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The neurokinin-1 antagonist activity of maropitant, an antiemetic drug for dogs, in a gerbil model

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Maropitant is a novel synthetic nonpeptide neurokinin type 1 (NK_1) selective receptor antagonist, recently developed for use in the dog as an antiemetic. The *in vivo* functional activity of maropitant was investigated in the gerbil foot-tapping model, to determine the ability of maropitant to penetrate the central nervous system and inhibit foot-tapping induced by the selective NK_1 agonist GR73632. In comparison with CP-122,721, a previously characterized NK_1 receptor antagonist, maropitant (1 mg/kg by s.c. injection) was found to inhibit foot-tapping for significantly longer ($P < 0.01$). Inhibition of foot-tapping by maropitant was 100% at 2 h and approximately 50% at 8 h postdosing. The mean brain:plasma concentration ratio at 8 h post-treatment was 3.59. These data demonstrate the central functional action of maropitant as a selective and potent NK_1 receptor antagonist and help to support and explain its clinical potential as a broad-spectrum antiemetic agent.

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INTRODUCTION

Vomiting is a familiar physiological reflex, elicited by a wide range of peripheral and central stimuli that include gastrointestinal irritation/inflammation, circulating toxins, cancer chemotherapy and other pharmacological agents, anxiety, pain and provocative motion (Koch, 1995). Emetogenic stimuli act either peripherally by triggering vagal and sympathetic afferents or centrally by stimulation of neurons in the chemoreceptor trigger zone (CTZ) (Koch, 1995). The CTZ is located in the area postrema on the floor of the fourth ventricle and lies outside the blood–brain barrier (BBB) facilitating exposure to circulating emetogens. Emesis is thought to be controlled via a series of nuclei located in the medulla oblongata, which include the nucleus tractus solitarius (NTS), and dorsal motor nucleus of the vagus, known collectively as the emetic centre (Bakowski, 1984; Leslie & Reynolds, 1992; Tattersall *et al.*, 1996). Several afferent pathways converge on the NTS including input from the vagus, sympathetic fibers, the CTZ, the cerebral cortex and the vestibular apparatus (Carpenter *et al.*, 1983; Koch, 1995; Tattersall *et al.*, 1996; Yates *et al.*, 1998). Neurotransmitter receptors found in those regions of the brain and peripheral neural pathways involved with the emetic response include dopaminergic (D_2), histaminergic (H_1 and H_2), serotoninergic ($5\text{-}HT_{1A}$, $5\text{-}HT_3$ and $5\text{-}HT_4$), and neurokinin type 1 (NK_1) receptors, as well as various endorphine (μ and δ), adrenergic (α_2), and cholinergic (M_1 and M_2) receptors (Washabau & Elie, 1995; Tonini *et al.*, 2004). The tachykinin, substance P, is thought to have a key controlling role within the emetic centre and has potent agonist activity at the NK_1 receptor. This role has been demonstrated in numerous investigations, including a key study in which emesis was rapidly elicited by directly injecting substance P into the brainstem of ferrets (Gardner *et al.*, 1995). Selective antagonism of NK_1 receptors in decerebrate dogs exposed to abdominal vagal stimulation has confirmed their involvement in the final common pathway that gives rise to vomiting in dogs (Fukuda *et al.*, 1999), and therefore NK_1 antagonists are likely to have potential as broad-spectrum antiemetics.

The NK_1 receptor is a G-protein coupled receptor that interacts with G_0, G_i, G_q and G_{11} to mediate its actions (Strader *et al.*, 1994; Quartara & Maggi, 1997). The mammalian neurotransmitters, neurokinin A and neurokinin B, which at high concentrations can also act as ligands for the NK_1 receptor, act more selectively as ligands at NK_2 and NK_3 receptors, respectively (Maggi, 1995). All three neurokinin receptors are distributed throughout both the central and peripheral nervous system, and are associated with a variety of physiological and pathological mechanisms (Maggi, 1995; Quartara & Maggi, 1998; Saria, 1999). In contrast to the natural tachykinins, synthetic agonists and antagonists are usually designed to be selective for only one receptor subtype (Hagan *et al.*, 1991;

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Maggi, 1995; Singh *et al.*, 1997; Tsuchiya *et al.*, 2002). Confirmation that emesis can be inhibited through selective antagonism at the NK_1 receptor was initially shown in the ferret, using the synthetic antagonists, CP-99,994 and L-741,671 (Bountra *et al.*, 1993; McLean *et al.*, 1993; Tattersall *et al.*, 1993; Diemunsch & Grelot, 2000). More recently, other NK_1 receptor antagonists, such as CP-122,721 and aprepitant (MK-869), have shown potential in the control of emesis induced either experimentally or during chemotherapy in human patients with emetogenic compounds, such as cisplatin (McLean *et al.*, 1996; Diemunsch & Grelot, 2000; Hesketh *et al.*, 2003; Patel & Lindley, 2003).

