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Pharmacological Characterization of LY303870: A Novel, Potent and Selective Nonpeptide Substance P (Neurokinin-1) Receptor Antagonist

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ABSTRACT

LY303870 [(*R*)-1-[N-(2-methoxybenzyl)acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl)piperidin-1-yl)acetyl)amino]-propane] is a new, potent and selective nonpeptide neurokinin-1 (NK-1) receptor antagonist. LY303870 bound selectively and with high affinity to human peripheral (K_i = 0.15 nM) and central (K_i = 0.10 nM) NK-1 receptors. LY303870 inhibited [^{125}I]substance P (SP) binding to guinea pig brain homogenates with similar affinity; however, it had approximately 50-fold lesser affinity for rat NK-1 sites. The less active enantiomer, LY306155 {(*S*)-1-[N-(2-methoxybenzyl)acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl)piperidin-1-yl)acetyl)amino]-propane}, was 1,000- to 15,000-fold less potent in all the species examined. LY303870 antagonized *in vitro* NK-1 receptor effects as demonstrated by blockade of SP-stimulated phosphoinositide turnover in UC-11 MG human astrocytoma cells (K_i = 1.5 nM) and interleukin-6 secretion from U-373 MG human astrocytoma cells (K_i = 5 nM). In addition, LY303870 inhibited SP-induced rabbit vena cava contractions (pA_2 = 9.4) with high (50,000-fold) selectivity *vs.* NK-2 or NK-3 receptor-mediated responses. *In vivo*, LY303870 inhibited [Sar^9,-Met(O$_2$)^{11}]-SP induced guinea pig bronchoconstriction (ED_{50} = 75 μg/kg i.v.) and pulmonary microvascular leakage in the bronchi (ED_{50} = 12.8 μg/kg i.v.) and trachea (ED_{50} = 18.5 μg/kg i.v.). Therefore, LY303870 is a potent and selective NK-1 receptor antagonist *in vitro* and *in vivo*. The use of LY303870 will facilitate a better understanding of NK-1 receptors in physiological processes.

The undecapeptide, SP is a member of the tachykinin family of neuropeptides (von Euler and Gaddum, 1931; Chang and Leeman, 1970; Chang *et al.*, 1971; Pernow, 1983; Maggio, 1988). These peptides act through three major receptor subtypes, denoted as NK-1, NK-2 and NK-3, having preferential affinity for SP, NK-A and NK-B, respectively (Henry, 1987; Regoli *et al.*, 1989; Guard and Watson, 1991). SP is released from primary sensory nerve fibers (unmyelinated) and elicits several biological activities, primarily *via* the activation of NK-1 receptors. As such, SP is a key mediator of pain in the peripheral and central nervous system. SP also stimulates smooth muscle contraction, vasodilation, plasma extravasation and the release of inflammatory mediators. These actions contribute to local inflammation and nociception. NK-1 (along with NK-2 and NK-3) receptors have been cloned and sequenced and are members of the seven transmembrane domain, G protein-coupled, family of receptors linked to phospholipase *C* and phosphoinositide turnover (Torrens *et al.*, 1986, 1989; Lee *et al.*, 1989; Nakanishi, 1991; Marriott *et al.*, 1991; Johnson and Johnson, 1992; Heuillet *et al.*, 1993). Additional information on the general physiology and pharmacology of NKs and their receptors can be found in the comprehensive reviews of Maggi *et al.* (1993) and Otsuka and Yoshioka (1993).

Recently, the pharmacological characterization of NK-1 receptors has been aided by the discovery of nonpeptide and cyclic peptide receptor antagonists (Snider *et al.*, 1991; Garret *et al.*, 1991; Fujii *et al.*, 1992; Morimoto *et al.*, 1992; Mclean *et al.*, 1993; Emonds-Alt *et al.*, 1993; Mills *et al.*, 1993; MacLeod *et al.*, 1993; Barrow *et al.*, 1994; Achard *et al.*, 1994; Tabart and Peyronel, 1994). The pharmacological profile of many of these compounds is species-dependent, resulting in

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ABBREVIATIONS: SP, substance P; NK, neurokinin; RPMI, Roswell Park Memorial Institute; EMEM, Eagles minimun essential medium; IL-6, interleukin-6; ELGV, excised lung gas volume; PI, phosphoinositide.

segregation of antagonist potencies into two major groups, human/guinea pig *vs.* rodent (Snider *et al.*, 1991; Gitter *et al.*, 1991; Beresford *et al.*, 1991; Watling *et al.*, 1991; Garret *et al.*, 1991; Fardin *et al.*, 1992).

Nonpeptide NK-1 receptor antagonists are particularly useful due to their improved stability and potential for greater p.o. bioavailability. It is expected that continued research resulting in the discovery of these antagonists will lead to a better understanding of the role of SP and NK-1 receptors in pathophysiological processes that underlie pain and inflammation. In the present study, we report on the discovery of LY303870 [(*R*)-1-[N-(2-methoxybenzyl)acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl)piperidin-1-yl)acetyl)amino]propane], a novel, highly potent and selective nonpeptide NK-1 antagonist with a species-dependent pharmacological profile.

