sub>1</sub> receptors of rat cortex, guinea pig, and gerbil ileum, human lymphoblast cells (IM9), and human astrocytoma cells (U373MG, STTG1); 2) binding of [<sup>125</sup>I]iodohistidyl-neurokinin A (or [<sup>125</sup>I]neuropeptide  $\gamma$ ) to tachykinin NK<sub>2</sub> receptors of rat or hamster urinary bladder or guinea pig ileum as well as to human receptors, stably expressed in CHO cells; and 3) binding of [<sup>125</sup>I]iodohistidyl-[MePhe<sup>7</sup>]neurokinin B (or [<sup>125</sup>I]eledoisin) to rat, guinea pig, and gerbil brain cortex tachykinin NK<sub>3</sub> receptors and human NK<sub>3</sub> receptor, cloned and stably expressed in CHO cells. All these binding assays were conducted and analyzed as previously described in detail (Emonds-Alt et al., 1993, 1995, 1997).

The affinity of SSR240600 for tachykinin receptors was also investigated on the binding of [<sup>3</sup>H]substance P to tachykinin NK<sub>1</sub> receptors of human brain cortex. Brain cortex was obtained from a 49-year-old man, 48 h after death due to pulmonary edema. Tissue was homogenized at 4°C in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM KCl and 10 mM EDTA. The homogenate was centrifuged at 28,000g for 15 min at 4°C. The pellet was homogenized and incubated for 30 min at 4°C in 50 mM Tris-HCl buffer, pH 7.4, containing 300 mM KCl and 10 mM EDTA. This homogenate was centrifuged at 40,000g for 15 min and the pellet



**Fig. 1.** Structure of SSR240600 [(*R*)-2-(1-[2-[4-(2-[3,5-bis(trifluoromethyl)phenyl]acetyl)-2-(3,4-dichlorophenyl)-2-morpholinyl]ethyl]-4-piperidinyl)-2-methylpropanamide].

was suspended in 50 mM Tris-HCl buffer, pH 7.4. Membranes were stored at -20°C until use. Before use in binding assays, the membranes were diluted at 4°C in 50 mM Tris-HCl buffer, pH 7.4, and centrifuged at 40,000g for 15 min. The final pellet was suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.2 mg/ml bovine serum albumin, 40  $\mu$ g/ml bacitracin, 4  $\mu$ g/ml leupeptin, 4  $\mu$ g/ml chymostatin, and 3 mM MnCl<sub>2</sub>. Binding assays were conducted in "low binding" tubes (NUNC A/S, Roskilde, Denmark). Human brain cortex membranes (10 mg) and [<sup>3</sup>H]substance P (0.5 nM) in 500  $\mu$ l of assay buffer (50 mM Tris-HCl buffer, pH 7.4, containing 0.2 mg/ml bovine serum albumin, 40  $\mu$ g/ml bacitracin, 4  $\mu$ g/ml leupeptin, 4  $\mu$ g/ml chymostatin, 3 mM MnCl<sub>2</sub>) were incubated at 25°C for 60 min with various concentrations of SSR240600. At the end of the incubation, separation of bound and free ligand was done after dilution [3 ml of cold (4°C) 50 mM Tris-HCl buffer, pH 7.4, containing 0.2 mg/ml bovine serum albumin] and rapid filtration on Whatman (Maidstone, Kent, UK) GF/C filters pretreated with 50 mM Tris-HCl buffer, pH 7.4, containing 0.2 mg/ml bovine serum albumin and 0.25% polyethylenimine. The filters were washed three times at 4°C with 50 mM Tris-HCl buffer, pH 7.4, containing 0.2 mg/ml bovine serum albumin. The radioactivity was counted in a  $\beta$  liquid scintillation counter. Specific binding was determined by subtraction of the nonspecific binding, which was determined in the presence of 1  $\mu$ M unlabeled [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P.

In addition, the selectivity of SSR240600 was evaluated in a large variety of ion channel- and receptor-binding assays as well as enzyme assays. This was performed by MDS Panlabs Pharmacology Services (Bothell, WA) and Cerep (Celles L'Evescault, France).

**In Vitro Functional Assays.** Antagonistic activity of SSR240600 was first determined by measuring inhibition of inositol phosphate-1 formation elicited by tachykinin NK<sub>1</sub> receptor activation with specific agonists ([Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P, septide, and GR73632) in human astrocytoma U373MG cells. The effect of SSR240600 on inositol phosphate-1 formation was also measured using CHO cells stably expressing either human tachykinin NK<sub>2</sub> or NK<sub>3</sub> receptor in response to [Nle<sup>10</sup>]neurokinin A-(4-10) or [MePhe<sup>7</sup>]neurokinin B, respectively. All these assays were conducted as previously described in detail (Oury-Donat et al., 1994, 1995).

The in vitro pharmacological profile of SSR240600 was then investigated by using several functional bioassays specific for the three tachykinin receptor subtypes (Regoli et al., 1994): [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-induced endothelium-dependent relaxation of rabbit pulmonary artery, precontracted by 0.1  $\mu$ M norepinephrine (specific for NK<sub>1</sub> receptors), [ $\beta$ Ala<sup>8</sup>]neurokinin A-(4-10)-induced contractions of endothelium-denuded rabbit pulmonary artery (specific for NK<sub>2</sub> receptors), and [MePhe<sup>7</sup>]neurokinin B-induced contractions of guinea pig ileum (specific for NK<sub>3</sub> receptors). All these assays were conducted and analyzed as previously described in detail (Emonds-Alt et al., 1993). As already reported and discussed for other nonpeptide antagonists of the tachykinin NK<sub>1</sub>, NK<sub>2</sub>, and NK<sub>3</sub> receptors (Emonds-Alt et al., 1993), preliminary experiments have indicated that full activity of SSR240600 was only observed after prolonged incubation with the tissue. Therefore, SSR240600 was added 120 min before the agonist in all experiments.

