[Lys⁵,MeLeu⁹,Nle¹⁰]-NKA_(4–10) Elicits NK2 Receptor–Mediated Micturition and Defecation, and NK1 Receptor–Mediated Emesis and Hypotension, in Conscious Dogs

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ABSTRACT

Tachykinin neurokinin 2 (NK2) receptor agonists may have potential to alleviate clinical conditions associated with bladder and gastrointestinal underactivity by stimulating contraction of visceral smooth muscle. The ability of [Lys⁵,MeLeu⁹,Nle¹⁰]-neurokinin A_(4–10) (LMN-NKA) to elicit micturition and defecation was examined after repeated administration in groups of 2–10 conscious dogs. Administration of 10–100 μg/kg, i.v., four times daily for six consecutive days, reliably elicited micturition after ≥90% of doses and defecation after ≥50% of doses. Voiding occurred <4 minutes after dosing and was short lasting (<10 minutes). LMN-NKA was well tolerated, with emesis after ~25% of doses at 100 μg/kg, i.v. Hypotension was induced by 100 μg/kg, i.v., of LMN-NKA but not by lower doses. Administration of 30–300 μg/kg, s.c., twice daily for seven consecutive days, reliably elicited both urination and

defecation after 88%–100% of doses, and was accompanied by a high rate of emesis (50%–100%). The onset of voiding was rapid (<7 minutes) but was more prolonged than after intravenous administration (30–60 minutes). Emesis induced by 30 or 300 μg/kg, s.c., of LMN-NKA was significantly reduced (from 58% to 8% and from 96% to 54%, respectively) by a 30-minute pretreatment with the neurokinin 1 (NK1) receptor antagonist, (2S,3S)-N-(2-methoxybenzyl)-2-phenylpiperidin-3-amine (CP-99,994; 1 mg/kg, s.c.). The ability of selective NK2 receptor agonists to elicit on-demand voiding could potentially address a major unmet need in people lacking voluntary control of micturition and/or defecation. LMN-NKA unexpectedly activated NK1 receptors at doses that stimulated voiding, causing emesis and hypotension that may limit the clinical utility of nonselective NK2 receptor agonists.

Introduction

Neurokinin 2 receptor (NK2R) agonists are under evaluation as a novel therapeutic approach to promote on-demand micturition and defecation in individuals who have lost voluntary control of voiding due to neurologic disease or trauma, such as spinal cord injury. Although the ability of NK2R agonists to contract bladder and gastrointestinal tissues in vitro via direct activation of NK2Rs expressed on smooth muscle is well known (Warner et al., 1999, 2003; Burcher et al., 2008), the potential to exploit this prokinetic activity therapeutically has not been explored until recently. [Lys⁵,MeLeu⁹,Nle¹⁰]-neurokinin A_(4–10) (LMN-NKA) was employed as a prototype NK2R agonist based on its >700-fold higher affinity for human cloned neurokinin 2 (NK2) over neurokinin 1 (NK1) receptors in radioligand binding assays, and >100-fold selectivity for NK2Rs than NK1Rs using intracellular calcium mobilization as an in vitro functional assay (submitted manuscript). In anesthetized, spinally intact, and acute spinal rats (Kullmann et al., 2017; Marson et al., 2018) and anesthetized dogs (Rupniak et al.,

2018), administration of LMN-NKA was able to increase colorectal and bladder pressure and elicit urinary voiding (defecation was not measured due to the placement of a balloon in the rectum to record colorectal pressure). The prokinetic effects of LMN-NKA on both bladder and rectum in both rats and dogs were rapid in onset (0.5–3 minutes) and short in duration (3–10 minutes) at efficacious doses, depending on the route of administration (intravenous or subcutaneous). Furthermore, the prokinetic effects were blocked by pretreatment with the NK2R antagonist, 5-fluoro-3-[2-[4-methoxy-4-[(R)-phenylsulphonyl]methyl]-1-piperidinyl]ethyl]-1H-indole (GR 159897), but not by the NK1R antagonists, spantide I or (2S,3S)-N-(2-methoxybenzyl)-2-phenylpiperidin-3-amine (CP-99,994).