Methods

Reagents and drugs. LY303870 [(*R*)-1-[N-(2-methoxybenzyl)-acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl)piperidin-1-yl)-acetyl)amino]propane] (and its enantiomer LY306155 [(*S*)-1-[N-(2-methoxybenzyl)acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl) piperidin-1-yl)acetyl)amino]propane]), (±)-CP-96,345 and (±)-RP 67580 were synthesized in the CNS Research Division (Lilly Research Laboratories, Indianapolis, IN). The structure of LY303870 is depicted in figure 1. [^{125}I]Bolton Hunter SP (2200 Ci/mmol) and myo-[^{3}H]inositol (10–20 Ci/mmol) were purchased from Dupont/New England Nuclear (Boston, MA) and American Radiolabeled Chemicals, Inc. (St. Louis, MO), respectively. SP, [Sar^{9},Met(O$_2$)^{11}]-SP, [β-Ala^{8}]NK-A$_{4-10}$, [MePhe^{7}]-NK-B and Senktide were purchased from Peninsula Laboratories, Inc. (Belmont, CA). Evans blue dye was purchased from Fisher Scientific Co. (Fairlawn, NJ). Unless indicated, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture. The human B lymphoblastoid cell line, IM-9, and the human astrocytoma cell line, U-373 MG (Payan *et al.*, 1984; Ponten and MacIntyre, 1968) were obtained from the American Type Culture Collection (Rockville, MD). The human astrocytoma cell line, UC-11 MG (Liwnicz *et al.*, 1986), was the kind gift of Dr. C. L. Johnson (University of Cincinnati). IM-9 and UC-11 MG cells were cultured in RPMI 1640, supplemented with 50 μg/ml of gentamicin sulfate (Whittaker M.A. Bioproducts, Inc., Walkersville, MD) and 10% heat inactivated fetal bovine serum (HyClone Laboratories, Logan, UT,) (RPMI-10%).

U-373 MG cells were grown in Eagle's minimum essential medium (Whittaker M.A. Bioproducts, Inc., Walkersville, MD) supplemented with 50 μg/ml of gentamicin sulfate, 4 mM L-glutamine and 10% heat inactivated fetal bovine serum (EMEM-10%). Confluent monolayers of U-373 MG and UC-11 MG cells were routinely passaged weekly by trypsinization (0.25% trypsin and 0.02% EDTA, in phosphate-buffered saline, pH 7.4) and plated in 175 mm^2 culture flasks.

NK-1 receptor binding. NK-1 radioreceptor binding assays were performed using IM-9 cells or various tissue homogenates. [^{125}I]SP binding to intact IM-9 cells was conducted according to Payan *et al.* (1984) with modifications. IM-9 cells (1 × 10^6/tube in RPMI-10%) were incubated with 20 pM [^{125}I]SP in the presence of increasing competitor concentrations for 45 min at 4°C. The reaction was terminated by filtration through a glass-fiber filter harvesting system (Brandel, Gaithersburg, MD) using filters soaked previously for 20 min in 0.1% polyethylenimine. Bound radioactivity was measured using a Pharmacia LKB gamma counter (Piscataway, NJ). Membranes from guinea pig, rat and mouse brains were prepared by homogenization of tissue in 30 volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl and 5 mM KCl) with a Polytron homogenizer for 15 sec, followed by centrifugation at 40,000 × *g* for 10 min. The pellets were resuspended in 50 mM Tris-HCl, pH 7.4, 300 mM KCl and 10 mM EDTA on ice for 30 min. The homogenate was centrifuged as above, and the pellet was washed 3 times by homogenization in 30 volumes of 50 mM Tris-HCl, pH 7.4, followed by centrifugation. The final pellet was resuspended in 50 mM Tris-HCl, pH 7.4, at a concentration of 5 to 10 mg of protein per ml and frozen at −80°C. Homogenate (400 μg/ml) in assay buffer (50 mM Tris-HCl, pH 7.4, with 3 mM $MgCl_2$, 2 μg/ml of chymostatin, 10 μM captopril, 4 μg/ml of leupeptin, 10 μM phosphoramidon and 40 μg/ml of bacitracin) were incubated with 20 pM [^{125}I]SP in the presence of increasing competitor concentrations for 1 hr at room temperature. Samples were filtered over GF/B filters soaked for 2 hr in 50 mM Tris-HCl, pH 7.7, with 0.1% w/v bovine serum albumin and 0.15% w/v polyethylenimine. Filters were washed 3 times with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.7, and counted as described above. Nonspecific binding was determined in the presence of 1,000-fold excess cold SP. Human caudate nuclei were obtained from ABS, Inc. (Wilmington, DE). These postmortem tissue samples were obtained from three patients aged 21, 55 and 67 years old. Homogenates were prepared by disrupting the tissue (1.7 g) in 30 ml of 50 mM Tris buffer (pH 7.4) with a Polytron homogenizer. After an initial centrifugation at 900 × *g*, the supernatant was transferred to a clean centrifuge tube and the membranes isolated by centrifugation at 38,000 × *g*. The homogenates were stored at −70°C until assayed. The membranes were resuspended in 6 ml of binding assay buffer (50 mM Tris, 3 mM $MnCl_2$, 0.02% bovine serum albumin, 40 μg/ml of bacitracin, 2 μg/ml of chymostatin, 4 μg/ml of leupeptin and 40 μg/ml of thiorphan, pH 7.4). Binding studies were conducted in a final volume of 200 μl containing 0.2 nM [^{125}I]SP and various concentrations of test compounds. Nonspecific binding was determined by incubating samples in the presence of 1 μM cold SP. The binding was initiated by adding 0.1 to 0.2 mg of protein per tube and terminated after a 60-min RT incubation by rapid filtration using a TOMTEC 96-well cell harvester (TOMTEC, Orange, CT) through GF/A filters that had been presoaked with 0.3% polyethylenimine for 1 hr. The filters were washed with 5 ml of ice-cold 50 mM Tris buffer (pH 7.4) and placed in a drying oven at 60°C. After drying, the radioactivity bound to the filters was measured using solid scintillation counting with a 1205-Betaplate counter (WALLAC, Inc., Gaithersburg, MD). IC_{50} values were determined using the Prism (Graphpad, Inc., San Diego, CA) software package.