The NTS lies within the BBB; therefore to abolish the propagation of the emetic reflex, NK_1 receptor antagonists must be able to cross the BBB (Rupniak & Williams, 1994; Tattersall *et al.*, 1996; Rupniak *et al.*, 1997; Smith *et al.*, 2001). It has previously been shown that intracerebroventricular (ICV) infusion of the highly selective NK_1 agonist GR73632, δ-Ava[L-Pro^9,*N*-MeLeu^{10}]Substance P(7–11), (Hagan *et al.*, 1991) in gerbils induces repeated, vigorous and readily quantifiable rhythmic tapping of the hindfeet (Bristow & Young, 1994; Rupniak & Williams, 1994). Inhibition of this effect, following systemic administration of a brain-penetrating antagonist, provides a simple assay for the central action of NK_1 receptor antagonists *in vivo* (Rupniak *et al.*, 1997; Singh *et al.*, 1997). Rupniak *et al.* (1997) found that inhibition of the gerbil foot-tapping response by NK_1 antagonists was highly predictive of their activity in preventing cisplatin-induced acute emesis in ferrets.

Maropitant (CJ-11,972) is a selective nonpeptide NK_1 receptor antagonist, that has been recently developed and formulated as a general antiemetic product (Cerenia™; Pfizer Animal Health) for use in dogs. The chemical structure of maropitant, (2*S*,3*S*)-2-benzhydryl-*N*-(5-*tert*-butyl-2-methoxybenzyl) quinuclidin-3-amine, is shown in Fig. 1. This manuscript presents the results of an investigation into the central NK_1 receptor antagonist activity of maropitant. The activity and duration of action of maropitant in the inhibition of gerbil foot-tapping were compared with the activity of CP-122,721, (+)-(2*S*,3*S*)-3-(2-methoxy-5-trifluoromethoxybenzyl)amino-2-phenylpiperidine (McLean *et al.*, 1996), a selective NK_1 receptor antagonist known to inhibit the gerbil foot-tapping response (Smith *et al.*, 2001). Subsequently, the brain disposition of maropitant and its major oxidative metabolite, CJ-18,518, produced by a single oxidation of the *t*-butyl methoxy benzyl group, was also investigated. This work was undertaken following ethical review by the Institutional Animal Care and Use Committee (IACUC) which issued an Animal Use Protocol Approval (ACUP #1226). The work was conducted under veterinary supervision in compliance with all relevant international legislation.

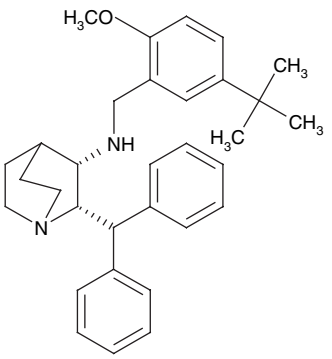

Fig. 1. The chemical structure of maropitant.

EXPERIMENTAL PROCEDURES

Materials

Maropitant (monohydrate citrate salt), CJ-18,518, CP-122,721 and internal standard were prepared at the Groton laboratories of Pfizer Global Research and Development (Pfizer Inc., Groton, CT, USA). GR73632 was purchased from Peninsula Laboratories (Belmont, CA, USA).

Animals

Male Mongolian gerbils (40–60 g) were sourced from Charles River Laboratories (Kingston, NY, USA). All animals were housed in standard accommodation with free access to food and water. Animal husbandry and welfare were maintained in accordance with established guidelines.