The ability of NK2R agonists to reliably elicit urination multiple times per day and/or defecation on a daily basis in conscious animals after repeated daily doses across multiple days, as would be needed in a clinical setting, has not yet been examined. Since there is evidence that the smooth muscle contractile effect of neurokinin A (NKA), the endogenous agonist for the NK2R, undergoes tachyphylaxis in some in vitro preparations (Daniel et al., 1989; Patacchini et al.,

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ABBREVIATIONS: CP-99,994, (2S,3S)-N-(2-methoxybenzyl)-2-phenylpiperidin-3-amine; LMN-NKA, [Lys⁵,MeLeu⁹,Nle¹⁰]-neurokinin A_(4–10); NKA, neurokinin A; NK1, neurokinin 1; NK1R, neurokinin 1 receptor; NK2, neurokinin 2; NK2R, neurokinin 2 receptor.

1997), but not others (Reynolds et al., 1998), it is important to examine whether NK2R agonists can consistently produce urinary and fecal voiding after repeated dosing in vivo. Thus, the aim of the present studies was to extend previous findings using anesthetized preparations to establish whether LMN-NKA elicits urinary and fecal elimination in conscious dogs after acute and repeated administration.

In addition to further examining these desired pharmacodynamic outcomes, examination of the unwanted effects of LMN-NKA in conscious dogs was also of interest because of the marked, but transient, hypotension that was seen in anesthetized dogs at the same intravenous doses that increased colorectal pressure (Rupniak et al., 2018). The mechanism underlying hypotension was distinct from the prokinetic activity since it was blocked by pretreatment with the NK1R antagonist, CP-99,994, and could be avoided by reducing the plasma exposure to LMN-NKA via subcutaneous administration while maintaining the prokinetic effect. The presence of hypotension after such low intravenous doses of LMN-NKA in anesthetized dogs was surprising since it was not seen after intravenous injection of prokinetic doses of LMN-NKA in anesthetized rats (Kullmann et al., 2017; Marson et al., 2018) or intravenous infusion of prokinetic doses of NKA in conscious humans (Evans et al., 1988; Lördal et al., 1997, 2001; Schmidt et al., 2003). Moreover, the overlap between NK2R-mediated colorectal contraction and NK1R-mediated hypotension in anesthetized dogs was not consistent with the >100-fold selectivity of LMN-NKA for NK2Rs over NK1Rs in vitro. This hypotensive response in anesthetized animals after acute intravenous administration of LMN-NKA was, therefore, further explored in conscious dogs implanted with a telemetric recording system.

Finally, because NK2Rs are expressed throughout the gastrointestinal tract and in respiratory smooth muscle, and NK1Rs are widely distributed throughout the body, it was also important to examine symptomatic, behavioral effects in conscious, unrestrained dogs that might be missed in anesthetized dogs.

Materials and Methods

Animals. Unless otherwise stated, naive dogs were housed in pairs in runs of up to 28 feet² that were divided during test sessions to create individual cages measuring 8.1–9.1 feet². Experiments conformed to Institutional Animal Care and Use Committee regulations, the Guidelines for the Care and Use of Laboratory Animals (2011), and were approved by the Animal Care and Use Committee of Calvert Laboratories Inc. (Scott Township, PA), Synchrony Laboratories (Durham, NC), and MPI Research (Mattawan, MI). For studies conducted at Calvert (studies 1–4) and MPI Research (study 7), water and food were available ad libitum. For studies conducted at Synchrony Laboratories (studies 5 and 6; Table 1), water was available ad libitum and food was provided twice daily at ~7.30 AM and 4 PM. Preliminary dose-ranging studies used small numbers of dogs to observe pharmacodynamic effects. Where possible, data from smaller studies were combined to increase the value of *N*. The total number of animals was based on regulatory and Institutional Animal Care and Use Committee guidelines and considered to be the minimum number needed to assess the tolerability, pharmacodynamic, and pharmacokinetic effects of LMN-NKA and CP-99,994 (*N* = 2–10 per dose group). The subjects were male and female beagles (6–16 kg; Marshall BioResources, North Rose, NY).