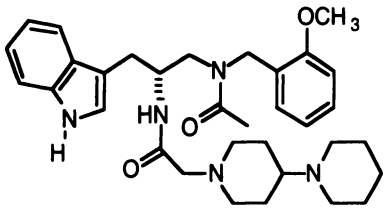

Fig. 1. Structure of LY303870: (*R*)-1-[N-(2 methoxybenzyl)acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl)piperidin-1-yl)-acetyl)amino]propane.

Non-NK-1 *in vitro* assays. Nineteen non-NK-1 receptor binding assays that were performed at Lilly Research Laboratories are listed, along with their corresponding methodological citations, in table 1. Table 2 lists an additional 46 non-NK-1 *in vitro* assays that were performed at Novascreen (Hanover, MD) according to their established proprietary protocols.

Inositol phosphate accumulation. UC-11 cells were split 1:5 into 6-well culture dishes 6 days before each experiment. Two days before the experiment, the medium was replaced with 1 ml of fresh medium containing 1 μCi of myo-[^{3}H]inositol. Incubations were initiated by washing monolayers twice with 1 ml of Tyrode's balanced salt solution containing 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) and 10 mM LiCl, followed by the addition of 1 nM SP (with or without increasing competitor concentrations) in 1 ml Tyrode's-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-LiCl. After a 1-hr incubation at 37°C, the reaction was terminated by removing the medium and adding 0.5 ml of 5% tri-

TABLE 1

Evaluation of LY303870 in non-NK-1 receptor binding assays

K_i values for LY303870 in all assays were >1.0 μM. ABBREVIATIONS: GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine; AMPA, α-amino-3-hydroxy-5-methybisoxazole-4-propionate; NMDA, N-methyl-D-aspartate; QNB, quinuclidinyl benzilate.

Receptor	Radioligand	Reference
Alpha-1 adrenergic	[^{3}H]Prazosine	Greengrass and Bremner, 1979
Alpha-2 adrenergic	[^{3}H]Rauwolscine	Boyajian and Leslie, 1987
Beta adrenergic	[^{3}H]Dihydroalprenolol	Bylund and Snyder, 1976
Dopamine D1	[^{3}H]SCH23390	Billard *et al.*, 1984
Dopamine D2	[^{3}H]Raclopride	Hall *et al.*, 1988
Benzodiazepine	[^{3}H]Flunitrazepam	Braestrup and Squires, 1978
Histamine H1	[^{3}H]Pyrilamine	Tran *et al.*, 1978
$GABA_A$	[^{3}H]Muscimol	Williams and Risley, 1979
Muscarinic (nonselective)	[^{3}H]QNB	Yamamura and Snyder, 1974
Muscarinic M1	[^{3}H]Pirenzepine	Potter *et al.*, 1988
NK-2	[^{125}I]NK-A	Gitter *et al.*, 1995
$5\text{-}HT_2$	[^{3}H]Ketanserine	Battaglia *et al.*, 1983
$5\text{-}HT_{1c}$	[^{3}H]Mesulergine	Wong *et al.*, 1991
$5\text{-}HT_{1d}$	[^{3}H]-5-HT	Wong *et al.*, 1991
Metabotropic glutamate	[^{3}H]Glutamate	Wright *et al.*, 1994
AMPA	[^{3}H]AMPA	Nielsen *et al.*, 1986
NMDA	[^{3}H]CGS19755	Murphy *et al.*, 1988
Kainate	[^{3}H]Kainate	Kamboj *et al.*, 1994
MK801	[^{3}H]MK801	Calligaro *et al.*, 1993

TABLE 2

Evaluation of LY303870 in non-NK-1 Novascreen assays

Assays performed at Novascreen according to their established proprietary protocols. K_i values for LY303870 in all assays were >1.0 μM. ABBREVIATIONS: CCK, cholecystokinin; VIP, vasoactive intestinal peptide; ANF, atrial natriuretic factor; CRF, corticotropin-releasing factor; TRH, thyrotropin-releasing hormone; NPY, neuropeptide Y.

