LY303870, a Centrally Active Neurokinin-1 Antagonist with a Long Duration of Action


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ABSTRACT

The selective neurokinin (NK)-1 antagonist LY303870 has high affinity and specificity for human and guinea pig brain NK-1 receptors labeled with $^{[125]i}$-substrate P. It has approximately 15- to 30-fold lower affinity for rat and mouse brain NK-1 receptors, consistent with previously reported species differences in the affinities of nonpeptide antagonists for NK-1 receptors. In vivo, LY303870 blocked the characteristic, caudally directed, biting and scratching response elicited by intrathecal administration of the selective NK-1 agonist Ac-[Arg$_6$Sar$_9$Met(O$_2$)$_{11}$]substance P$_{6-11}$ in conscious mice. The administration of the selective NK-1 agonist Ac-[Arg$_6$Sar$_9$Met(O$_2$)$_{11}$]substance P$_{6-11}$ in conscious mice. The potentiation of the tail-flick response elicited by intrathecal administration of the NK-1 agonist [Sar$_9$Met(O$_2$)$_{11}$]substance P$_{6-11}$ in rats was also selectively blocked by LY303870. When tested in a model of persistent nociceptive activation induced by tissue injury (the formalin test), LY303870 blocked licking behavior in the late phase of the formalin test, in a dose-dependent manner. After oral administration of 10 mg/kg, the blockade of the late-phase licking behavior was evident for at least 24 hr. Ex vivo binding studies in guinea pigs showed that orally administered LY303870 potently inhibited binding to central and peripheral NK-1 receptors labeled with $^{[125]i}$-substrate P. This inhibition was long-lasting, consistent with other in vivo activities. LY306155, the opposite enantiomer of LY303870, was less active in all of the functional assays. In rodents, LY303870 did not exhibit any neurological, motor, cardiovascular, gastrointestinal or autonomic side effects at doses of $\leq$50 mg/kg p.o. Thus, LY303870 is a potent, centrally active, NK-1 antagonist in vivo, with long-lasting oral activity.

SP is a member of the tachykinin family of neuropeptides, which includes NKA and NKB. These tachykinin peptides act via three major classes of G protein-coupled receptors, NK-1, NK-2 and NK-3 (Henry, 1987; Guard and Watson, 1991; Regoli et al., 1994). All of these have been cloned (Hershey and Krause, 1990; Nakanishi, 1991; Maggi et al., 1993; Otsuka and Yoshioka, 1993). SP, acting via NK-1 receptors, has been implicated in a variety of physiological functions, including inflammation and the transmission of nociceptive information from the periphery to the central nervous system via primary afferent neurons (Maggi et al., 1993; Otsuka and Yanagisawa, 1990).

In the central nervous system, SP is synthesized in dorsal root ganglion neurons, in interneurons and projection neurons within the spinal cord and in supraspinal sites that may mediate pain transmission. SP/NK-1 receptors are also widely distributed at these spinal and supraspinal sites and have been found in a variety of brain regions, including amygdala, bed nucleus of the stria terminalis, caudate putamen and, to a lesser extent, cortex and limbic regions (Shults, et al., 1984; Dam and Quirion, 1986; Danks et al., 1986; Saffroy et al., 1988; Gehlert et al., 1996). This unique distribution, coupled with other functional studies, further supports the role of SP, acting via NK-1 receptors, as a key mediator of the processing of nociceptive information in the central nervous system. NK-1 receptors are located in the superficial laminae of the spinal cord dorsal horn (Yashpal et al., 1990; Moussaoui et al., 1992; Mantyh et al., 1995), the site of termination of SP-containing afferent neurons and the location of intrinsic spinal neurons (Hökfelt et al., 1975; Barber et al., 1979; Jessell et al., 1979). SP-containing terminals appose nociception-specific dorsal horn neurons, and SP selectively activates dorsal horn cells that normally receive nociceptive inputs (Henry, 1976), causing a powerful sustained excitation of second-order sensory neurons in the spinal cord. SP also facilitates the activation of these neurons by noxious peripheral stimuli (Henry, 1976; Randic and Milentie, 1977; Radhakrishnan and Henry, 1991, 1995; Dougherty et al., 1994). SP administered i.t. induces licking behaviors (Hylden and Wilcox, 1981; Piercey et al., 1981; Matsumura et al., 1985), and noxious peripheral stimuli evoke the release of SP (Kuraishi et al., 1983, 1989; Duggan and Hendry, 1986;
Go and Yaksh, 1987; McCarson and Goldstein, 1991). Thus, SP and NK-1 receptors appear to be physiologically involved in noxious stimulation and the manifestation of persistent pain. An efficacious and selective NK-1 antagonist that crosses the blood-brain barrier would allow further characterization of the involvement of central NK-1 receptors in pain and could thus be of therapeutic relevance.