Cardiovascular Parameters. Arterial blood pressure and heart rate were monitored in four non-naive male dogs that had previously been implanted with telemetry probes following a drug wash-out period of at least 30 days since the last test compound. At least 24 hours prior to surgery, a DURAGESIC fentanyl transdermal patch (2.5 mg for dogs under 10 kg; 5 mg for dogs over 10 kg) obtained from Janssen Pharmaceutica (Beerse, Belgium) was applied for continuous analgesia. On the day of surgery, dogs received atropine (0.02–0.3 mg/kg, i.m.; Vedco Inc., St Joseph, MO) and induction anesthesia with telazol (2.2–4.4 mg/kg, i.v.; Zoetis, Parsippany, NJ). Following endotracheal intubation, anesthesia was maintained by inhalation of isoflurane. The antibiotic cefazolin (20–25 mg/kg; Kefzol, Pfizer, NY) was administered by intravenous infusion. An incision was made extending ~4 inches from the dorsal scapula to the fifth rib, and another incision was made in the abdomen just below the diaphragm through which the radio telemetry module (Integrated Telemetry Systems version 3.0.1; Mistral Solutions Inc., Fremont, CA) was inserted. The body of the telemetry device was placed in an intramuscular pocket between the internal and external oblique muscles and sutured under the first muscle layer; cables carrying

TABLE 1
Pharmacodynamic and pharmacokinetic studies using conscious dogs

Study Number	Description	Doses and Route	Subjects	Design
1 (DT-044)	Single dose cardiovascular telemetry	Vehicle, 1, 10, or 100 µg/kg, i.v.	One group of four non-naive males	All dogs received each treatment with ≥3 days between doses
2 (DT-052)	Single dose pharmacodynamics and pharmacokinetics	10 and 100 µg/kg, i.v., or 10, 30, and 100 µg/kg, s.c.	One group of four non-naive females	All dogs received each treatment with ≥1 day between doses
3 (DT-017)	Single and repeat dose pharmacodynamics	Vehicle, 1, 10, or 30 µg/kg, i.v.	Four groups of two naive dogs (one of each sex)	Separate groups received each treatment up to four times daily for 5 days
4 (DT-042)	Single and repeat dose pharmacodynamics	Vehicle, 1, 10, or 100 µg/kg, i.v.	Four groups of 10 naive dogs (five of each sex)	Separate groups received each treatment up to four times daily for 7 days
5 (DT-076)	Single dose pharmacodynamics	Vehicle, 3, 10, or 30 µg/kg, s.c.	One group of four naive and six non-naive dogs (five of each sex)	All dogs received vehicle and 30 µg/kg; three dogs also received 3 or 10 µg/kg. Doses were separated by at least 3 days.
6 (DT-085)	Single and repeat dose pharmacodynamics	30 and 300 µg/kg, s.c.	Two groups of six naive dogs (three of each sex)	Separate groups received 300 µg/kg on days 1 and 7 and 30 µg/kg on days 2–6, with or without CP-99,994 (1 mg/kg, s.c.)
7 (DT-049)	Single dose pharmacokinetics	100 and 300 µg/kg, s.c.	Two groups of 10 non-naive dogs (five of each sex)	Separate groups received each treatment on 1 day