Radioreceptor Binding Assays	Ion Channels and Reuptake Sites
Adenosine	Calcium (T & L) channel; calcium N channel
Benzodiazepine (central and peripheral)	ATP modulated-potassium channel
Sigma	Low conductance Ca^{++} activated-potassium channel
Opiate (*mu*, *delta*, *sigma* and *kappa*)	Voltage-dependent-potassium channel
Nicotinic	Sodium channel (site 1); sodium channel (site 2)
Angiotensin I; angiotensin II	Dopamine reuptake (cocaine site)
Vasopressin I; vasopressin II	Norepinephrine reuptake site
Bombesin	Serotonergic reuptake site
CGRP1; CGRP2	
CCK_A; CCK_B	
Endothelin A; endothelin B	
Neurotensin	
Somatostatin	
VIP	
ANF_1; ANF_2	
CRF	
Oxytocin	
TRH	
NK-3	
NPY_1	
Leukotriene B_4	
Leukotriene D_4	
Thromboxane A_2	
Basic fibroblast growth factor	
Inositol triphosphate	

chloroacetic acid. Total [^{3}H]inositol phosphates were measured by the method of Berridge *et al.* (1993). All samples were run in triplicate.

IL-6 induction and immunoassay. Stimulation of IL-6 secretion and the IL-6 immunoassay were performed essentially as described previously (Gitter *et al.*, 1994). Freshly detached U-373 MG cells were washed 3 times in serum-free EMEM, plated at 5×10^5 cells/well in 24-well culture dishes and incubated (37°C, 5% CO_2) overnight to facilitate adherence. Attached cells were washed 3 times with serum-free EMEM and treated with 20 nM SP with or without increasing competitor concentrations (prepared in EMEM with 5% fetal calf serum and 12.5 μg/ml of polymyxin B, in a total volume of 1 ml). Triplicate preparations were incubated for an additional 18 to 24 hr and cell-free supernatant samples were collected and stored at −20°C before cytokine assay. Levels of immunoreactive IL-6 in these samples were determined using a specific and sensitive ELISA kit (R & D Systems, Minneapolis, MN). This competitive "sandwich" assay utilizes monoclonal antihuman IL-6 antibody-coated microtiter wells and peroxidase conjugated polyclonal (goat) anti-IL-6 antibody.

Smooth muscle pharmacology. The smooth muscle experiments for the NK-1, NK-2 and NK-3 receptor-mediated responses were performed according to previously published methods (Rouissi *et al.*, 1991).

[Sar^9,Met(O$_2$)^{11}]-SP-induced pulmonary gas trapping. Pulmonary function studies were conducted using barrier-maintained, male outbred Hartley guinea pigs obtained from Charles River Breeding Laboratories, Inc. (Portage, MI). Approximately 1 hr before challenge with [Sar^9,Met(O$_2$)^{11}]-SP, each guinea pig was anesthetized with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane, Halocarbon Laboratories, North Augusta, SC) and a catheter (S-54-HL, microbore tubing, 0.76 mm outside diameter) was inserted in the saphenous vein (2% lidocaine hydrochloride was applied to the leg before catheter placement). Animals were allowed to regain consciousness and were placed in plastic restraining tubes (Research and Consulting Company AG, Basel, Switzerland). Once animals recovered from anesthesia, they were dosed with an i.v. injection of LY303870 (0.01, 0.1 or 1.0 mg/kg), LY306155 (1.0 mg/kg) or drug-vehicle (1.0 ml/kg). Drug-vehicle was 0.05 ml of 1 N HCl in 0.95 ml of saline. An i.v. bolus injection of [Sar^9,Met(O$_2$)^{11}]-SP (10.0 nmol/kg) in saline was delivered 5 min after administration of the vehicle. Sham-treated animals were dosed i.v. with drug-vehicle and given saline (1.0 ml/kg) 5 min later. All guinea pigs were sacrificed by i.v. administration of 0.2 ml of Euthanasia-5 solution (Veterinary Laboratories, Inc., Lenexa, KS) 3 min after the [Sar^9,Met(O$_2$)^{11}]-SP challenge.

After death, the abdomen was opened, the diaphragm punctured and the lungs allowed to deflate. The trachea was exposed and cut 10 to 12 rings below the larnyx; then a 2-cm long section of polyethylene-240 tubing was inserted to one-half its length. The trachea and lungs were removed and carefully trimmed of nonpulmonary tissue. The ELGV, *i.e.*, postmortem pulmonary gas trapping, was deter-

mined by Archimedes' principle (Stengel and Silbaugh, 1986; Silbaugh *et al.*, 1987). Briefly, the lungs were attached by the tracheal cannula to a 15-gauge needle fitted into a brass anchor. The lungs and brass anchor were then placed in a plastic cup, immersed in a beaker of saline and suspended from a hook at the top of a Mettler AE160 balance (the weight in saline of the brass anchor and plastic cup was tarred before each experiment). Because lung tissue density approximates that of saline, the volume of gas trapped in the lungs could be measured.