Finally, SSR240600 antagonist activity for tachykinin NK<sub>1</sub> receptors was evaluated by measuring inhibition of [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-induced contractions of human isolated small bronchi (diameter <1 mm) as described by Naline et al. (1996). Bronchial tissues were removed from patients (25 men, previous smokers, mean age 64  $\pm$  2 years) with lung cancer at the time of the surgical operation. Just after resection, segments of bronchi with an inner diameter of 0.5 to 1 mm were taken from an area as far as possible from the malignancy and stored overnight at 4°C in Krebs-Henseleit solution.

**In Vivo Assays.** All protocols have been approved by the Comité d'Expérimentation Animale (Animal Care and Use Committee) of Sanofi-Synthelabo Recherche and are in accordance with the principles of the Declaration of Helsinki. The in vivo pharmacological profile of SSR240600 was investigated in three animal models in which the role of tachykinin NK<sub>1</sub> receptor has been well characterized: hypotension, bronchoconstriction, and plasma extravasation

TABLE 1

Inhibition constants ( $K_i$ ) of SSR240600 in radioligand binding assays for tachykinin receptors

Radioligands: [<sup>125</sup>I]Bolton-Hunter-labeled substance P for NK<sub>1</sub> receptors, except for human brain cortex where [<sup>3</sup>H]substance P was used; [<sup>125</sup>I]iodohistidyl-neurokinin A for NK<sub>2</sub> receptors, except for guinea-pig ileum where [<sup>125</sup>I]neuropeptide  $\gamma$  was used; [<sup>125</sup>I]iodohistidyl-[MePhe<sup>7</sup>]neurokinin B for NK<sub>3</sub> receptors, except for rat brain cortex where [<sup>125</sup>I]leleodoisin was used. Tachykinins: substance P, neurokinin A, and [MePhe<sup>7</sup>]neurokinin B, respectively, for NK<sub>1</sub>, NK<sub>2</sub>, and NK<sub>3</sub> receptors, except for human brain cortex where [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P was used. Values are means  $\pm$  S.D. obtained from at least three independent experiments performed in triplicate.

	Receptors	$K_i$ (nM)	
		Tachykinins	SSR240600
Human brain cortex	NK1	0.65 $\pm$ 0.23	0.0043 $\pm$ 0.0012
IM9 (human)	NK1	0.044 $\pm$ 0.008	0.0061 $\pm$ 0.0004
U373MG (human)	NK1	0.31 $\pm$ 0.06	0.10 $\pm$ 0.01
STTG1 (human)	NK1	0.33 $\pm$ 0.03	0.09 $\pm$ 0.01
Rat ileum	NK1	0.054 $\pm$ 0.006	1.07 $\pm$ 0.07
Gerbil ileum	NK1	0.042 $\pm$ 0.008	1.15 $\pm$ 0.02
Guinea pig ileum	NK1	0.059 $\pm$ 0.007	0.23 $\pm$ 0.03
Human (CHO cells)	NK2	0.48 $\pm$ 0.01	24 $\pm$ 2
Guinea pig ileum	NK2	2.30 $\pm$ 0.49	71 $\pm$ 5
Rat urinary bladder	NK2	0.76 $\pm$ 0.03	34 $\pm$ 3
Human (CHO cells)	NK3	0.34 $\pm$ 0.05	206 $\pm$ 9
Guinea pig brain cortex	NK3	0.43 $\pm$ 0.05	21 $\pm$ 2
Gerbil brain cortex	NK3	0.16 $\pm$ 0.03	544 $\pm$ 77
Rat brain cortex	NK3	0.053 $\pm$ 0.001	>10,000

induced by substance P or specific agonists of the tachykinin NK<sub>1</sub> receptor (Regoli et al., 1994; Quartara and Maggi, 1998). Furthermore, the activity of SSR240600 was studied in a model of cough provoked by inhalation of citric acid, where endogenous tachykinins and their receptors play an important role (Advenier et al., 1993; Ujiie et al., 1993; Girard et al., 1995; Yasumitsu et al., 1996; Daoui et al., 1998).

**Hypotension in Dogs.** Mongrel dogs of either sex (10–20 kg) were anesthetized with sodium pentobarbital (30 mg/kg by intravenous route), and the anesthetic was infused throughout the experiments at a rate of 5 mg/kg per hour. The animals were intubated with an endotracheal cannula and allowed to breathe spontaneously. After an equilibration period, [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P (5 ng/kg) was repeatedly injected via the femoral vein at 15-min intervals before and after intravenous or intraduodenal administration of SSR240600. Mean blood pressure was calculated on the basis of systolic and diastolic blood pressure values recorded with a Honeywell PC 156 transducer at the carotid artery. In control experiments, repeated injections of [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P (5 ng/kg) produced a reproducible hypotension of 30 to 40 mm Hg (Emonds-Alt et al., 1993).

**Bronchoconstriction in Guinea Pigs.** Male tricolored guinea pigs (200–250 g) were anesthetized with urethane (1.25 g/kg) administered by the intraperitoneal route and were pretreated with atropine (0.5 mg/kg), diphenhydramine (1 mg/kg), and indomethacin (2 mg/kg) injected intravenously. Bronchoconstriction was induced with GR73632 (a tachykinin NK<sub>1</sub> receptor agonist) (5 ng/kg), administered intravenously at 20-min intervals before and after intravenous administration of SSR240600. In control experiments, repeated injections of GR73632 produced a reproducible bronchoconstriction. Bronchoconstriction, quantified as a reduction of tidal volume, was evaluated according to a modified Konzett-Rössler method (Emonds-Alt et al., 1993).

**Plasma Extravasation in Guinea Pigs.** Tricolored, male or female guinea pigs (250–400 g) were anesthetized with urethane (1.25 g/kg by the intraperitoneal route) and prepared for cannulation of the jugular vein. SSR240600 was administered by the intraperitoneal route 30 min before intravenous injection of Evans blue dye (30 mg/kg), used as a marker for plasma extravasation. One minute later, plasma extravasation was provoked by intravenous administration of substance P (0.3  $\mu$ g/kg). The animals were killed 5 min later, and tissues (trachea, main bronchi, esophagus, urinary bladder) were removed and weighed. Evans blue dye was extracted by incubating the tissues in formamide at 60°C for 18 h and measured photometrically. Plasma extravasation was expressed as nanograms of dye per milligram wet-weight tissue (Qian et al., 1993).