Toward this end, the discovery of LY303870, \( \text{N}-(2-	ext{methoxybenzyl})\text{acetylaminol}-3-(1H-\text{indol-3-yl})-2-[N-(2-(4-(\text{piperidin-1-yl})\text{piperidin-1-yl})\text{acetylaminol})\text{propane} \) (fig. 1), has recently been reported (Gitter et al., 1995; Hipkinskld et al., 1996). This drug is highly selective for NK-1 receptors and binds with very high affinity to human NK-1 sites labeled by \( ^{125}\text{I}-\text{SP}, \) both in the periphery (IM9 cells, \( K_{i} = 0.15 \text{nM} \)) and in brain (cortex, \( K_{i} = 0.10 \text{nM} \)). It binds with similar potency to guinea pig brain membranes (\( K_{i} = 0.31 \text{nM} \)) but with lower potency to rat and mouse brain membranes (\( K_{i} = 8.7 \text{nM} \) and 7.5 nM, respectively). This difference in affinities for NK-1 receptors across species is consistent with previously identified species differences in binding of nonpeptide antagonists to human/guinea pig receptors vs. rodent receptors (Gitter et al., 1991; Snider et al., 1991; Beresford et al., 1991; Watling et al., 1991; Garret et al., 1991; Fardin et al., 1992). The opposite (−)-enantiomer, LY306155, \( (S)-[N-(2-\text{methoxybenzyl})\text{acetylaminol}-3-(1H-\text{indol-3-yl})-2-[N-(2-(4-(\text{piperidin-1-yl})\text{piperidin-1-yl})\text{acetylaminol})\text{propane} \) was 1,000 to 15,000 less potent in all species examined (human IM9 cells, \( K_{i} = 0.42 \mu\text{M} \); human cortex, \( K_{i} = 1.5 \mu\text{M} \); guinea pig cortex, \( K_{i} = 7.6 \mu\text{M} \); rat brain, \( K_{i} = 23.5 \mu\text{M} \); mouse brain, \( K_{i} = 57 \mu\text{M} \)). That LY303870 was a potent functional NK-1 antagonist in vivo was demonstrated by the ability of the drug to block SP-induced interleukin-6 secretion from UC-11 MG human astrocytoma cells (\( K_{i} = 5.5 \text{nM} \)), SP-induced phosphoinositide turnover (\( K_{i} = 1.2 \text{nM} \)) and SP-induced rabbit vena cava contractions (\( p_{\text{A}_{2}} = 9.4 \)) (Gitter et al., 1995). In vivo, LY303870 inhibited [\( \text{Sar}^{\text{a}},\text{Met(O}_{2})^{\text{b}} \text{SP}] \) SP-induced guinea pig bronchoconstriction (ED_{50} = 75 \mu\text{g/kg i.v.}) and pulmonary microvascular leakage in the bronchi (ED_{50} = 12.8 \mu\text{g/kg i.v.}) and trachea (ED_{50} = 18.5 \mu\text{g/kg i.v.}), suggesting systemic availability as well as potent activity at NK-1 receptors (Gitter et al., 1995). Additionally, SP-induced salivation (IC_{50} = 126 \mu\text{g/kg i.v.}) and hypotension (IC_{50} = 336 \mu\text{g/kg i.v.}) in urethane-anesthetized rats (Cellier et al., 1996) was blocked by LY303870. Localization of [\( ^{3}H \)]LY303870 to NK-1 binding sites in brain and spinal cord was found to be similar to that of [\( ^{125}\text{I} \)]SP (Gehlert et al., 1996).

In the following series of studies, functional central activity of LY303870 was further evaluated after parenteral and oral administration. The ability of LY303870 to block NK-1 agonist-mediated spinal nociceptive reflexes was measured in both rats and mice. Analgesic activity of LY303870 was tested in the formalin test in rats, as a model of persistent nociceptive activation induced by tissue injury. In addition, binding of LY303870 to central and peripheral NK-1 receptors was studied by ex vivo binding after oral administration in guinea pigs. The potential secondary pharmacological activity was profiled using behavioral, cardiovascular and gastrointestinal assays.

**Materials and Methods**

**Animals**

**Functional activity tests.** Male Swiss Webster mice (18–21 g; Harlan Labs, Indianapolis, IN) were used in the NK-1 agonist-driven nociceptive behavioral response studies. Male Sprague-Dawley rats (200–250 g; Charles River, Portage, MI) were used in the formalin test and the NK-1 agonist-driven potentiation of the tail-flick test. Male guinea pigs (175–225 g; Charles River) were used for ex vivo radioligand binding studies.

**Secondary pharmacological evaluation.** Adult male CD-1 mice (Crl:CD-1(ICR), 22–25 g; Charles River) and male Sprague-Dawley rats (300–400 g; Hilltop Laboratories, Scottdale, PA) were used in the formalin test and the NK-1 agonist-driven potentiation of the tail-flick test. Male guinea pigs (175–225 g; Charles River) were used for ex vivo radioligand binding studies.