[Sar^9,Met(O$_2$)^{11}]-SP-induced microvascular leakage. A catheter was inserted in the saphenous vein to allow i.v. administration of all test agents. The guinea pigs were allowed to recover from anesthesia for 1 hr and then placed in plastic restraining tubes as described above. Evans blue dye (30.0 mg/kg in saline) was given to each animal 2 min before administration of LY303870 (0.001, 0.01 or 0.1 mg/kg), LY306155 (0.1 mg/kg) or drug-vehicle (1.0 ml/kg). A bolus injection of [Sar^9,Met(O$_2$)^{11}]-SP (0.3 nmol/kg) was delivered 5 min later. Evans blue dye-treated sham animals were dosed with drug-vehicle and given saline (1.0 ml/kg) 5 min later. All guinea pigs were sacrificed 3 min after the [Sar^9,Met(O$_2$)^{11}]-SP challenge with 0.2 ml of Euthanasia-5 solution.

Once the animal died, the trachea and mainstem bronchi were dissected from the lungs, separated at the carina and trimmed of adventitia. The technique of Katayama *et al.* (1978) was altered to examine vascular permeability changes in the trachea and mainstem bronchi. The leakage of plasma protein bound Evans blue dye was proven to be highly correlated with extravasation of [^{125}I]human serum albumin in guinea pig airways (Rogers *et al.*, 1989). The trachea and bronchi were opened longitudinally along the posterior side, the serosal and mucosal surfaces rubbed with a saline-soaked gauze and then patted with a dry gauze. Each tissue segment was weighed, placed into 2 ml of saline and homogenized. Two milliliters of acetone were then added and the tubes vortexed. The tissues were centrifuged at 800 × *g* for 25 min. After centrifugation, the top layer was discarded and the supernatant carefully transferred to new tubes. The supernatant was centrifuged at 800 × *g* for 15 min. Optical absorbance of the supernatant was measured at 620 nm (Beckman DU-64 spectrophotometer) and normalized by wet tissue weight. To adjust for non-Evans blue-related absorbance, trachea and bronchi were taken from naive guinea pigs and absorbance of the tissue measured. The mean background absorbance per milligram of trachea and bronchi was subtracted from each experimental value to obtain the final Evans blue absorbance. Evans blue extravasation was calculated from a standard curve generated from varying concentrations of dye in an acetone-saline (50%:50%) solution. The results are expressed as the mean ± S.E.M. of four to seven animals per group. All ELGV measurements were normalized on a body weight basis (milliliters per kilogram). Analysis of variance was used to compare rank-transformed data of ELGV and extravasated Evans blue dye values with P-values computed from the analysis of variance. Analyses were run using SAS (SAS Institute Inc., Cary, NC) on an IBM 3081 computer. Comparisons were considered significant for P-values of .05 or less.

Results

NK-1 receptor binding. LY303870 [(*R*)-1-[N-(2-methoxybenzyl)acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl)piperidin-1-yl)acetyl)amino]propane] was a potent ligand for NK-1 receptors expressed by human peripheral (IM-9 lymphoblasts) and central (caudate homogenate) tissues (fig. 2; table 3) with affinities similar to SP and previously described nonpeptide NK-1 receptor antagonists (McLean *et al.*, 1993) (table 3). The less active enantiomer, LY306155 [(*S*)-1-[N-(2-methoxybenzyl)acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl)piperidin-1-yl)acetyl) amino]propane], had 1,000- to 15,000-fold less affinity for

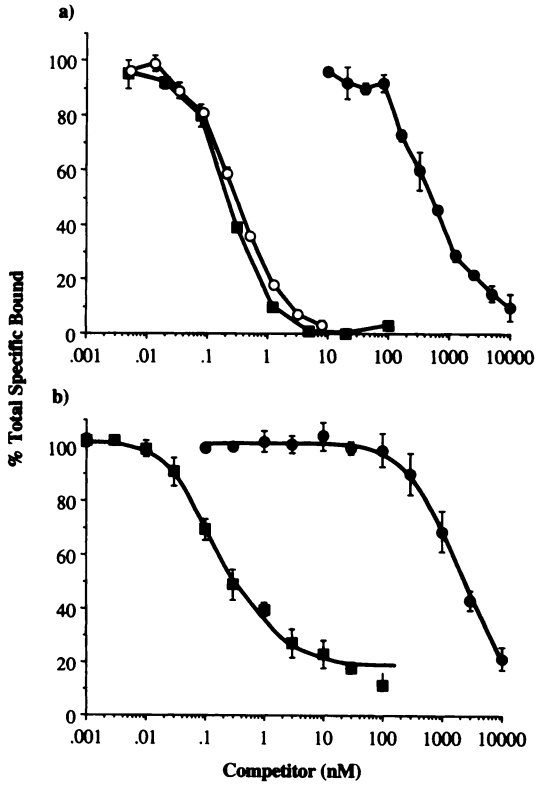

Fig. 2. Inhibition of [^{125}I]SP binding to IM-9 cells (a) and human caudate homogenate (b) by LY303870 and LY306155. Binding inhibition curves were established using various concentrations of cold SP (○, a only), LY303870 (■) and LY306155 (●). Specific bound radioligand was determined as described under "Methods." Values are the mean ± S.E.M. of triplicate samples.