**Citric Acid-Induced Cough in Guinea Pigs.** Tricolored, awake, unrestrained male or female guinea pigs (250–400 g) were placed in a body plethysmograph. They were then exposed for 10 min to an aerosol of either an aqueous solution of citric acid (0.4 M) or saline as a control. SSR240600 was administered by the intraperitoneal route at various times before the aerosol challenge. Coughs were counted by a trained observer, and recognized by the characteristic animal posture and the pressure variation in the body plethysmograph (Advenier et al., 1993; Girard et al., 1995; Daoui et al., 1998).

**Chemicals.** SSR240600 (Fig. 1) was synthesized at Sanofi-Synthélabo Recherche and was used as its hydrochloride salt. It was dissolved in organic solvents (ethanol or dimethyl sulfoxide) and diluted in distilled water when interference from organic solvents was observed. For oral and intraduodenal administration, it was suspended in water with 0.6% carboxymethylcellulose. Radioactive ligands were purchased from Amersham Biosciences Inc. (Les Ulis, France) and PerkinElmer Life Sciences (Paris, France). All peptides were obtained from Bachem (Bubendorf, Switzerland), and were dissolved in organic solvents (ethanol or dimethyl sulfoxide) and then diluted in water.

## Results

**Binding Studies.** The inhibition constants ( $K_i$ ) for SSR240600 obtained in the different binding assays for tachykinin receptors are shown in Table 1. SSR240600 inhibited the binding of radioactive substance P to tachykinin NK<sub>1</sub> receptors with subnanomolar  $K_i$  values, using established human cell lines as well as human brain membranes. SSR240600 also displayed a high affinity for tachykinin NK<sub>1</sub> receptors from various animal species, especially guinea pigs. In binding assays for tachykinin NK<sub>2</sub> and NK<sub>3</sub> receptors, SSR240600 slightly interfered with the binding of their respective ligands, with  $K_i$  values always above 10 nM in all species studied, including human. Finally, SSR240600 was assayed in 100 (mainly human) receptor-binding, ion channel-binding, and enzyme assays including adenosine (A<sub>1</sub>, A<sub>2A</sub>, A<sub>3</sub>), adrenergic ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$ ,  $\beta_2$ ) dopamine (D<sub>1</sub>, D<sub>2</sub>), nicotinic, muscarinic (M<sub>1</sub>, M<sub>2</sub>, M<sub>3</sub>, M<sub>4</sub>, M<sub>5</sub>), opiate ( $\mu$ ,  $\kappa$ ,  $\delta$ , opioid receptor-like receptor 1) serotonin (5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>3</sub>, 5-HT<sub>5A</sub>, 5-HT<sub>6</sub>, 5-HT<sub>7</sub>), angiotensin (AT<sub>1</sub>, AT<sub>2</sub>), bradykinin (B<sub>1</sub>, B<sub>2</sub>), calcitonin gene-related peptide, cholecystokinin (CCK<sub>1</sub>, CCK<sub>2</sub>), corticotropin-releasing factor (CRF<sub>1</sub>,

TABLE 2

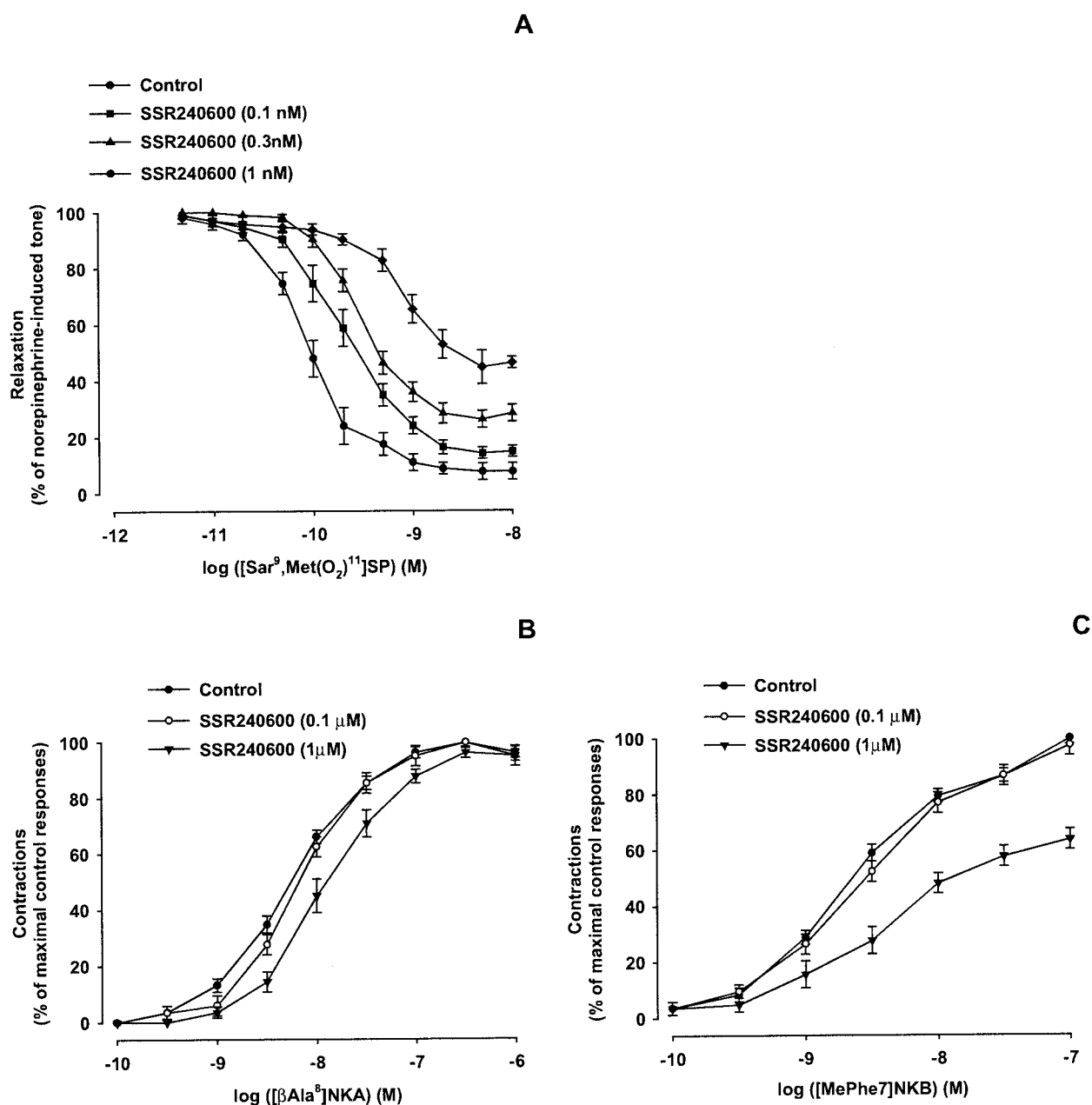
Inhibition by SSR240600 of tachykinin receptor-mediated inositol phosphate-1 formation in human astrocytoma U373MG cells (NK<sub>1</sub> receptors) and in CHO cells expressing either human NK<sub>2</sub> or NK<sub>3</sub> receptors