**Drugs and Injections**

LY303870 and LY306155 were synthesized at Lilly. Antagonists were dissolved in distilled water, and the pH was adjusted to neutrality with 1 N NaOH. SP, Ac-[\( \text{Arg}^{\text{a}},\text{Sar}^{\text{b}},\text{Met(O}_{2})^{\text{c}},\text{SP}_{\text{a},-11} \) , and [\( \text{Sar}^{\text{a}},\text{Met(O}_{2})^{\text{b}} \text{SP}_{\text{a},-11} \) SP were purchased from Peninsula Laboratories (Belmont, CA). All peptides were dissolved in 0.9% saline or in artificial cerebrospinal fluid (128.6 mM NaCl, 2.6 mM KCl, 2.0 mM MgCl\(_2\), 1.4 mM CaCl\(_2\), PH 7.2) for i.t. application. Drugs or vehicles were administered i.t. (as described below), i.p. or p.o. by gavage.

**NK-1 Agonist-mediated Potentiation of the Nociceptive Behavioral Response (Mice)**

Exogenous SP or NK-1 agonists, when injected into the spinal cord of conscious mice, elicit a characteristic, caudally directed, nociceptive behavioral response consisting of intense biting and scratching episodes (Hylden and Wilcox, 1981). This behavioral test was adapted to evaluate the central activity of LY303870 and several other compounds from this series, in vivo. In this test, a selective NK-1 agonist, Ac-[\( \text{Arg}^{\text{a}},\text{Sar}^{\text{b}},\text{Met(O}_{2})^{\text{c}},\text{SP}_{\text{a},-11} \) , was injected i.t. (into the spinal cord) in the L5/L6 intravertebral space in conscious mice (male, 18–21 g; Harlan), according to previously published procedures (Hylden and Wilcox, 1981). Briefly, a volume of 5 µl of a given concentration of the agonist was injected i.t. with a 30-gauge needle connected to a 50-µl Hamilton microsyringe, while the animal was lightly restrained to maintain the position of the needle. Puncture of the dura was indicated by the flick of the tail, which was evident throughout the duration of the infusion of the agonist. Only animals that showed this response were included in the study (the success rate was approximately 95%). The number of caudally directed scratching and biting events induced by the NK-1 agonist were...
scored for 5 min after the i.t. injections. LY303870 or LY306155 was either coinjected i.t. or administered i.p. 15 min before agonist challenge.

**NK-1 Agonist-mediated Potentiation of the Tail-flick Response (Rats)**

The tail-flick latency was measured in conscious rats (male, Sprague-Dawley, 200–250 g, placed in a plastic restraining holder) and taken as the reaction time required to remove the tail from a source of radiant heat. The intensity of the heat stimulus was set to elicit a tail-flick response with a latency of 10 to 15 sec. This was the base-line reaction time. Each testing trial consisted of several measurements of reaction time, spaced at 5-min intervals. The initial three measurements were used to determine base-line reaction time. One minute before the fourth reading, 20 µl of artificial cerebrospinal fluid was given i.t. via an indwelling catheter (PE10) that had been implanted 1 week before, at the vertebral midlumbar level (Picard et al., 1993). Three subsequent readings were taken to determine the effect of vehicle (artificial cerebrospinal fluid) on the base-line reaction time. After this, 6.5 nmol of the NK-1 agonist [Sar9,Met(O2)11]SP was injected i.t. At 1 min after injection, the NK-1 agonist decreased the reaction time to the noxious heat stimulus, thus potentiating the tail-flick response (in figure 4A, this potentiated response is calculated as 100% and the base-line reaction time is shown as 0%). LY303870 and LY306155 were tested for their ability to block this potentiated response when administered either i.v. 5 min before the agonist or p.o. 60 min before the agonist.

**Formalin Test (Rats)**

The formalin test was performed in custom-made Plexiglas boxes (25 × 25 × 20 cm). A mirror placed at the back of the cage allowed unhindered observation of the formalin-injected paw. Rats were individually placed in the cubicles at least 1 hr before the experiment. All testing was conducted between 8:00 A.M. and 2:00 P.M., and the animals were dosed either 15 or 30 min before the formalin injection. Formalin (50 µl of a 5% solution in saline) was injected s.c. into the dorsal lateral surface of the rat’s right hind paw with a 27-gauge needle. Observation started immediately after the formalin injection. Formalin-induced pain was quantified by recording the number of formalin-injected paw-licking events and the number of seconds each licking event lasted, in 5-min intervals. The pain scoring was measured for 50 min after the formalin injection. Two phases of pain-related behavior were observed, as previously described (Shibata et al., 1989; Wheeler-Aceto et al., 1990; Codere et al., 1993a; Abbott et al., 1995); the first or early phase started immediately after the formalin injection and lasted approximately 5 min, followed by the second or late phase, which started between 10 and 15 min, with a maximum response typically being observed approximately 25 to 35 min after the formalin injection. After the 50-min observation period, animals were sacrificed with an overdose of barbiturate mixture (Euthanasia-5; Henry Schein, Inc.)

**Data Collection**

After a careful review of the current literature, time spent licking the injected paw was chosen as the pain behavior parameter most relevant to evaluating NK-1 receptor function. The collected data for the first phase were obtained by adding the total number of seconds spent licking the injected paw for the 0–5-min interval, and data for the second phase were obtained by adding the total number of seconds spent licking the injected paw from min 15 to min 40 of the observation period. This area under the curve (total time spent licking the injected paw) was taken as a data point. Data are presented as means ± S.E.M. from several sets of experiments.