human NK-1 receptors (fig. 2; table 3). LY303870 was approximately 50-fold more potent at human and guinea pig NK-1 receptors than mouse and rat receptors. LY303870 was essentially inactive in an extensive profile of 65 additional *in vitro* (receptor, ion channel reuptake) assays, including NK-2 and NK-3 receptors and T-, L- and N-type calcium channels (tables 1 and 2). In all assays, LY303870 had K_i values greater than 1 μM. Thus, LY303870 was a highly potent, stereospecific and selective NK-1 receptor ligand.

PI turnover and IL-6 secretion. UC-11 MG and U-373 MG human astrocytoma cells express a single class of high affinity NK-1 receptors linked to PI turnover and IL-6 secretion (Lee *et al.*, 1989; Johnson and Johnson, 1992; Gitter *et al.*, 1994). LY303870 inhibited SP-induced PI accumulation in UC-11 MG cells with a K_i of 1.5 nM. In comparison, LY306155 was approximately 4,000-fold less active (fig. 3). In additional studies, LY303870 blocked SP-induced IL-6 secretion from U-373 MG cells with a K_i of 5 nM (fig. 4). LY306155 was inactive in this system (fig. 4).

Smooth muscle pharmacology. LY303870 was characterized further using "monoreceptor" smooth muscle systems (Rouissi *et al.*, 1991). LY303870 blocked SP-induced rabbit vena cava smooth muscle responses (NK-1 receptor-mediated) with a pA_2 of 9.4. LY303870 was approximately 50,000-fold less effective in NK-2 (rabbit pulmonary artery) or NK-3 (rat portal vein) receptor-mediated responses (table 4).

NK-1 receptor agonist-induced pulmonary gas trapping and microvascular leakage. The dose-related effect of LY303870 on airway obstruction produced by [Sar^9,Met(O$_2$)^{11}]-

TABLE 3

Species differences in affinities of nonpeptide antagonists for NK-1 receptors

Compound	K_i[a]				
	Human (IM-9)[b]	Human caudate[c]	Guinea pig brain[c]	Rat brain[c]	Mouse brain[c]
			nM		
LY303870	0.15 ± 0.01	0.10 ± 0.03	0.31 ± 0.07	8.7 ± 0.15	7.5 ± 0.65
LY306155	420 ± 62	1,545 ± 400	763 ± 245	23,557 ± 5,630	5,735 ± 2,489
SP	0.18 ± 0.02	ND[d]	0.02 ± 0.003	0.18 ± 0.03	0.17 ± 0.03
(±)-CP-96,345	0.35 ± 0.01	ND	0.16 ± 0.08	31.5 ± 0.30	28.6 ± 3.8
(±)-RP 67580	30 ± 4.9	ND	32.3 ± 3.2	6.7 ± 2.0	8.7 ± 3.5

[a] Values represent the mean ± S.E. of 3 to 14 separate experiments.
[b] Intact cell line.
[c] Tissue homogenate.
[d] ND, not determined.

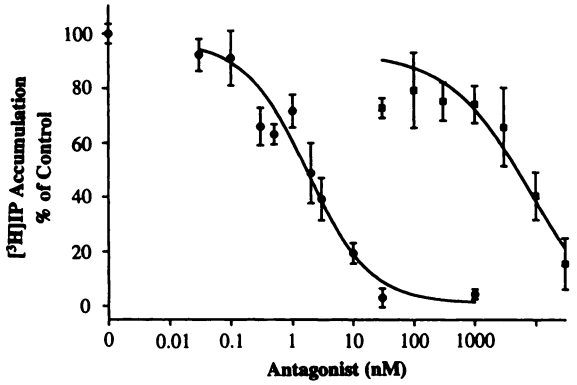

Fig. 3. Inhibition of SP-induced PI turnover by LY303870 (●) and LY306155 (■) in UC-11 MG astrocytoma cells. Total [^{3}H]inositol phosphates in SP (1 nM)-stimulated UC-11 MG cells were measured as described under "Methods." Values represent the mean ± S.E.M. of sextuplicate samples.