Gerbil foot-tapping

The study was conducted as previously described with minor modifications (Rupniak & Williams, 1994; Smith *et al.*, 2001). Gerbils were treated by s.c. injection with maropitant at 1.0 mg/kg (free-base equivalents) or CP-122,721 (1.0 mg/kg) in a vehicle of 5% dimethyl sulfoxide/5% Emulphor/90% phosphate buffered saline. At 2, 4 and 8 h postdose, gerbils ($n = 3$ for maropitant, $n = 2$ for CP-122,721) were anaesthetized using isoflurane in oxygen and a dorsal midline incision was made to expose the skull. GR73632 at 3 pmol/5 μL was administered in a volume of 5 μL directly into the lateral ventricles (ICV) through a 25-gauge needle inserted vertically to a depth of 4.5 mm below the cranium. Three control gerbils were administered GR73632 only and a further two received vehicle alone. The skin incision was closed using tissue adhesive (Vet-Bond™, 3M, Minneapolis, MN, USA). Animals were placed in individual cages to regain consciousness. Once righting reflex occurred, animals were videotaped for 15 min. The foot-tapping response was later scored every 30 sec over a 12-min period by an observer blinded to treatment allocation. A positive response was recorded if the gerbil foot-tapped for a span of 5 sec or more within the 30 sec period.

Analysis of maropitant in plasma and brain

Immediately after foot-tapping responses had been videotaped (nominally 2, 4 and 8 h postantagonist dose), gerbils were killed

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in a carbon dioxide chamber and whole blood was collected via cardiac puncture. Brain samples were collected and stored at −20 °C until processed. Plasma was harvested following centrifugation of the whole blood at 2616 ***g*** for 15 min. Whole brain was homogenized 1:1 with 100 mM sodium phosphate (pH 6.7) prior to solid phase extraction (SPE). Aliquots of 100 μL plasma were placed into a 96-deep well polypropylene plate. To each well was added 10 μL of a 1-μg/mL internal standard stock solution (a tri-deuterated analog of CJ-11,972), with 100 μL of 1% formic acid in water. Samples were vortex mixed for 1 min. An MCX 96-well SPE plate was conditioned with 200 μL methanol followed by 200 μL water under a gentle vacuum and 200 μL of sample were loaded onto the SPE plate. Analyte and internal standard were eluted from the SPE plate into a new 96-deep well polypropylene plate using 400 μL 5% ammonium hydroxide in methanol. The eluent was evaporated to a residue under nitrogen at 50 °C. Sample residues were reconstituted in 200 μL methanol and vortex mixed. A 10-μL aliquot of each sample was injected onto the HPLC/MS/MS system for analysis. A Keystone BDS C_8 column (2.1 × 30 mm; particle size 3 μm) was used, with flow rate of 0.5 mL/min and run-time of 2.5 min. The effluent was analyzed by a triple quadrupole mass spectrometric detector (Applied Biosystems/Sciex API 3000, Foster City, CA, USA) and the data were analyzed using ANALYST v.1.2 software. Retention times for maropitant, CJ-18,518, CP-122,721 and internal standard were 0.99, 0.39, 0.42 and 1.00 min, respectively. Maropitant, CJ-18,518, CP-122,721 and internal standard were detected in the positive ion mode by monitoring the M + H ion conversions: 469.3 → 177.1, 485.3 → 193.2, 381.0 → 160.0, and 472.3 → 180.2, respectively. Integrated peak areas for drug and internal standard were determined, and corresponding drug: internal standard ratios were calculated. Drug concentrations for each sample were then calculated based on a linear regression analysis of a standard curve. The dynamic range of the assay was 1–800 ng/mL for plasma (1–800 ng/g for brain homogenate). For gerbil plasma, the ranges for intra-assay mean accuracy and precision were 89–107% and 3–13% for maropitant, 82–97% and 2–11% for CJ-18,518 and 76–96% and 4–13% for CP-122,721, respectively. For gerbil brain, intra-assay mean accuracy and precision ranged from 106–130% and 18–21% for maropitant, to 127–129% and 2–12% for CJ-18,518 and 120–121% and 7–25% for CP-122,721, respectively.

Data analysis

Gerbil foot-tapping data were expressed as 'percent time tapping' compared with the agonist (GR73632) alone, and the percent reversal of the response to agonist was calculated. The results for maropitant were compared with those for CP-122,721 using analysis of variance with a one-way ANOVA with Dunett's as the *post hoc* test. A computer curve-fitting program (Labstats™ MS-Excel™ plug-in; Computer Lab Solutions LLC, Farmington, UT, USA) was used to calculate the time points that gave 90% and 50% efficacy as the antagonist effect diminished over time.

RESULTS

Pretreatment of gerbils with maropitant resulted in a significant ($P < 0.01$) reversal of tachykinin agonist GR73632-induced foot-tapping at each time point tested (2, 4 and 8 h postdose). Following a single s.c. dose of maropitant at 1 mg/kg, approximately 100% reversal of tapping behavior was obtained by 2 h (Fig. 2), declining over time to 90% at 4.3 h (range 3.38–5.37 h) and 50% reversal of tapping at 8.0 h (range 7.4–8.65 h). In comparison, following CP-122,721 administration, 90% reversal was observed at 2.2 h (range 1.09–4.29 h), 50% at 4.4 h (range 2.9–6.7 h) but only 12% at 8 h postdose. Statistical analysis showed that the effect of maropitant was significantly greater than that of CP-122,721 at both 4 and 8 h postdose ($P < 0.01$).