**Secondary Pharmacological Evaluation (Rats and Mice)**

The various tests used, routes, species and relevant methods are summarized in table 2. A detailed description of all behavioral, cardiovascular and gastrointestinal tests was previously published (Helton et al., 1996a,b). Briefly, clinical observations were based on the method of Irwin (1968), as described by Helton et al. (1996a). Animals were observed for changes in autonomic function (respiration rate, lacrimation, salivation, piloerection and urination/diarrhea), awareness (hyperreactivity, hyporeactivity, aggressiveness and vocalization), motor activity (atalepsy, hypoactivity, hyperreactivity and stereotypy) and motor coordination/tone (hypotonic gait, ataxic gait and tremor). Spontaneous activity of individual mice was recorded using MultiVarimax activity monitors (Columbus Instruments, Columbus, OH), and sensorimotor activity (auditory startle) was evaluated using a SDI startle system (San Diego Instruments, San Diego, CA). Blood pressure and heart rate were recorded on a Beckman recorder and by the Masscomp computer. Gastrointestinal motility, convulsive models, central nervous system depression/hexobarbital-induced sleep times, body temperature, grip strength, Rotarod performance and sensorimotor activity/auditory startle were also tested based on the methods of Helton et al. (1996a).

**Ex Vivo Binding of LY303870 to NK-1 Receptors (Guinea Pigs)**

Male guinea pigs (175–225 g) were administered an oral bolus dose of LY303870. Animals were sacrificed at various time points. Lungs and striatum were dissected out. The striatum was homogenized in 6 ml and the lung in 45 ml of 50 mM Tris buffer (pH 7.5); the tissues were incubated at 37°C for 15 min to remove endogenous SP. Protein concentration was estimated using the Bradford reagent (Bradford, 1976). [125I]-SP (2200 Ci/mmol; DuPont-NEN, Boston, MA) binding was conducted in a 200-µl volume containing 50 mM Tris buffer (pH 7.5), 3 mM MnCl2, 0.02% bovine serum albumin, 40 μg/ml bacitracin, 2 μg/ml chymostatin, 2.5 μg/ml thiorphan and 4 μg/ml leupeptin. Nonspecific binding was assessed with 1 μM SP. After a 1-hr incubation at room temperature, the binding reaction was terminated by filtration through glass microfiber filters using a Tomtec 96-well filtration apparatus (Tomtec Inc., Orange, CT). Filters were pretreated for 2 hr with 0.3% polyethyleneimine (Sigma Chemical Co., St. Louis, MO). The filters were washed with 5 ml of cold 50 mM Tris buffer (pH 7.4). After drying, the radioactivity retained on the filters was quantitated by scintillation counting (Wallac, Orange, CT). Specific binding was defined as the difference between total and nonspecific binding and is expressed as dpm per milligram of protein.

**Data Analysis**

For all data, means and S.E.M. were calculated using Microsoft Excel. For all tests except behavioral pharmacological, cardiovascular and gastrointestinal tests, data were evaluated by one-way analysis of variance and post hoc comparisons were analyzed by Dunnett’s test (Dunnett, 1964) for two-sided comparisons. Statistical analyses were performed using JMP for MacIntosh. For behavioral pharmacological, cardiovascular and gastrointestinal tests, data were analyzed by analysis of variance, followed by a Tukey’s range test for post hoc comparison of group means. Statistical analysis was performed using statistical analysis systems. For all tests, effects with a probability of P < .05 were considered to be significant.

**Results**

**Antagonism by LY303870 of NK-1 agonist-mediated causally directed scratching and biting behavior in mice.** Intrathecally administered Ac-[Arg5,Sar9,Met(O2)11]SP was tested in a dose-dependent increase in causally directed biting and scratching behavior (fig. 2), with a maximal effect occurring at 0.5 pmol. Selective NK-2 and NK-3 agonists did not elicit this response (data not shown), suggesting that this behavioral response was selectively mediated via NK-1 receptors. The effect of LY303870 on this NK-1 agonist-induced, causally directed, biting and scratching response was
tested in subsequent experiments after direct i.t. or systemic (i.p.) administration. For these experiments, caudally directed biting and scratching behavior was elicited by a 0.5-pmol dose of the NK-1 agonist.

Intrathecal NK-1 agonist-induced, caudally directed, biting and scratching behavior was found to be potently and efficaciously antagonized by LY303870, in a dose-dependent manner, after both i.t. and i.p. administration (fig. 3). The opposite stereoisomer, LY306155, was markedly less active. These data indicated that LY303870 blocked the activation of central NK-1 receptor-mediated biting and scratching behavior. Data further indicated that LY303870 crossed the blood-brain barrier in mice after systemic administration.