Results are given as IC<sub>50</sub> values. Values are means ± S.E.M. from at least three independent experiments performed in triplicate.

Receptors	Agonists (Concentration)	IC <sub>50</sub>
		nM
NK1	[Sar <sup>9</sup> ,Met(O <sub>2</sub> ) <sup>11</sup> ]Substance P (0.1 μM)	0.66 ± 0.11
	GR73632 (0.1 μM)	0.57 ± 0.08
	Septide (0.1 μM)	0.45 ± 0.07
NK2	[βAla <sup>8</sup> ]Neurokinin A(4-10) (10 nM)	140 ± 7
NK3	[MePhe <sup>7</sup> ]Neurokinin B (10 nM)	1760 ± 100

CRF<sub>2</sub>), galanin (GAL<sub>1</sub>, GAL<sub>2</sub>), neurotensin (NT<sub>1</sub>), vasopressin (V<sub>1A</sub>), hormones (glucocorticoid, estrogen, progesterone, testosterone), ion channels (sodium, calcium, potassium and chloride), cyclooxygenases (COX<sub>1</sub>, COX<sub>2</sub>), phosphodiesterases (III and IV), acetylcholinesterase. SSR240600, at concentrations up to 1 μM, was inactive (inhibition less than 50%), except in σ receptor (IC<sub>50</sub> = 0.21 μM) and sodium channel site 2 (IC<sub>50</sub> = 0.18 μM) assays (data not shown).

**In Vitro Functional Studies.** Antagonistic activity of SSR240600 at human tachykinin NK<sub>1</sub> receptors was studied by measuring inhibition of inositol phosphate-1 formation provoked by NK<sub>1</sub> receptor activation in human astrocytoma U373MG cells. Activation of tachykinin NK<sub>1</sub> receptors in U373MG cells by three different specific agonists

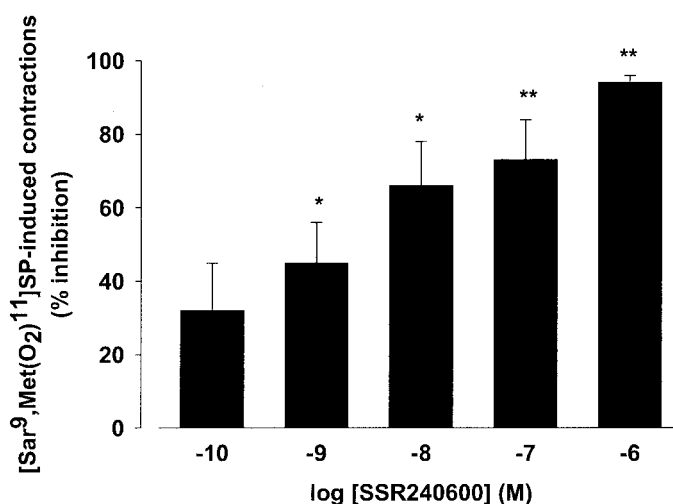


**Fig. 2.** Concentration-response curves for [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-induced endothelium-dependent relaxation of rabbit pulmonary artery precontracted with 100 nM norepinephrine (A), [βAla<sup>8</sup>]neurokinin A-induced contractions of endothelium-deprived rabbit pulmonary artery (B), and [MePhe<sup>7</sup>]neurokinin B-induced contractions of guinea pig ileum (C) in the absence and in the presence of SSR240600. Values are means ± S.E.M. (n = 6).

([Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P, septide, and GR73632) provoked the formation of inositol phosphate-1, which was concentration dependently inhibited by SSR240600 regardless of the agonist used. In contrast, SSR240600 showed a much lower potency to block inositol phosphate-1 formation following the activation of human tachykinin NK<sub>2</sub> and NK<sub>3</sub> receptors stably expressed in CHO cells. The IC<sub>50</sub> values are given in Table 2.

In a classical tachykinin NK<sub>1</sub> receptor assay using isolated tissues, [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-induced endothelium-dependent relaxation of rabbit pulmonary artery previously contracted by norepinephrine, SSR240600 produced a concentration-dependent rightward shift of the concentration-response curves of [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P (Fig. 2A). However, SSR240600 also induced a reduction of the maximal response to the agonist, suggesting that SSR240600 antagonism was not purely competitive. The slope of the Schild plot (0.65) was significantly different from unity and the apparent affinity of SSR240600 was thus calculated in terms of pD'<sub>2</sub> (negative logarithm of the molar concentration of antagonist that produces a 50% reduction of the maximal response to the agonist). The pD'<sub>2</sub> value was 8.67 ± 0.08 (*n* = 18). The activity of SSR240600 was then examined on tissue preparations containing tachykinin NK<sub>2</sub> and NK<sub>3</sub> receptors. At concentrations up to 0.1 μM, it had no effect in bioassays for NK<sub>2</sub> ([βAla<sup>8</sup>]neurokinin A-induced contractions of endothelium-deprived rabbit pulmonary artery) (Fig. 2B) or NK<sub>3</sub> ([MePhe<sup>7</sup>]neurokinin B-induced contractions of guinea pig ileum) (Fig. 2C) receptors. At a concentration of 1 μM, SSR240600 produced a rightward shift of the agonist concentration-response curve in the two bioassays, with a reduction of maximal response to the agonist in bioassay for NK<sub>3</sub> receptors. Finally, in an assay using an isolated human tissue, SSR240600 potently inhibited contractions of human isolated small bronchi (diameter <1 mm) induced by [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P (100 nM) with a pIC<sub>50</sub> of 8.6 (Fig. 3).