Concentrations of maropitant, CJ-18,518 and CP-122,721 in plasma and brain samples are shown in Table 1. Gerbil brain

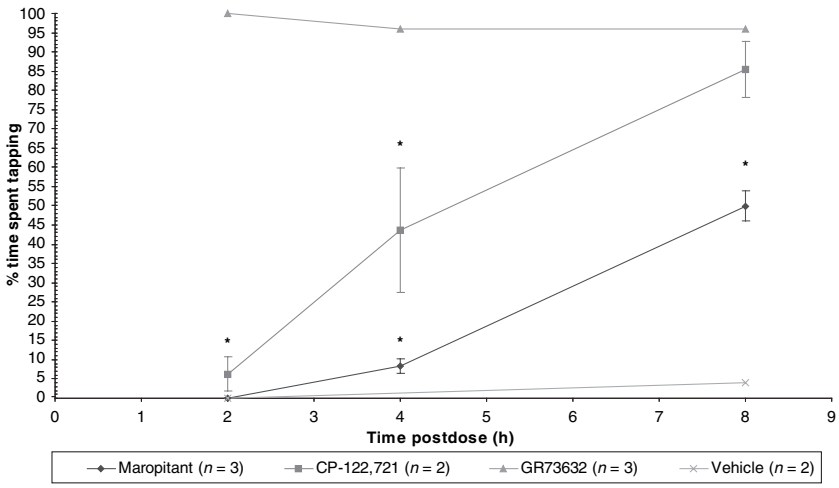

Fig. 2. NK_1 antagonism of GR7362-induced gerbil foot-tapping: mean percentage of time spent foot-tapping.

Error bars represent SEM. *n* = number of animals at each time point. *Significant difference ($P < 0.01$) compared with agonist (GR73632). At 4 and 8 h postdose there was a significant difference ($P < 0.01$) between maropitant and CP-122,721 according to a Student–Neuman Keul's test.

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Table 1. Concentrations of maropitant and the major metabolite CJ-18,518 in gerbil plasma and brain samples following a 1-mg/kg s.c. dose of maropitant ($n = 3$ for maropitant; $n = 2$ for CP-122,721)

Nominal time (h)	Brain (ng/g)			Plasma (ng/mL)			Brain:plasma ratio	
	Maropitant	CJ-18,518	CP-122,721	Maropitant	CJ-18,518	CP-122,721	Maropitant	Mean
2	36.5	ND	141.0	25.4	1.2	10.6	1.44	1.76
2	33.1	ND	105.3	15.8	1.1	23.5	2.10	
2	43.2	ND	–	24.6	1.3	–	1.75	
4	14.5	ND	17.5	22.5	2.5	5.6	0.65	1.49
4	20.2	0.9	19.4	10.0	1.8	9.5	2.02	
4	26.7	ND	–	14.8	0.9	–	1.80	
8	5.2	ND	7.9	1.2	ND	7.2	4.40	3.59
8	5.9	ND	4.8	1.8	ND	4.6	3.34	
8	8.8	ND	–	2.9	ND	–	3.05	

ND, not detected; LLOQ = 1 ng/g.

concentrations of maropitant in this study were generally higher than plasma levels, especially at the 8-h time point. Plasma maropitant concentrations of 16–25 ng/mL were achieved by 2-h postdose, with corresponding brain concentrations of 33–43 ng/g. Over the 2–8 h time interval, the mean brain:plasma ratios were 1.76, 1.49, and 3.59 at 2, 4 and 8 h postdose, respectively. For CJ-18,518, plasma concentrations of 0.9–2.5 ng/mL were attained between 2 and 4 h postdose with no detectable concentrations by 8 h postdose. Only one animal had a detectable amount of CJ-18,518 in a brain sample.