Antagonism of NK-1 agonist-mediated potentiation of tail-flick latency in rats. The tail-flick latency was measured in conscious rats and taken as the reaction time required to remove the tail from a source of radiant heat. The NK-1 agonist [Sar⁹,Met(O₂)¹¹]SP, administered i.t., reduced this reaction time at 1 min after injection, thereby potentiating the tail-flick response (fig. 4). This NK-1-potentiated nociceptive response was blocked by LY303870 in a dose-dependent manner, after i.t. (ED₅₀ = 21 nmol, 3-min pretreatment; data not shown), i.v. and p.o. administration (fig. 5), without altering the base-line tail-flick response latency. (There was no statistical difference in base lines in the presence of LY303870 at any of the doses tested by all three routes; figure 4B shows this lack of effect with LY303870, 30 mg/kg p.o., and data with other doses/routes are similar and not shown.) In comparison with LY303870, its opposite stereoisomer, LY306155, was less active by both routes of administration (fig. 5). These data further indicated that LY303870 blocked NK-1 receptor-mediated spinal responses in rats after systemic administration, again suggesting central nervous system penetration of the drug.

Antagonism of late-phase licking behavior after formalin challenge in rats. Formalin administered s.c. produced a distinct biphasic behavioral response involving licking of the injured paw (fig. 6A). This response showed an early phase of 5 min, followed by a more exaggerated late phase, suggesting persistent activation of nociceptive neurons. The ability of LY303870 to block this licking behavior in rats was tested by two different routes of administration. Paw-licking behavior in the late phase (second phase) was blocked in a dose-dependent manner by LY303870, after both i.p. (fig. 6B) and p.o. administration (fig. 7A). The opposite stereoisomer, LY306155, was less active than LY303870 at the doses tested (fig. 7B). After p.o. administration of 10 mg/kg, the blockade of the licking behavior was evident for 24 hr, with the base-line returning to vehicle levels by 48 hr (fig. 8). Early-phase licking behavior showed a tendency toward attenuation. However, this was not dose-dependent and was significantly different from vehicle only at the highest dose tested with both routes (table 1). The variability was higher. The magnitude of blockade at the highest dose in the first phase was much less than in the corresponding second phase as well. Early-phase licking behavior was not blocked significantly over a 48-hr period after p.o. administration of 10 mg/kg LY303870 (table 1). There was no blockade of the early-phase response after LY306155 administered p.o. at the doses tested.

Lack of general behavioral effects of LY303870 in rodents. The activity of LY303870 in several acute behavioral tests for motor dysfunction or behavioral depression in rats and mice was tested (table 2). Animals dosed with LY303870 did not exhibit any neurological, muscular or autonomic changes or behavioral depression after i.p. (≥30 mg/kg) or p.o. (≤50 mg/kg) administration. The only effect ob-
served was increased awareness or responsiveness in the auditory startle test after p.o. administration, which was not dose-dependent. The drug also did not show changes in gastrointestinal function after oral administration at doses up to 50 mg/kg. There was a slight but significant increase in heart rate only at the 50 mg/kg oral dose group. However, this increase was not considered to be biologically relevant.

Antagonism by orally administered LY303870, ex vivo, of NK-1 receptor binding in guinea pigs. The ability of LY303870 to inhibit 125I-SP binding to peripheral and central NK-1 receptors after oral administration in guinea pigs was characterized by ex vivo binding studies. The binding of 125I-SP to both lung and striatum was significantly inhibited at very low doses (fig. 9). The time course of this effect was further studied at a fixed dose (10 μg/kg p.o.), and the inhibition of binding was assessed at various times up to 48 hr. A maximal reduction in binding was observed at 2 hr and persisted for up to 24 hr. The levels of binding returned to near control levels 48 hr after administration (fig. 10). Thus, LY303870 produced long-lasting inhibition of NK-1 binding to the striatum after oral administration, indicating that LY303870 appeared to readily cross the blood-brain barrier to interact with central NK-1 receptors.

Discussion

The present studies suggest that LY303870 blocked NK-1 receptors in vivo, in rats, mice and guinea pigs, after systemic administration (parenteral and/or oral routes). In these studies, LY303870 blocked central NK-1 receptor-mediated nociceptive behavioral responses in both rats and mice and exhibited long-lasting analgesic activity in the formalin test at doses that were consistent with its affinity for rodent NK-1 receptors. Moreover, the drug did not produce motor dysfunction, behavioral depression or autonomic deficits. Consistent with its high affinity for human/guinea pig receptors, very low oral doses of LY303870 also exerted potent and long-lasting inhibition of 125I-SP binding to guinea pig striatal and lung membranes ex vivo.

The functional activity of LY303870 was evaluated in various rodent nociceptive models because of the widely implicated role of SP as a mediator of pain transmission (for reviews, see Otsuka and Yanagisawa, 1990; Maggi et al., 1993). The assays then chosen were measures of NK-1 receptor activation in central nociceptive pathways. One such assay, the caudally directed biting and scratching behavioral response in mice, was based on the observation that SP and its analogs, when injected i.t. into mice, caused a characteristic nociceptive behavior (Hylden and Wilcox, 1981; Takahashi et al., 1987), consisting of intense, caudally directed, scratching, biting and licking episodes. Based on the rank order of potency of the endogenous ligands SP, NKA and NKB, Sakurada et al. (1991) suggested that the receptor mediating this nociceptive behavioral response may be the NK-1 receptor; they further supported this by demonstrating blockade of the SP-induced behavior by a selective peptide NK-1 antagonist, d-Trp7-sendide (Sakurada, et al., 1994). However, peptide antagonists in some cases have caused motor dysfunction after i.t. administration (Couture et al., 1987; Vaught, 1988), precluding unequivocal demonstration of the receptor selectivity of this nociceptive behavioral response. The NK-1 selectivity of this response in mice was confirmed in the present experiments with the use of the selective NK-1 agonist Ac- [Arg6,Sar9,Met(O2)11]SP6–11 to elicit the biting and scratching response and by the stereoselective and dose-dependent blockade of this response by i.t. administration of LY303870. Similar blockade was also achieved after i.p. administration of LY303870, suggesting that this antagonist was penetrating the blood-brain barrier and acting at central NK-1 receptors.