the pressure and ECG sensors were routed subcutaneously to the thorax, and the transmission antenna was routed subcutaneously toward the head. An atraumatic vascular clamp was applied to the descending aorta for incision and insertion of a pressure transducer; the incision was closed with sutures and the clamp released. The ECG spur was tethered near the sternum in the sixth intercostal space. Bupivacaine (Marcaine, Pfizer, NY) was applied for local anesthesia and incisions were closed in layers using sutures and staples. After recovery from anesthesia, dogs received an anti-inflammatory (Rimadyl, 4.4 mg/kg, s.c.; Zoetis) and an antibiotic (Baytril, 2.5 mg/kg, s.c.; Bayer, Leverkusen, Germany). Rimadyl was continued at 2.2 mg/kg by mouth twice daily for 7 days, and cephalexin was continued twice daily (20–25 mg/kg by mouth) for 14 days. The fentanyl patch was removed 3 days after surgery; skin sutures or staples were removed by day 14, and animals were placed on study at least 4 weeks after surgery. Dogs were individually housed and tested in a dedicated telemetry area with a controlled environment. On test days, cardiovascular parameters were collected for up to 22 hours after intravenous administration of LMN-NKA (1, 10, or 100 $\mu\text{g/kg}$) or vehicle (study 1).

Observation of Pharmacodynamic Effects. Pharmacodynamic data were captured in six studies, as summarized in Table 1. Unless otherwise stated, urination, defecation, diarrhea, salivation, and emesis induced by LMN-NKA were recorded by direct visual observation of animals in their home cage. Events were captured on a score sheet by one or two observers monitoring two or three dogs simultaneously. On test days, dogs were gently restrained for intravenous injection of test compounds into a cephalic vein, alternating between injection sites and veins in repeat dose studies; subcutaneous injections were given near the shoulder blades. Dogs were dosed between 9:00 and 9:30 AM and at designated time intervals thereafter as specified subsequently.

The pharmacodynamic effects of a single administration of vehicle or LMN-NKA (1, 10, and 100 $\mu\text{g/kg}$, i.v.) were recorded in four separate studies in which either the same animals received each dose separated by a washout period or separate groups received different doses as detailed in Table 1. Dogs were observed continuously thereafter for 10–20 minutes, and the number of animals voiding and the latency to the first void were recorded. Events were counted only once after each dose to estimate incidence (i.e., responder rate). There were two repeat dose intravenous studies. In study 3, four daily injections were given 4 hours apart, for five consecutive days. In study 4, injections were given 30 minutes apart on day 1 and 4 hours apart on days 2–7. Dogs were observed for at least 20 minutes after each injection.

The incidence of pharmacodynamic effects after a single subcutaneous dose of vehicle or LMN-NKA (3, 10, 30, or 100 $\mu\text{g/kg}$) was determined by combining data from three studies, each using 3–10 dogs per dose. Study 5 used dogs that had been acclimated to handling and placement in individual metabolism cages (10.4 feet²) for at least 3 days prior to study. In this study, dogs were observed in the metabolism cage for up to 60 minutes, and the volume of voided urine was recorded. However, since water was available ad libitum, the predose bladder volume was not measured, and open pan cages were employed in most studies, voided volumes were not quantified in all studies. Study 6 included a 7-day repeat dose study in which six naive dogs (three of each sex) received twice daily doses of 300 $\mu\text{g/kg}$, s.c., LMN-NKA on days 1 and 7 and twice daily doses of 30 $\mu\text{g/kg}$, s.c., on days 2–6. A separate group of six dogs received the same treatment but were pretreated with the selective NK1R antagonist CP-99,994 (1 mg/kg, s.c.) 30 minutes before each dose of LMN-NKA (30 or 300 $\mu\text{g/kg}$, s.c.) on days 1–7. Animals were observed for 30 minutes after each dose of LMN-NKA.