DISCUSSION

In the present study, the gerbil foot-tapping model was used, as described previously, with minor modifications (Rupniak & Williams, 1994; Smith *et al.*, 2001). Central (ICV) administration of the highly selective tachykinin NK_1 receptor agonist, GR73632 [truncated substance P(7–11)] induces a predictable foot-tapping response, inhibition of which has been used to demonstrate centrally mediated NK_1 receptor antagonism *in vivo* (Rupniak *et al.*, 1997; Singh *et al.*, 1997). As this model determines the ability of pharmaceutical agents to inhibit NK_1 receptors located in the brain, it also identifies the potential for antiemetic efficacy. An NK_1 receptor antagonist that does not cross the BBB will have poor or nonexistent antiemetic activity *in vivo* (Rupniak *et al.*, 1997; Smith *et al.*, 2001). Systemically administered maropitant was found to be highly effective in inhibition of GR73632-induced foot-tapping in gerbils, indicating that maropitant does indeed penetrate the BBB and acts by binding to and antagonizing central NK_1 receptors. Further evidence in support of this conclusion was derived from the concentrations of maropitant detected in brain samples, which confirmed that whilst maropitant generally achieved higher levels in the brain than in plasma at each time point, the metabolite CJ-18,518 was not present to an appreciable degree (Table 1) in brain tissue. Furthermore, whilst CP-122,721 achieved much higher initial concentrations in brain than maropitant, these appeared to decline more rapidly which may, in part, explain the shorter duration of antagonist effect observed in the foot-tapping phase of the experiment.

The specificity of maropitant has been further demonstrated by *in vitro* binding studies conducted during the development of the registration database (Cerenia® European Public Assessment Report at http://www.emea.eu.int). In radioligand binding studies, maropitant has been found to be a potent antagonist at the canine NK_1 receptor as shown by inhibition of ^{3}H-substance P binding in canine striatum ($K_i = 0.5$ nM). In contrast, maropitant showed a low affinity for the NK_2 and NK_3 receptors, with IC_{50} values of >1000 nM for both receptor subtypes. The selectivity of maropitant for the NK_1 receptor has been further confirmed by a lack of activity when tested at 1–10 μM against a broad range of other neurotransmitter receptors including adrenergic (α_1, α_2 and β), adenosine$_1$, benzodiazepine, GABA, H_1, 5-HT_{1A} and 5-HT_2, muscarinic, D_2 and μ-endorphine. The major hepatic metabolite of maropitant (CJ-18,518) also showed binding affinity at the human NK_1 receptor in human lymphoblast (IM-9) cells ($K_i = 0.4$ nM). However, as CJ-18,518 does not penetrate the gerbil BBB to an appreciable extent, it is highly unlikely that this metabolite would contribute to the antiemetic activity of maropitant following administration to dogs. In other studies investigating the functional antagonism of maropitant to NK_1 receptors in dog carotid artery tissue, maropitant showed evidence of high-affinity, slow dissociation binding to the NK_1 receptor, with sustained agonist activity at the NK_1 receptor despite substance P concentrations of >1 μM (data not shown).

NK_1 receptor antagonists have been found to have similar binding and functional activity in humans, dogs, ferrets, and guinea pigs but a lower apparent activity in rats and mice. These differences are believed to be due to a species-dependent heterogeneity in NK_1 receptor amino acid sequences, leading to differential binding affinity (Sachais *et al.*, 1993; Singh *et al.*, 1997; Saria, 1999). NK_1 receptors are found throughout the central and peripheral nervous system and appear to be associated with a variety of physiological functions. In the spinal cord, NK_1 receptors are thought to be involved in producing nociceptive responses, particularly the hyperalgesia and hypersensitivity that

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accompany persistent peripheral inflammation (Quartara & Maggi, 1998). Peripherally, NK_1 receptors are present around arteries and veins throughout the body providing a means for the local regulation of blood flow and vascular permeability by triggering vasodilatation and plasma protein extravasation. High densities of NK_1 receptors are present in the smooth muscle, submucosa and intestinal epithelium, where they are involved in the regulation of gut motility (Quartara & Maggi, 1998). In the bladder, NK_1 receptors also appear to regulate smooth muscle tone and vascular permeability, and in the respiratory tract, mucus secretion and bronchomotor tone are mediated by NK_1 receptors (Quartara & Maggi, 1998; Chapman *et al.*, 1999).

In the CNS, involvement of NK_1 receptors has been demonstrated in a number of behavioral responses, such as locomotion, grooming, wet-dog shaking, hind leg tapping in gerbils and defensive rage in cats (Shaikh *et al.*, 1993; Quartara & Maggi, 1998). However, acute blockage of central NK_1 receptors does not appear to induce gross behavioral changes. NK_1 receptors also play a role in the emetic and micturition reflex pathways and in the mediation of stress-induced activation of ascending central pathways (Doi *et al.*, 1999; Mantyh, 2002). They also regulate cardiovascular (hypertension and tachycardia) and respiratory (tachypnoea) parameters through activation of efferent sympathetic activity (Quartara & Maggi, 1998). At doses that prevent emesis, there is no evidence that maropitant produces undesired physiological or behavioral responses, including inhibition of gut motility.