Nociceptive behavioral responses, similar to those de-
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LY303870 or LY306155 injected i.v. 5 min before i.t. administration of 6.5 nmol of \([\text{Sar}^9,\text{Met(O2)}^{11}]\text{SP}\) on reaction time was elicited with LY303870. Base-line reaction time was not affected by LY303870, further supporting the notion that NK-1 receptors selectively modulate ongoing spinal nociceptive reflexes. The data also demonstrate that LY303870 crosses the blood-brain barrier after i.v. and p.o. administration, to block NK-1 receptors in the spinal cord, and that this occurs at doses that do not cause motor dysfunction or neurological deficits.

The selective blockade of NK-1 agonist-potentiated noxious stimulation of spinal neurons also supports the hypothesis that LY303870 can interfere with NK-1 mechanisms related to pain sensitivity. In recent years, NK-1 receptors have been linked to mechanisms of sensitization (Coderre et al., 1993b; Urban et al., 1994; Rang and Urban, 1995). SP appears to produce noiceptive effects with prolonged noxious thermal, mechanical and chemical stimuli (Henry, 1993). SP, acting via NK-1 receptors, may specifically mediate long-term noiception. In the normal physiological state, the activity of SP neuronal systems during prolonged activation may be important for the continued presentation of physiologically relevant information to higher brain centers. However, in pathological states, continuous activation of the nociceptive input to the central nervous system could potentially lead to persistent pain. Although the pathophysiological processes that contribute to persistent pain are not completely understood, recent studies suggest that both peripheral and central mechanisms are involved (Wooll, 1983; Dubner, 1991; Dubner and Ruda, 1992; Coderre et al., 1993b). Thus, peripheral mechanisms such as inflammation or injury lead to abnormal excitation of peripheral nociceptive afferent fibers, whereas central mechanisms such as sensitization result in facilitated transmission (wind-up) in the dorsal horn as well as second-order sensory neurons, as a direct consequence of increased C-fiber input. SP, acting via NK-1 receptors, is implicated in both processes. Central sensitization and hyperexcitation appear to be involved in humans as well (Gracey et al., 1992) and have been shown to involve SP-containing primary afferents (LaMotte et al., 1992; Torebjork et al., 1992). Several studies have shown that noxious stimulation or injury affects the physiological activity of SP. Resulting changes include increased SP synthesis and release (Yaksh, 1988; Schaible et al., 1990; Sluka and Westlund, 1993) and up-regulation of tachykinin mRNA expression (Noguchi et al., 1988; Noguchi and Ruda, 1992; McCarson and Krause, 1994). Concomitant changes in NK-1 receptor function have been reported, such as increased immunoreactivity (Mantyh et al., 1995) and up-regulation of NK-1 receptor mRNA (McCarson and Krause, 1994) and binding sites (Yashpal et al., 1991; Aanonsen et al., 1992). Up-regulation of early activation gene products such as c-fos occurs simultaneously (Noguchi and Ruda, 1992), suggesting involvement of NK-1 receptors in a cascade of central events. Recent evidence suggests that SP affects spinal hyperexcitability via NK-1 receptors (Radhakrishnan and Henry, 1991, 1995; Toda and Hayashi, 1993), either directly or indirectly, by modulating glutamate release in the spinal cord (Heapy et al., 1987; Hylden et al., 1989; Dubner, 1991; Wilcox, 1991; Henry, 1993). This hyperexcitability could be dynamically maintained by ongoing stimulation from the peripheral nociceptors that are sensitized by inflammation or injury, in which SP has also been shown to play a

Fig. 5. A, LY303870 or LY306155 (its opposite enantiomer) injected i.v. 5 min before i.t. administration of 6.5 nmol of \([\text{Sar}^9,\text{Met(O2)}^{11}]\text{SP}\). B, LY303870 or LY306155 injected p.o. 1 hr before i.t. administration of \([\text{Sar}^9,\text{Met(O2)}^{11}]\text{SP}\). LY303870 blocked NK-1-induced lowering of reaction time after both i.v. and p.o. administration, in a dose-dependent and stereoselective manner. In this figure, the potentiated latency is shown as 100% and the base-line latency is 0%. *P < .05, compared with its own vehicle control.
role (Schaible et al., 1990). Thus, NK-1 receptors may be unique in that they can modulate the key pain processes implicated in the transmission of long-lasting pain, probably via sensitization of nociceptors in the periphery, sensitization and hyperexcitation of spinal neurons and mechanisms that maintain the sensitized state. Based on the activity of LY303870 on NK-1 agonist-mediated nociceptive responses, this antagonist was thought to be a useful tool to further delineate the functional role of NK-1 receptors in chronic pain and may be of therapeutic relevance to the treatment of persistent pain.