Pharmacokinetics. In studies 2 and 7, six or seven blood samples, respectively (~2 ml), were collected from the jugular vein of four non-naive dogs prior to and at 0.5, 1, 2, 4, 6, 10, and 20 minutes after intravenous administration and at 2, 5, 10, 20, 40, and 60 minutes after subcutaneous administration of 100 $\mu\text{g/kg}$ of LMN-NKA. Samples were collected in tubes containing EDTA anticoagulant (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ), gently mixed

by inversion, and 1.8 ml immediately transferred to a tube containing 200 μl of cold 10% ascorbic acid to yield a final concentration of 1% ascorbic acid. The solution was gently mixed by inversion and kept on ice until centrifugation within 30 minutes of collection at 2–3000 rpm for ~10 minutes at 4°C. Plasma aliquots were flash frozen in liquid nitrogen and stored at –80°C until assay. Samples were analyzed in 100 μl aliquots using a solid-phase extraction procedure followed by liquid chromatography–tandem mass spectrometry. Concentrations of LMN-NKA were calculated with a $1/x^2$ linear regression over a qualified concentration range of 1–1000 ng/ml using [¹³C, ¹⁵N]-substituted LMN-NKA as an internal standard. An API 5000 platform was operated under optimized conditions for detection of LMN-NKA positive ions formed by electrospray ionization Applied Biosystems/MDS Analytical Technologies, Ontario, Canada.

Preparation of Test Compounds. LMN-NKA [Asp-Lys-Phe-Val-Gly-Leu(NMe)-Nle-NH₂] was manufactured using solid-phase synthesis (Bachem, Torrance, CA) to a purity $\geq 95\%$. Stock solutions (10 mg/ml) of LMN-NKA and the NK1R antagonist CP-99,994 (Monomerchem, Durham, NC) were prepared in saline or saline:water (3:1), respectively, sterilized by passing through a 0.2 μm polyethersulfone membrane syringe filter (ThermoFisher Scientific, Carlsbad, CA) and stored in aliquots at –20°C until use. Dilutions were made using sterile saline.

Data Analysis and Statistics. Mean blood pressure and heart rate data were calculated in 1-minute time bins and expressed as a percentage of the mean baseline value (recorded for 15 minutes prior to dosing) using Excel (Microsoft, Redmond, WA) and Prism 6 (GraphPad Software Inc., San Diego, CA). Mean values were compared with vehicle control values using two-way analysis of variance followed by Bonferroni multiple comparisons test (Prism 7; GraphPad Software Inc.). Differences of $P \leq 0.05$ were considered statistically significant. Values are expressed as the mean + S.D. For pharmacodynamic studies, the responder rate (number of animals responding after any injection of vehicle or LMN-NKA) for pharmacodynamic events was expressed as a fraction of the total number of animals treated. Nonparametric incidence data were compared using logistic regression. Since some analyses contained levels of zero responses, models were estimated using the exact method. Pairwise comparisons of doses versus control group were performed using Fisher's exact test using an adjusted alpha level (0.01) to account for multiple comparisons. Pharmacokinetic data were collected using Analyst (MDS Sciex, Framingham, MA) and parameters were calculated using Phoenix WinNonlin 6.3 (Certara, Princeton, NJ).

Results

Cardiovascular Parameters

The cardiovascular effects of single intravenous injections of LMN-NKA were monitored in four male dogs with implanted radio telemetry probes. During the 15-minute pretreatment baseline periods, mean arterial blood pressure was 132.13 ± 11.15 mm Hg (mean $\pm$ S.D.). The mean arterial blood pressure remained stable during the first 10 minutes after intravenous injection of vehicle or 1 $\mu\text{g/kg}$ LMN-NKA. Blood pressure was highly variable after 10 $\mu\text{g/kg}$, with no consistent change across animals. After 100 $\mu\text{g/kg}$ of LMN-NKA, hypotension was reliably seen in all dogs compared with administration of vehicle. The mean arterial blood pressure fell up to 32 mm Hg below baseline 3–5 minutes after dosing (Fig. 1). Blood pressure returned to baseline levels within 10 minutes.