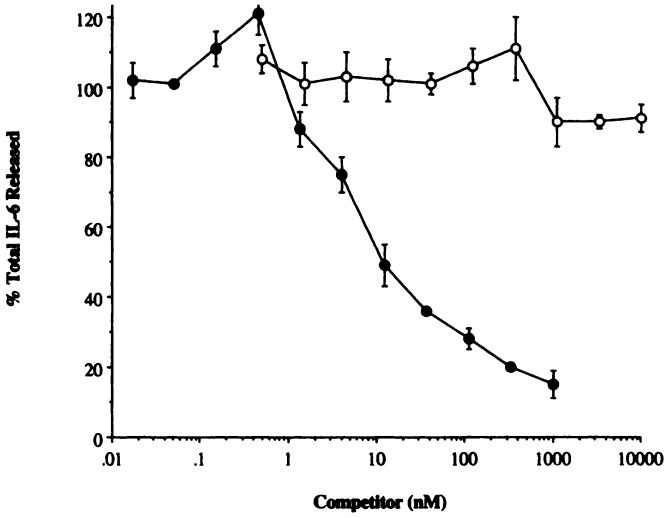

Fig. 4. Inhibition of SP-induced IL-6 secretion by LY303870 (●) and LY306155 (○). SP (20 nM)-stimulated U-373 MG astrocytoma cells were treated simultaneously with various concentrations of competitors; 18- to 24-hr conditioned media were assayed for immunoreactive IL-6 by ELISA. Values are mean ± S.E.M. of triplicate samples.

SP is shown in figure 5. ELGV values of [Sar^9,Met(O$_2$)^{11}]-SP-exposed, drug vehicle-treated guinea pigs were 6 times that of sham animals. LY303870 at 1 mg/kg produced nearly complete inhibition of [Sar^9,Met(O$_2$)^{11}]-SP-induced increases in ELGV. The ED_{50} value of 303870 needed to inhibit [Sar^9,Met(O$_2$)^{11}]-SP-induced pulmonary gas trapping was 75.0 μg/kg. LY306155

TABLE 4

Demonstration of NK-1 receptor selectivity using monoreceptor smooth muscle assays

Compound	pA_2[a]		
	Rabbit vena cava (NK-1)[b]	Rabbit pulmonary artery (NK-2)[c]	Rat portal vein (NK-3)[d]
LY303870	9.4	4.7	4.7
LY306155	5.3	4.7	6.7
(±)-CP-96,345	9.5	4.3	5.6

[a] pA_2 values were calculated by the method of Schild (1947).
[b] SP was used as NK-1 receptor agonist.
[c] NK-A was used as NK-2 receptor agonist.
[d] NK-B was used as NK-3 receptor agonist.

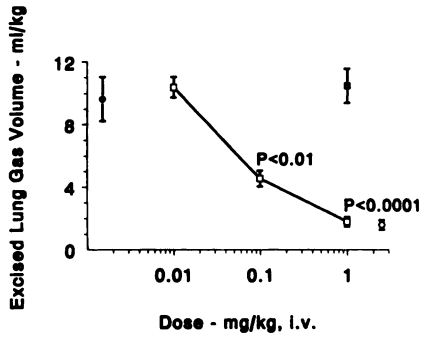

Fig. 5. Effects of LY303870 (□), LY306155 (■), drug-vehicle (●) and sham treatment (○) on [Sar^9,Met(O$_2$)^{11}]-SP-induced pulmonary gas trapping. Each point represents the mean ± S.E.M. (n = 5–7 guinea pigs per group). The P-value comparison is between drug and drug-vehicle groups.

had no effect on airway obstruction produced by [Sar^9,Met-(O$_2$)^{11}]-SP.

The dose related effect of LY303870 on [Sar^9,Met(O$_2$)^{11}]-SP-induced microvascular leakage of trachea and mainstem bronchi is shown in figure 6. [Sar^9,Met(O$_2$)^{11}]-SP markedly increased the amount of extravasated Evans blue dye in the trachea and mainstem bronchi of drug vehicle-treated guinea pigs compared to sham animals. LY303870 at 0.1 mg/kg resulted in almost complete inhibition of [Sar^9,Met(O$_2$)^{11}]-SP-induced microvascular leakage. The ED_{50} values of LY303870 for inhibition of [Sar^9,Met(O$_2$)^{11}]-SP-induced-microvascular leakage from trachea and mainstem bronchi were 18.5 and 12.8 μg/kg, respectively. LY306155 had no effect on microvascular leakage in these tissues produced by [Sar^9,Met(O$_2$)^{11}]-SP.

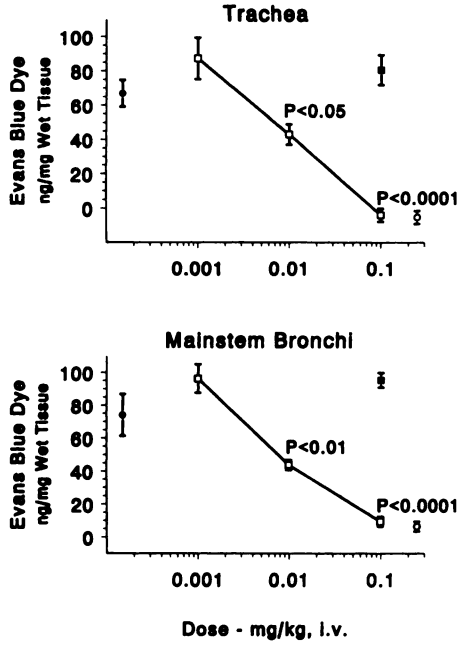

Fig. 6. Effects of LY303870 (□), LY306155 (■), drug-vehicle (●) and sham treatment (○) on [Sar^9,$Met(O_2)^{11}$]-SP-induced microvascular leakage in the bronchi and trachea. Each point represents the mean ± S.E.M. (n = 4 guinea pigs per group). The P-value comparison is between drug and drug-vehicle groups.