To further investigate this potential, LY303870 was evaluated in an animal model of pain involving aspects of ongoing activation and sensitization that may be related to persistent pain. The injection of formalin, with the resulting activation of nociceptive pathways, is widely used as a model of chemogenic nociception (Dubuisson and Dennis, 1977; Tjølsen et al., 1992). The formalin test for nociception involves moderate continuous pain generated by the injured tissue. It thus differs from most traditional tests of nociception that rely on brief stimuli of threshold intensity. Subcutaneous injection of formalin into the hind-paw produces a persistent activation of low-intensity activity in primary C-afferent pain fibers. This stimulation is known to release SP and increase excit-
ability at the spinal cord level (Dickensen and Sullivan, 1987; McCarson and Goldstein, 1990, 1991). NK-1 receptor immunoactivity (Mantyh et al., 1995), as well as mRNA expression (Schäfer et al., 1993) and mRNA levels (McCarson and Krause, 1994, 1995), are increased in the dorsal horn after formalin- or adjuvant-induced nociception, providing evidence that NK receptor expression and turnover may be acutely up-regulated as a consequence of nociceptive activation of the hind paw. The formalin test involves an early first phase and a late second phase, in which the behavioral response (paw-licking) is exaggerated. Previous studies have suggested that the early phase seems to be caused predominantly by C-fiber activation due to the peripheral stimulus, whereas the late phase appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord. These functional changes seem to be initiated by the C-fiber discharge during the early phase (Tjølsen et al., 1992). The pain rating scale has been previously simplified (Shibata et al., 1989) for this test, suggesting that licking behavior with the formalin-injured paw is a reliable indicator of pain response and appears to be a good behavioral measure of sensitization due to persistent activation of the nociceptive fi-

### TABLE 1

**Effects of LY303870 on formalin-induced early-phase licking behavior**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Early-phase Licking (sec)</th>
<th>Pretreatment Time</th>
<th>Early-phase Licking (10 mg/kg p.o.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.p.</td>
<td>p.o.</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>23.0 ± 4.2</td>
<td>33.0 ± 7.5</td>
<td>Vehicle</td>
</tr>
<tr>
<td>1.0</td>
<td>33.5 ± 5.2</td>
<td>37.4 ± 10.6</td>
<td>30 min</td>
</tr>
<tr>
<td>3.0</td>
<td>11.2 ± 2.6</td>
<td>17.0 ± 6.3</td>
<td>60 min</td>
</tr>
<tr>
<td>10.3</td>
<td>12.2 ± 4.0</td>
<td>32.0 ± 6.7</td>
<td>120 min</td>
</tr>
<tr>
<td>30.0</td>
<td>4.7 ± 4.7*</td>
<td>21.5 ± 2.3*</td>
<td>24 hr</td>
</tr>
</tbody>
</table>

* P < .05, compared with control.

**Fig. 8.** Time course of the effect of LY303870 on total time spent licking in the late phase. The effect of LY303870 on formalin-induced licking behavior was long-lasting. At 10 mg/kg p.o., this effect was evident up to 24 hr after administration; values returned to baseline by 48 hr. *P < .05, compared with its vehicle control. Mean control values were 240 ± 24 sec total licking time in the late phase.

**Fig. 9.** Inhibition by LY303870, administered orally to guinea pigs (n = 4–7), of 125I-SP binding to both the lung (A) and the striatum (B), which appeared to be maximal after 1 μg/kg. No further reduction in binding was seen at doses up to 1000 μg/kg. *P < .05, compared with vehicle control.
long-acting and stereoselective, because LY306155 was less active. These data support the contention that NK-1 receptors mediate persistent activation of spinal neurons in this test, as has been previously suggested by early studies using less selective NK-1 antagonists (Yamamoto and Yaksh, 1991; Garret et al., 1991; Coderre and Melzack, 1992; Xu et al., 1992; Sakurada et al., 1995; Seguin et al., 1995). That this activity occurred at the NK-1 receptor was additionally supported by the similar dose ranges required to block the formalin response and the NK-1 agonist-mediated potentiation of the tail-flick response in rats. Moreover, the duration of action in this assay was similar to that observed for inhibiting NK-1 receptor binding after oral administration in the guinea pig striatum, further strengthening the proposal that the observed analgesic activity was being mediated via NK-1 receptor blockade. The effect of LY303870 on early-phase licking behavior was not as clear as that on the late phase. Only the highest doses of LY303870 (i.p. and p.o.) significantly blocked licking behavior. The magnitude of blockade was somewhat less than at the corresponding late-phase behavior as well. There was greater variation in response during this time period. To examine the possibility that the lower efficacy was due to pharmacokinetic mechanisms, the effect of LY303870 was also evaluated over a 48-hr period after a dose of 10 mg/kg p.o. Early-phase licking behavior was not blocked significantly at any time tested. These results suggest that NK-1 receptors may play a less important role in the early-phase response, consistent with previous observations by Yamamoto and Yaksh (1991).