During the 15-minute pretreatment baseline periods, mean heart rate was 102.53 ± 10.13 beats per minute (mean $\pm$ S.D.). Heart rate was generally stable during the 10-minute postinjection period and there was no consistent change

Fig. 1. Effect of single intravenous injection of LMN-NKA (1, 10, or 100 µg/kg) on arterial blood pressure. Values are the mean percentage change in mean arterial blood pressure (MAP) from baseline $\pm$ S.D. from four dogs in study 1. Filled circles: vehicle; open circles: LMN-NKA. Data were subjected to two-way analysis of variance followed by Bonferroni multiple comparisons test: $F(10,132) = 1.0$, $*P \leq 0.01$ compared with vehicle.

after administration of vehicle or any dose of LMN-NKA (Fig. 2).

Urination, Defecation, and Other Pharmacodynamic Effects

Intravenous Administration. Urination and defecation were the most consistent and prominent effects of a single intravenous administration of LMN-NKA, and their incidence increased in a dose-related manner that was similar in males and females. While urination and defecation were not seen after injection of vehicle or 1 µg/kg, urination was almost always elicited after 10 or 100 µg/kg and was accompanied by defecation on about one-half of those occasions after 100 µg/kg (Table 2). Salivation was the next most frequently observed effect of LMN-NKA at 100 µg/kg followed by emesis and occasionally lacrimation at lower rates that did not reach statistical significance (Table 2). After intravenous injection of LMN-NKA (10 or 100 µg/kg), the mean latencies to the first urination and defecation were 3.5 ± 8.4 and 3.8 ± 5.1 minutes, respectively. Other pharmacodynamic effects also had a rapid

onset (2–5 minutes after dosing), and all were short lasting, with behavior returning to normal within 10 minutes of dosing.

In an exploratory, multiple dose study in which four groups of two dogs (male and female) received vehicle and 1, 10, or 30 µg/kg, i.v., of LMN-NKA four times per day with 4 hours between doses for five consecutive days, for a total of 40 injections per treatment group, similar rates of urination and defecation were observed (Table 3). Doses of 10 and 30 µg/kg consistently elicited urination and defecation after almost every dose throughout the study. The latency was not formally documented in the repeated dose studies, but appeared similar to that after a single dose. Salivation was the next most frequently observed effect of the highest dose of LMN-NKA, followed by emesis and lacrimation. The response rates for urination and defecation, as well as adverse events, remained consistent across days 1–5.

In a larger, repeated dose study to determine the maximum tolerated dose for chronic administration, vehicle or LMN-NKA

Fig. 2. Effect of single intravenous injection of LMN-NKA (1, 10, or 100 µg/kg) on heart rate (HR). Values are the mean percentage change in HR from baseline $\pm$ S.D. from four dogs in study 1. Filled circles: vehicle; open circles: LMN-NKA. Data were subjected to two-way analysis of variance followed by Bonferroni multiple comparisons test. $F(10,132) = 2.5$, $*P < 0.05$ compared with vehicle.

TABLE 2

Incidence of urination, defecation, emesis, lacrimation, and salivation after single intravenous injection of LMN-NKA (1–100 $\mu\text{g}/\text{kg}$)

Values are the number of observations from 16 to 20 dogs per group from studies 1–4. Equal numbers of male and female dogs were examined and the data were pooled since no difference between sexes was apparent. Animals were observed continuously for 10 minutes after intravenous injection of LMN-NKA.

Treatment and Dose $\mu\text{g}/\text{kg}$, i.v.	Response Rate (Number of Responders/ <i>N</i> per Group) and Percentage (%)				
	Urination	Defecation	Retching and Emesis	Lacrimation	Salivation
Vehicle	0/16 (0)	0/16 (0)	0/16 (0)	0/16 (0)	0/16 (0)
1	0/16 (0)	0/16 (0)	0/16 (0)	0/16 (0)	0/16 (0)
10	18/20 (90.0)*	7/20 (35.0)	2/20 (10.0)	0/20 (0)	0/20 (0)
100	18/18 (100)*	9/18 (50.0)*	3/18 (16.7)	1/18 (5.5)	6/18 (33.3)

* $P \leq 0.01$ compared with vehicle, Fisher's exact test.