**In Vivo Studies.** The in vivo activity of SSR240600 was first investigated using typical pharmacological responses to tachykinin NK<sub>1</sub> receptor agonists ([Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P, GR73632, or substance P). In anesthetized dogs, intravenous injection of [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P (5 ng/kg) provoked a reproducible hypotension of 30 to 40 mm Hg. SSR240600 administered either intravenously (3–10 μg/kg) or intraduodenally (30–300 μg/kg) produced a dose- and time-dependent inhibition of this [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-induced hypotension (Fig. 4). At the doses tested, SSR240600 itself had no effect on mean blood pressure. SSR240600 also potently antagonized GR73632-induced bronchoconstriction in anesthetized guinea pigs (Fig. 5). Intravenous injection of GR73632 (0.5 ng/kg) produced a reproducible bronchoconstriction that was dose dependently inhibited by pretreatment with intravenously administered SSR240600 (50 and 100 μg/kg). Furthermore, 3 h after a single oral administration, SSR240600 (3 mg/kg) inhibited GR73632-induced bronchoconstriction by 83 ± 5% (*n* = 5), indicating both oral bioavailability and a long-lasting effect of the compound. SSR240600 itself did not modify the resting bronchial tone at the doses tested. Finally, intravenous administration of substance P (0.3 μg/kg) induced plasma extravasation in different guinea pig tissues. After intraperitoneal administration, 30 min before substance P, SSR240600 at doses equal to or greater than 1 mg/kg inhibited the plasma extravasation (Fig. 6).

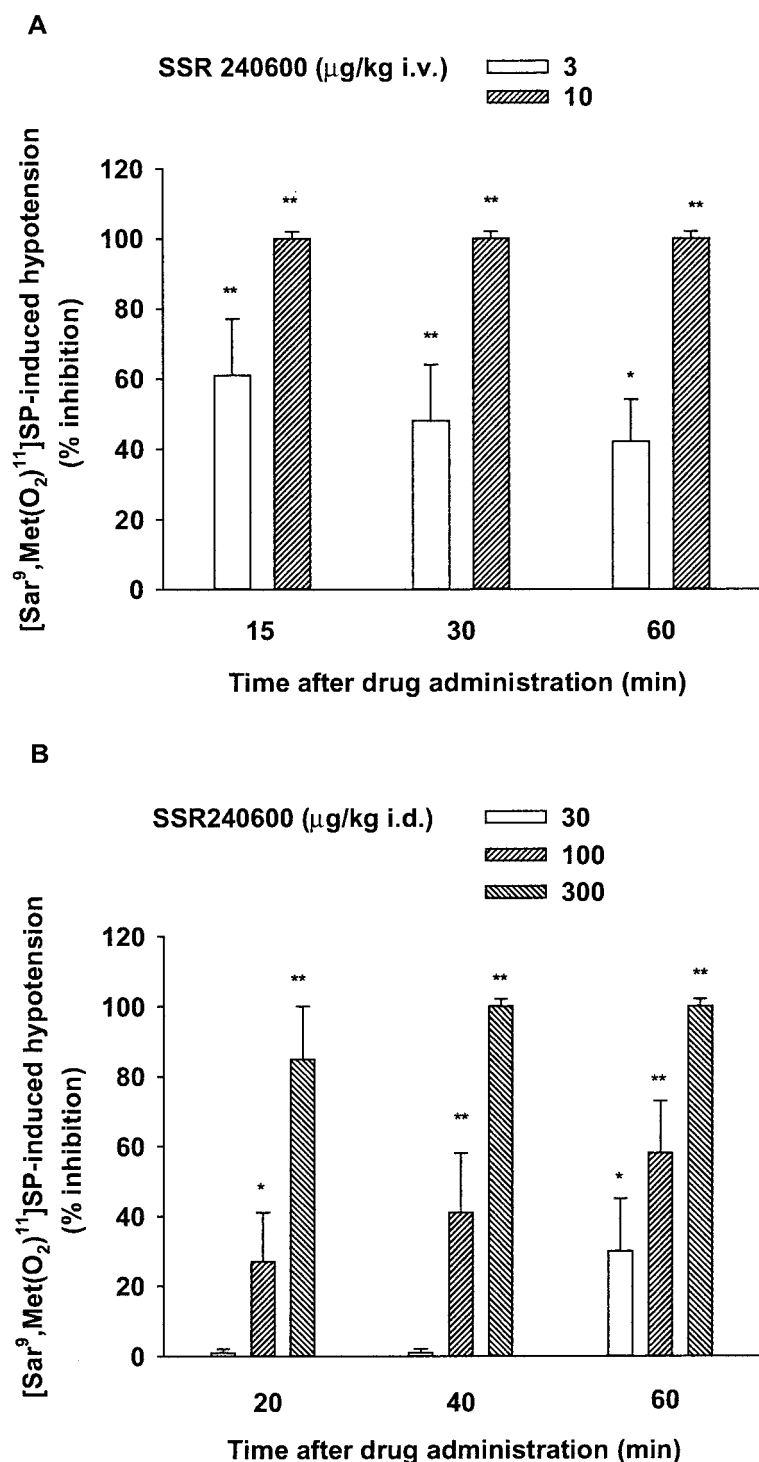


**Fig. 3.** Inhibition by SSR240600 of [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]substance P-induced contractions of human isolated small bronchi (diameter <1 mm). [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]Substance P concentration was 100 nM. Results are expressed as percentage inhibition of control and values are means ± S.E.M. (*n* = 7–8). Significant variations from control are shown as \* for *P* < 0.05 and \*\* for *P* < 0.01 (ANOVA followed by Dunnett's *t* test).