Overall, the functional activity of LY303870 in the different assays was consistent with earlier suggestions of NK-1 receptor mediation of selective nociceptive reflexes, using NK-1 receptor antagonists that were less selective, less potent, less bioavailable or shorter acting (Yamamoto and Yaksh, 1991; Radhakrishnan and Henry, 1991, 1995; Naga-hisa et al., 1992; Birch et al., 1992; Xu et al., 1992; Toda and Hayashi, 1993; Yashpal et al., 1993; Smith et al., 1994; Seguin et al., 1995). Moreover, the inactivity of LY303870 on the base-line tail-flick response further supported the observation that NK-1 antagonists may not affect traditional measures of acute nociception (Garces et al., 1992; Seguin et al., 1995). In summary, the ability of LY303870 to block central NK-1 receptors in areas of importance to sensitization processes and its selective oral analgesic activity, without other central effects, suggests potential utility of this drug for the treatment of persistent pain.

The binding of LY303870 and [³H]LY303870 to guinea pig brain membranes in vitro was extensively characterized previously (Gitter et al., 1995; Gehlert et al., 1996). Because LY303870 was more selective for guinea pig/human NK-1 receptors, the efficacy of LY303870 for displacing binding of ¹²⁵I-SP to guinea pig tissues, ex vivo, was also tested. The ability of LY303870 to inhibit ¹²⁵I-SP binding to striatal and lung membranes ex vivo after low oral doses, over an extended period of time, further supports the utility of LY303870 as a tool to evaluate the function of NK-1 receptors in humans.

The characterization of functional activity mediated by NK-1 receptors has been greatly aided in recent years by the introduction of selective nonpeptide receptor antagonists that are systematically available, have high affinity for NK-1 receptors and are more selective than peptide antagonists (Snider 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 studies described here with LY303870 distinguish it from the other antagonists described to date. The ex vivo binding studies in guinea pigs confirmed the ability of LY303870 to potently bind to NK-1 receptors in both the periphery and the central nervous system after oral administration. Additionally, these studies provided confirmation of the functional importance of the long duration of action of LY303870 in the formalin test. The lack of neurological, behavioral, motor or autonomic side effects of LY303870 in other central nervous system and cardiovascular tests reinforces the selectivity and safety of LY303870 in vivo. The receptor selectivity of LY303870 was

**TABLE 2**

**Effects of LY303870 on secondary pharmacological evaluation**

There was no activity in any of the tests listed at doses of LY303870 of ≤30 mg/kg i.p., for the free base or 50 mg/kg p.o. For the dihydrochloride trihydrate salt, when tested in mice or rats (n = 10, except n = 4 for cardiovascular function), with the exception of 50 mg/kg p.o., which produced a slight but significant increase in heart rate only. However, this increase was not considered to be physiologically relevant. These results suggest no evidence of neurological deficits, behavioral depression or disruption of autonomic function at the doses tested.

<table>
<thead>
<tr>
<th>Test</th>
<th>Species (Route)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Startle responsiveness (sensorimotor activity)</td>
<td>Mouse (i.p., p.o.)</td>
</tr>
<tr>
<td>Spontaneous activity (behavioral depression)</td>
<td>Mouse (i.p., p.o.)</td>
</tr>
<tr>
<td>Grip strength (neuromuscular function)</td>
<td>Mouse (i.p., p.o.)</td>
</tr>
<tr>
<td>Hexobarbital-induced sleep times (central nervous system depression)</td>
<td>Mouse (p.o.)</td>
</tr>
<tr>
<td>Electroshock- or pentlenetetrazol-induced convulsions (convulsive liability)</td>
<td>Mouse (p.o.)</td>
</tr>
<tr>
<td>Rotarod performance (coordination)</td>
<td>Rat (i.p., p.o.)</td>
</tr>
<tr>
<td>Body temperature</td>
<td>Mouse (p.o.)</td>
</tr>
<tr>
<td>Cardiovascular function</td>
<td>Rat (p.o.)</td>
</tr>
<tr>
<td>Clinical signs (behavioral observations)</td>
<td>Mouse (i.p., p.o.)</td>
</tr>
<tr>
<td>Charcoal meal transit (gastrointestinal function)</td>
<td>Mouse (p.o.)</td>
</tr>
</tbody>
</table>

*Helton et al. (1996a).
*Helton et al. (1996b).
previously demonstrated in vitro, and LY303870 was shown to have no significant affinity for NK-2 receptors, NK-3 receptors, any of 70 other neuropeptide/neurotransmitter receptor sites, calcium channels or other ion channel or uptake sites (Gitter et al., 1995).

In conclusion, these data suggest that LY303870 is a structurally novel, centrally active, NK-1 receptor antagonist in vivo. It is potent, efficacious, highly selective, safe, long-acting and orally active. Thus, LY303870 may be a useful tool to evaluate the therapeutic relevance of NK-1 receptors in inflammation, pain and other central pathophysiological processes.

References


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