(1, 10, or 100 $\mu\text{g}/\text{kg}$, i.v.) was administered four times daily for seven consecutive days to groups of 10 dogs (five of each sex). On day 1, injections were initially given 30 minutes apart, but this dosing interval was poorly tolerated in dogs receiving 10 or 100 $\mu\text{g}/\text{kg}$; on the second to fourth doses, these animals often strained and vocalized but did not urinate or defecate. Accordingly, on days 2–7, injections were given 4 hours apart for a total of 240 doses (4/day per dog $\times$ 10 dogs $\times$ 6 days). As in the previous study, urination and defecation were elicited after almost every injection of 10 $\mu\text{g}/\text{kg}$ in both sexes; this dose was generally well tolerated, with occasional episodes of emesis (Table 4). When given four times daily, 100 $\mu\text{g}/\text{kg}$ was also tolerable and again elicited urination and defecation after almost every dose; however, the incidence of adverse effects was higher, with emesis or salivation occurring after $\sim 23\%$ of injections. Urination and defecation response rates remained consistent from day 2 to day 7. Straining to defecate and vocalization was seen in 8% of doses, and reddening of the skin on the ears and muzzle was noted in two dogs after one injection. These events were distributed throughout the course of the study.

Subcutaneous Administration. LMN-NKA also caused a dose-related increase in the incidence of urination and defecation after subcutaneous administration to male and female dogs (Table 5). After a single subcutaneous injection of vehicle or LMN-NKA (3 or 10 $\mu\text{g}/\text{kg}$), no pharmacodynamic effects were observed. At 30 $\mu\text{g}/\text{kg}$, urination was seen after almost 70% of injections, while defecation occurred at a lower rate (25%) and was accompanied by diarrhea in two dogs; emesis was observed after 50% of injections. After all injections of 100 $\mu\text{g}/\text{kg}$, urination and defecation were seen, usually

with emesis ($>70\%$ of occasions). All doses of 300 $\mu\text{g}/\text{kg}$ caused urination, defecation, and emesis (Table 5); two out of eight dogs strained to defecate and vocalized, and one of these also exhibited flushing of the ears. Lacrimation and salivation were not observed after subcutaneous administration of LMN-NKA.

Urination, defecation, and emesis generally occurred within 10 minutes of subcutaneous administration of LMN-NKA (30, 100, and 300 $\mu\text{g}/\text{kg}$) with latencies to the first event of 5.6 ± 6.0 , 6.3 ± 6.9 and 8.8 ± 7.8 minutes, respectively. Recovery from the pharmacodynamic effects of 30 $\mu\text{g}/\text{kg}$ LMN-NKA was complete after 30 minutes, while after 300 $\mu\text{g}/\text{kg}$, the effects were generally not complete until 60 minutes postdose. In this study, the volume of urine voided was measured after administration of the 30 $\mu\text{g}/\text{kg}$ dose and ranged from 0 to 58 ml with a mean of 15.64 ± 14.14 ml ($N = 14$). The volume of urine in the dogs' bladder prior to dosing was not ascertained.