SSR240600 was then studied on citric acid-induced cough in awake guinea pigs, a model in which endogenous tachykinins and their receptors are suspected to play an important role. Cough was provoked by exposure of animals to an aerosol of aqueous citric acid solution (0.4 M) for 10 min. Intraperitoneal administration of SSR240600 (1–10 mg/kg), 30 min before the citric acid challenge, resulted in a dose-dependent inhibition of cough (Fig. 7A). This inhibition was highly time dependent (Fig. 7B), increasing with the length of the pretreatment. The cough inhibition following 120 min pretreatment with 1 mg/kg i.p. SSR240600 was comparable with that observed after 30 min pretreatment with 10 mg/kg i.p.

## Discussion

This paper describes biochemical and pharmacological activities of SSR240600, a new, selective and highly potent nonpeptide antagonist of the tachykinin NK<sub>1</sub> receptor. In binding experiments, SSR240600 potently inhibited binding of radioactive substance P to human tachykinin NK<sub>1</sub> receptors with inhibition constants (*K<sub>i</sub>*) in the subnanomolar range. Of particular interest is its very high affinity for the native tachykinin NK<sub>1</sub> receptor present in human brain cortex membrane preparation. The potency of SSR240600 was comparable with that previously observed with another chemically related tachykinin NK<sub>1</sub> receptor antagonist, SR140333 (Emonds-Alt et al., 1993), except that its affinity was typically species-dependent. Contrary to SR140333, it was more active on tachykinin NK<sub>1</sub> receptors of guinea pigs than of rats and gerbils. Such species-dependent affinities have been observed for other nonpeptide and peptidomimetic NK<sub>1</sub> receptor antagonists (McLean et al., 1993, 1996; Aramori et al., 1994; Beattie et al., 1995; Cellier et al., 1996; Quartara and Maggi, 1997). The potent antagonism of SSR240600 at tachykinin NK<sub>1</sub> receptors has been further demonstrated in different in vitro functional assays for tachykinin receptors. First, like SR140333, it blocked with high efficacy both tachykinin NK<sub>1</sub> receptor-mediated inositol phosphate-1 formation in human astrocytoma U373MG cells

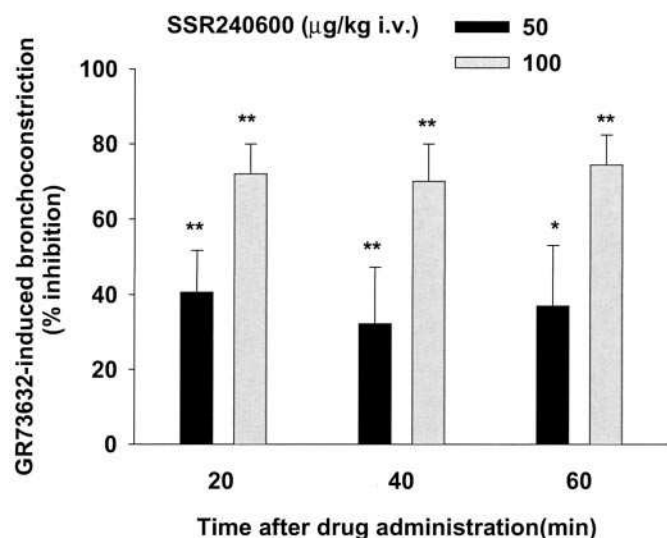


**Fig. 4.** Inhibition by SSR240600 of  $[\text{Sar}^9, \text{Met}(\text{O}_2)^{11}]$ substance P-induced hypotension in anesthetized dogs. SSR240600 was administered by the intravenous (A) or intraduodenal (B) route.  $[\text{Sar}^9, \text{Met}(\text{O}_2)^{11}]$ Substance P (5 ng/kg) was injected intravenously at various times after SSR240600 administration. Results are expressed as percentage inhibition of the reduction of mean blood pressure (about 30–40 mm Hg) induced by  $[\text{Sar}^9, \text{Met}(\text{O}_2)^{11}]$ substance P before SSR240600 administration. Values are means  $\pm$  S.E.M. ( $n = 3-5$ ). Significant variations from control are shown as \* for  $P < 0.05$  and \*\* for  $P < 0.01$  (ANOVA for repeated measures followed by Dunnett's  $t$  test).

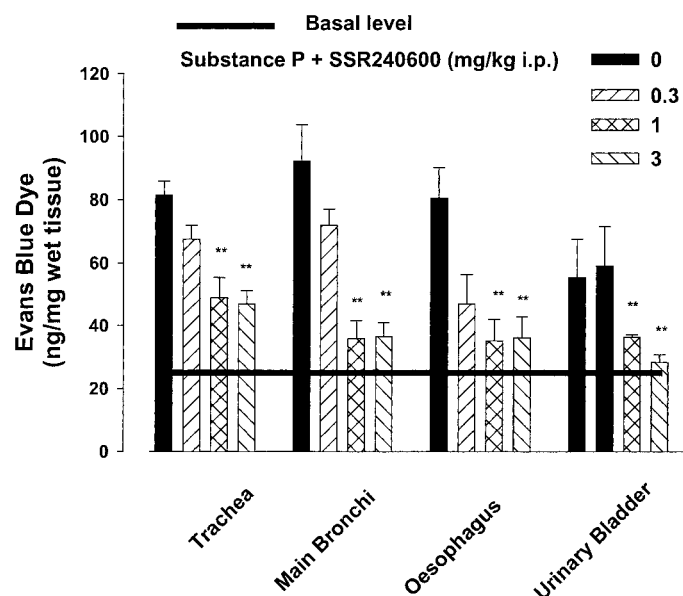
(Oury-Donat et al., 1994) as well as contractions of isolated human small bronchi (Naline et al., 1996). Second, it also potently antagonized  $[\text{Sar}^9, \text{Met}(\text{O}_2)^{11}]$ substance P-induced endothelium-dependent relaxation of rabbit pulmonary artery precontracted by norepinephrine, a typical tachykinin  $\text{NK}_1$  receptor assay (Regoli et al., 1994). Like SR140333 (Emonds-Alt et al., 1993), SSR240600 antagonism was not purely competitive. A similar profile was reported with other peptidomimetic or nonpeptide tachykinin  $\text{NK}_1$  receptor antagonists (Beattie et al., 1995; Cirillo et al., 1998).