Discussion

Attempts to understand the role of SP and its receptor in the pathophysiology of pain and inflammation (with the potential for providing novel and improved treatment modalities) have led to the recent discovery of several potent and selective nonpeptide NK-1 receptor antagonists (Snider *et al.*, 1991; Garret *et al.*, 1991; Fujii *et al.*, 1992; Mclean *et al.*, 1993; Emonds-Alt *et al.*, 1993; Mills *et al.*, 1993; MacLeod *et al.*, 1993; Achard *et al.*, 1994; Tabart and Peyronel, 1994). Nonpeptide antagonists have advantages over peptides as potential drugs because of their increased metabolic stability and likelihood of improved p.o. bioavailability. In this report, we describe the pharmacological properties of LY303870 [(*R*)-1-[N-(2-methoxybenzyl)acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl)piperidin-1-yl)acetyl)amino]propane], a new, potent and selective nonpeptide NK-1 receptor antagonist.

LY303870 was a potent ligand for human and guinea pig NK-1 receptors with binding affinities similar to SP, (±)-CP-96,345 (table 3) and CP-99,994 (McLean *et al.*, 1993). In addition, LY303870, (±)-CP-96,345 and (±)-RP67580 showed potency differences between species as was reported previously for nonpeptide NK-1 receptor ligands (table 1) (Snider *et al.*, 1991; Gitter *et al.*, 1991; Beresford *et al.*, 1991; Watling *et al.*, 1991; Garret *et al.*, 1991; Fardin *et al.*, 1992). Recently, structural evidence was provided to explain species differences in affinities of NK-1 receptor antagonists. Of the 22 amino acid residues that differ between the sequence of human and rat NK-1 receptors, positions 116 (valine in human and leucine in rat) and 290 (isoleucine in human and serine in rat) were identified as key sites that afford high affinity binding for (±)-CP-96,345 (Fong *et al.*, 1992). It is not clear, at present, whether the affinity of LY303870 for human and rat NK-1 receptors is similarly influenced by these amino acid residues. In addition, LY303870 was a highly selective ligand for NK-1 receptors because affinities less than 1 μM were not observed at a number of other binding sites (including NK-2, NK-3 and Ca^{++} channel systems). Thus, the potency, specificity and selectivity of LY303870 for NK-1 receptors suggests the opportunity for fewer side effects. The *S*-enantiomer, LY306155 {(*S*)-1-[N-(2-methoxybenzyl)acetylamino]-3-(1H-indol-3-yl)-2-[N-(2-(4-(piperidin-1-yl)piperidin-1-yl)acetyl)amino]propane}, was 1,000- to 15,000-fold less potent in the NK-1 receptor binding assays (table 3; fig. 2). The 3- to 4-fold difference in the affinities of LY306155 at the two human (IM-9; human caudate) NK-1 receptor preparations (table 3) may suggest intraspecies subtypes; however, these K_i values were not statistically significant. Further evaluation of selected compounds (*e.g.*, LY303870 and LY306155) in NK-1 receptor-driven activities *in vitro* demonstrated enantioselective antagonist effects (figs. 3–4; table 4).

The *in vivo* NK-1 receptor antagonist effects of LY303870 were determined by the stereospecific blockade of NK-1 receptor agonist-driven bronchoconstriction (measured by ELGV) and plasma extravasation (figs. 5 and 6). The pulmonary gas trapping measurement used in this study is a sensitive indicator of *in vivo* airway obstruction in the guinea pig (Stengel and Silbaugh, 1989; Watson *et al.*, 1990; Frazer *et al.*, 1993). Use of the ELGV measurement in this investigation allowed us to minimize anesthesia and surgical interventions. The elevated ELGV values after [Sar^9,$Met(O_2)^{11}$]-SP most likely produced airway narrowing by increasing bronchomotor tone (Orehek, 1980). We have found postmortem pulmonary gas trapping to correlate strongly with bronchoconstrictor-related decreases in dynamic compliance and increases in total pulmonary resistance measured immediately before death in the guinea pig (Stengel *et al.*, 1987; Stengel and Silbaugh, 1989). However, airway hypersecretions and/or interstitial edema also may have contributed to the airway obstruction. Because the trachea and mainstem bronchi were visually open at the time measurements were made, the location of airway obstruction must have been distal to these airways. Our data are consistent with previous studies demonstrating the effects of NK-1 receptor antagonists in NK-1 receptor agonist-driven guinea pig airway functions (Murai *et al.*, 1993; Solway *et al.*, 1993).

Because NK-1 receptor-mediated activities may contribute to the pathophysiology of chronic airway diseases, such as asthma (Solway and Leff, 1991), nonpeptide NK-1 receptor antagonists, such as LY303870, may provide a novel therapeutic approach for the treatment of this disease and others in which neurogenic inflammation plays an important role.

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