The distribution of NK_1 receptors in the brainstem has been identified by tritiated substance P-binding in the NTS, dorsal motor nucleus of the vagus and the hypoglossal nucleus and at much lower densities in the area postrema (Watson *et al.*, 1995). The emetic centre receives peripheral input from the gastrointestinal tract and other abdominal viscera via vagal and sympathetic afferent nerve fibers (Andrews *et al.*, 2001). The NTS situated within the BBB also receives direct input from other brain areas including the vestibular apparatus, the cerebral cortex, and from outside the BBB from the CTZ located in the area postrema. Neurotransmitter receptors in the CTZ are stimulated by toxins of bacterial, uraemic or hepatic origin present in the circulation, resulting in centrally initiated emesis. Other emetogenic stimuli active at the CTZ include the classical emetogens (e.g. apomorphine and loperamide), as well as a number of pharmaceuticals (such as cardiac glycosides, α_2-agonist sedatives and opiates) that may cause emesis as an unwanted side-effect, and probably also radiation. There is also a link between the vagal pathway and the CTZ (Carpenter *et al.*, 1983; Andrews *et al.*, 2001; Tonini *et al.*, 2004) and, although the functional role of this link is not clear at present, it may be involved with clinical signs of nausea. Important neuroreceptors in the CTZ involved in the transmission of impulses to the emetic centre include dopaminergic (D_2), serotoninergic ($5\text{-}HT_3$), histaminergic (H_1), and muscarinic receptors (Carpenter *et al.*, 1983; Koch, 1995).

Thus, neurons in the NTS receive and integrate sensory stimuli from the abdominal viscera, higher cortical areas and the vestibular system, as well as input via the CTZ from chemical stimuli in the circulation and cerebrospinal fluid (Yates *et al.*, 1998). Central vasopressinergic pathways, resulting in the release of vasopressin from the pituitary, are also thought to play a role in motion sickness, as levels increase in affected individuals (Koch, 1995; Yates *et al.*, 1998). Although different stimuli may initiate vomiting at disparate anatomical sites and/or via diverse etiologies, activation of the emetic centre leads to a predictable series of clinical events concluding with vomiting. Efferent impulses from the emetic centre travel to the stomach and upper small intestine via the vagus, and to the diaphragm, oesophagus, pharynx and glossal musculature, resulting in prodromal signs, such as mouth opening, salivation, gastric relaxation, and increased respiration, part of the constellation of clinical signs referred to as nausea, as well as the generation of rhythmic abdominal contractions that culminate in the expulsion of gastric contents (Koch, 1995). The consequence of the co-ordination of vomiting by the same region of the CNS regardless of the initiating stimulus, means that control can be achieved through the precise targeting of the key neurotransmitter receptors, located in the NTS (Andrews *et al.*, 2001). There is convincing evidence confirming that NK_1 receptors play a key role in the co-ordination of emesis (Fukuda *et al.*, 1999; Diemunsch & Grelot, 2000). The administration of substance P results in vomiting and compounds which block the central NK_1 receptor result in broad-spectrum prevention of vomiting (Diemunsch & Grelot, 2000). Studies in ferrets, dogs and man have demonstrated that the administration of NK_1 receptor antagonists capable of penetrating the BBB result in the prevention of vomiting induced by both central and peripheral emetogens (Watson *et al.*, 1995; Gardner *et al.*, 1996; Rudd *et al.*, 1996; Tattersall *et al.*, 1996; Singh *et al.*, 1997; Diemunsch & Grelot, 2000; Tsuchiya *et al.*, 2002) as well as motion sickness in the house-musk shrew (*Suncus murinus*) and cats (Gardner *et al.*, 1996; Lucot *et al.*, 1997).

CONCLUSION

Maropitant was shown to be a potent and selective *in vitro* NK-1 antagonist, binding selectively at NK_1 receptors with long-lasting centrally mediated effects when administered *in vivo* to gerbils. The results of this study demonstrate that maropitant penetrates the BBB and produces functional anatagonism of central NK_1 receptors in the gerbil, helping to model and explain its potential as a broad-spectrum antiemetic agent for clinical use in dogs.

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