The selectivity of SSR240600 for tachykinin  $\text{NK}_1$  receptors

has also been clearly demonstrated in our binding and in vitro functional studies. Indeed, the affinities measured in binding assays for tachykinin  $\text{NK}_2$  and  $\text{NK}_3$  receptors remained very low compared with tachykinin activities or activities displayed by specific antagonists of tachykinin  $\text{NK}_2$  (SR48968, SR144190) (Emonds-Alt et al., 1992, 1997) or  $\text{NK}_3$  (SR142801, SSR146977) (Emonds-Alt et al., 1995, 2002) receptors at these receptors. The selectivity of SSR240600 for tachykinin  $\text{NK}_1$  receptors was further evidenced in different in vitro functional assays for these tachykinin receptors. In assays using isolated organ preparations typical for tachyki-

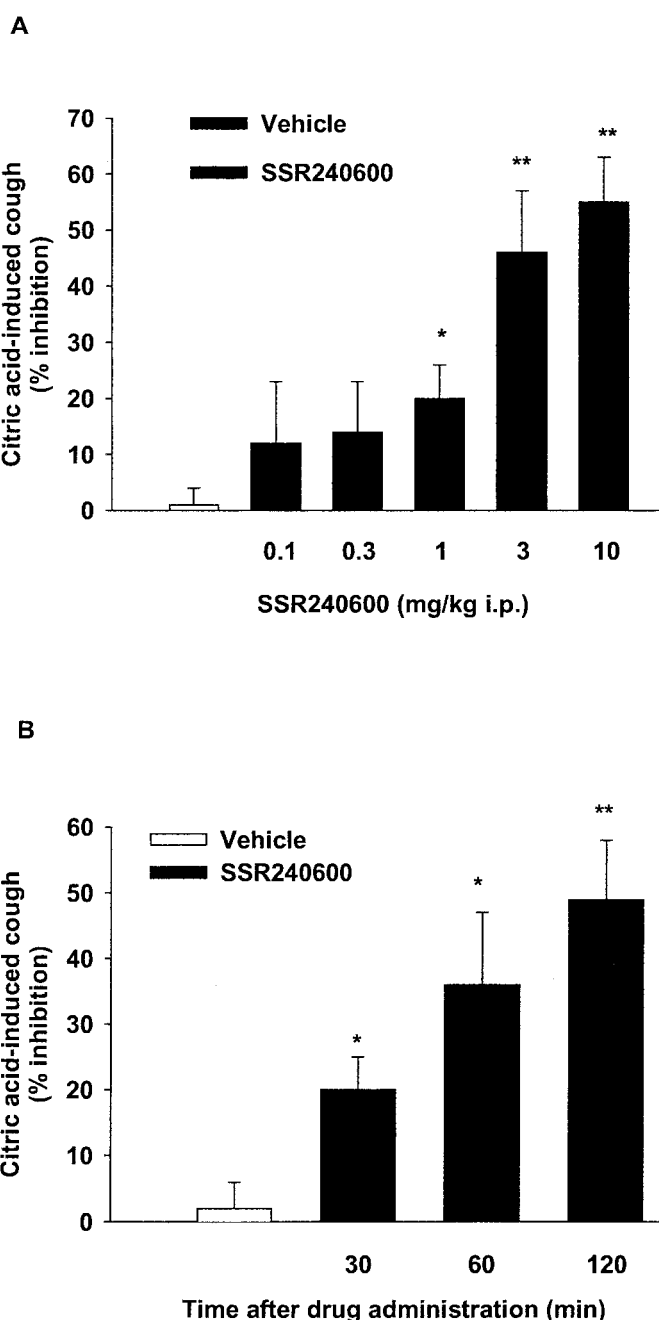


**Fig. 5.** Inhibition by SSR240600 of GR73632-induced bronchoconstriction in anesthetized guinea pigs. SSR240600 was administered by the intravenous route at various doses. GR73632 (0.5 ng/kg) was injected intravenously at the indicated times after SSR240600 administration. Results are expressed as percentage inhibition of bronchoconstriction induced by GR73632 before administration of SSR240600. Values are means  $\pm$  S.E.M. ( $n = 5$ ). Significant variations from control bronchoconstriction before SSR240600 administration are shown as \* for  $P < 0.05$  and \*\* for  $P < 0.01$  (ANOVA for repeated measures followed by Dunnett's  $t$  test).



**Fig. 6.** Inhibition by SSR240600 of substance P-induced microvascular leakage in anesthetized guinea pigs. Saline (control) or SSR240600 was administered by the intraperitoneal route at various doses, 30 min before Evans blue dye (30 mg/kg i.v.). One minute after dye administration, plasma extravasation was provoked by substance P (0.3  $\mu$ g/kg i.v.). Basal level was determined in the absence of substance P. Results are expressed as tissue content of Evans blue dye. Values are means  $\pm$  S.E.M. ( $n = 4-6$ ). Significant variations from control are shown as \*\* for  $P < 0.01$  (ANOVA followed by Dunnett's  $t$  test).

nin NK<sub>2</sub> and NK<sub>3</sub> receptors (Regoli et al., 1994), it did not interact with these receptors, at concentrations up to 0.1  $\mu$ M. Its antagonist activity at these receptors at a concentration of 1  $\mu$ M remained limited compared with the activities of specific antagonists of these receptors in the same assays (Emonds-Alt et al., 1992, 1995, 1997, 2002). This limited



**Fig. 7.** Inhibition by SSR240600 of cough provoked by exposure of conscious guinea-pigs to an aerosol of citric acid solution (0.4 M) for 10 min. A, SSR240600 was administered at different doses by the intraperitoneal route, 30 min before the citric acid challenge. B, SSR240600 (1 mg/kg i.p.) was administered at different times before the citric acid challenge. Results are expressed as percentage inhibition of control and are means  $\pm$  S.E.M. ( $n = 4-10$ ). Significant variations from control are shown as \* for  $P < 0.05$  and \*\* for  $P < 0.01$  (ANOVA followed by Dunnett's  $t$  test).

effect of SSR240600 on tachykinin receptors other than NK<sub>1</sub> receptors was confirmed in functional assays using CHO cells stably expressing human tachykinin NK<sub>2</sub> and NK<sub>3</sub> receptors. In these assays, the inhibition of tachykinin NK<sub>2</sub> or NK<sub>3</sub> receptor-mediated inositol phosphate-1 formation by SSR240600 was only observed at IC<sub>50</sub> values about 200-fold higher than those obtained in similar experimental conditions for specific NK<sub>2</sub> (SR 48968, SR144190) (F. Oury-Donat, O. Thurneysen, and P.

Soubrié, unpublished results) or NK<sub>3</sub> (SR142801, SSR146977) (Oury-Donat et al., 1995; Emonds-Alt et al., 2002) receptor antagonists. Finally, the selectivity of SSR240600 for tachykinin NK<sub>1</sub> receptors was also shown by its lack of activity at concentrations up to 1 μM in almost all binding assays for various ion channels and for neurotransmitter, neuropeptide, and hormone receptors as well as in enzyme assays. In the few cases where an interaction was detected, this was always with IC<sub>50</sub> values above 0.1 μM.

In vivo, SSR240600 has been shown to exert a highly potent antagonism at tachykinin NK<sub>1</sub> receptors. Its potency was first demonstrated in animal models where direct activation of tachykinin NK<sub>1</sub> receptors was provoked by substance P or specific agonists of these receptors. Like SR140333 and other tachykinin NK<sub>1</sub> receptor antagonists (Emonds-Alt et al., 1993; Hirayama et al., 1993; Cellier et al., 1996; McLean et al., 1996; Cirillo et al., 1998), SSR240600 very potently inhibited tachykinin NK<sub>1</sub> receptor-mediated hypotension, bronchoconstriction, and plasma extravasation, three typical effects of substance P and its analogs (Regoli et al., 1994; Quartara and Maggi, 1998). It was active by the oral route and had long-lasting effects.

SSR240600 was then studied in an animal model where endogenous tachykinins through their respective receptors are suspected to play a major role: citric acid-induced cough in unanesthetized guinea pigs (Widdicombe, 1995; Advenier and Emonds-Alt, 1996; Advenier et al., 1997). Either tachykinin NK<sub>2</sub> (Advenier et al., 1993; Girard et al., 1995; Yasumitsu et al., 1996; Emonds-Alt et al., 1997) or NK<sub>3</sub> (Daoui et al., 1998; Emonds-Alt et al., 2002) receptor antagonists have been reported to have potent antitussive activity in this model. Regarding the effect of nonpeptide tachykinin NK<sub>1</sub> receptor antagonists in this model, the results are controversial. No inhibitory activity was observed for a nonpeptide antagonist such as SR140333 (Girard et al., 1995), whereas an antitussive effect was reported for a peptidomimetic antagonist (FK888) (Yasumitsu et al., 1996). However, another nonpeptide tachykinin NK<sub>1</sub> receptor antagonist, CP-99,994 (McLean et al., 1993), was shown to block cough induced by capsaicin in unanesthetized guinea pigs as well as by mechanical stimulation of trachea in anesthetized cats (Bolser, 1996; Bolser et al., 1997). In the present study, SSR240600 was clearly shown to potently inhibit citric acid-induced cough in unanesthetized guinea pigs. Moreover, in the same model and under the same experimental conditions used for SR140333 and SSR240600, another tachykinin NK<sub>1</sub> receptor antagonist, GR205171 (Gardner et al., 1996), also displayed potent antitussive activity (C. Advenier, E. Naline, and S. Daoui, unpublished results).

The antitussive activity of SSR240600 in guinea pigs may be related to its ability to readily penetrate into the brain. Indeed, the antitussive activity of CP-99,994 was reported as probably mainly mediated by a central action (Bolser, 1996; Bolser et al., 1997). On the other hand, there is some parallelism between antitussive activity and other centrally mediated activities of the tachykinin NK<sub>1</sub> receptor antagonists. SR140333 was shown to have several activities in the rat central nervous system (Jung et al., 1994), but it was also reported to lack activity in some models, in particular, models for emesis, in which brain penetration of the compound is essential (Rupniak et al., 1997; Diemunsch and Grélot, 2000). On the contrary, GR205171 was shown to have a potent

broad-spectrum anti-emetic activity (Gardner et al., 1996; Diemunsch and Grélot, 2000). Similarly, CP-99,994 also showed potent anti-emetic activity as well as other typical centrally mediated effects (Rupniak et al., 1997). Moreover, a potent antidepressant-like activity of SSR240600 in guinea pigs was clearly demonstrated (Steinberg et al., 2002) as for centrally active tachykinin NK<sub>1</sub> receptor antagonists (Rupniak and Kramer, 1999). A preliminary pharmacokinetic study (C. Briot, unpublished results) also showed an efficient brain penetration of the compound in guinea pigs with slow kinetics (peak plasma level of 650 ng/ml obtained at 1 h, brain tissue level of 70 ng/g reached at 4 h, and stable between 4 and 8 h after oral administration at 10 mg/kg), explaining its time-dependent antitussive activity. However, the results reported for the evaluation of FK888 brain penetration are opposite (Yasumitsu et al., 1996; Rupniak et al., 1997), but together, they suggest a low brain penetration, if any. The antitussive activity of FK888 in guinea pigs could be due to a peripheral effect related to its peptidomimetic structure since a low brain-penetrant nonpeptide antagonist, SR140333, was completely inactive.

In conclusion, SSR240600 is a novel, highly potent nonpeptide antagonist of the tachykinin NK<sub>1</sub> receptor. It is active by the oral route with long-lasting effects and can penetrate into the brain.

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