A multiple dose tolerability study was initiated with the intention of administering 300 $\mu\text{g}/\text{kg}$, s.c., of LMN-NKA twice daily for 7 days; however, after the first day the dose was lowered to 30 $\mu\text{g}/\text{kg}$ on days 2–6 because of the high rate of emesis at the higher dose. On the last day, the dose was again increased to examine sensitization or desensitization on efficacy or side effects after 5 days of repeated dosing. Thus, two groups of six dogs received twice daily subcutaneous injections of 300 $\mu\text{g}/\text{kg}$ of LMN-NKA on days 1 and 7 (total number of doses = 24) and 30 $\mu\text{g}/\text{kg}$ twice daily on days 2–6 (total number of doses = 60). The first group received LMN-NKA only; the second group was pretreated with the NK1R antagonist CP-99,994 (1 mg/kg, s.c.) 30 minutes before each administration of LMN-NKA.

TABLE 3

Incidence of urination, defecation, emesis, lacrimation, and salivation after repeated intravenous injection of LMN-NKA (1–30 $\mu\text{g}/\text{kg}$) four times daily for 5 days

Values are the total number of observations from two dogs per group (one of each sex) over 5 days of treatment in study 3. Animals were observed for 20 minutes after each dose.

Treatment and Dose $\mu\text{g}/\text{kg}$, i.v.	Response Rate (Number of Responses/Number of Injections) and Percentage (5%)				
	Urination	Defecation	Retching and Emesis	Lacrimation	Salivation
Vehicle	0/40 (0)	0/40 (0)	0/40 (0)	0/40 (0)	0/40 (0)
1	2/40 (5.0)	3/40 (7.5)	0/40 (0)	0/40 (0)	0/40 (0)
10	39/40 (97.5)*	33/40 (82.5)*	1/40 (2.5)	2/40 (5.0)	5/40 (12.5)
30	40/40 (100)*	39/40 (97.5)*	11/40 (27.5)*	9/40 (22.5)*	17/40 (42.5)*

* $P \leq 0.01$ compared with vehicle, Fisher's exact test.

TABLE 4

Incidence of urination, defecation, emesis, lacrimation, and salivation after repeated intravenous injection of LMN-NKA (1–100 $\mu\text{g}/\text{kg}$) four times daily for 6 days

Values are the total number of observations from 10 dogs per group over days 2–7 of treatment in study 4. Equal numbers of male and female dogs were examined and the data were pooled since no difference between sexes was apparent. Animals were observed for 20 minutes after each dose.

Treatment and Dose $\mu\text{g}/\text{kg}$, i.v.	Response Rate (Number of Responses/Number of Injections) and Percentage (%)				
	Urination	Defecation	Retching and Emesis	Lacrimation	Salivation
Vehicle	0/240 (0)	0/240 (0)	1/240 (0)	0/240 (0)	0/240 (0)
1	10/240* (4.2)	5/240 (2.1)	0/240 (0)	0/240 (0)	0/240 (0)
10	227/240* (94.6)	183/240* (76.3)	12/240* (5.0)	0/240 (0)	0/240 (0)
100	230/240* (95.8)	183/240* (76.3)	55/240* (22.9)	2/240 (0.8)	53/240* (22.1)

* $P \leq 0.01$ compared with vehicle, Fisher's exact test.

After injections of 300 $\mu\text{g}/\text{kg}$, s.c., urination and defecation occurred after almost every dose and typically had a rapid onset (within 5 minutes) and were usually accompanied by emesis. On days 2–6, twice daily administration of 30 $\mu\text{g}/\text{kg}$ LMN-NKA elicited urination and defecation after most injections and caused emesis on 58% of occasions (Table 6). Full recovery from these effects of LMN-NKA was complete within ~45 minutes after each dose.

In the second group of dogs, on days 1 and 7 pretreatment with CP-99,994 (30 minutes before 300 $\mu\text{g}/\text{kg}$ of LMN-NKA) had no effect on the rate of urination or defecation but reduced the incidence of emesis after each dose from 96% to 54%. Similarly, on days 2–6, pretreatment with CP-99,994 had no effect on urination or defecation but markedly reduced the rate of emesis from 58% to 8% (Table 6).

Pharmacokinetics

Plasma samples were obtained at seven time points per dog from 0.5 to 20 minutes after intravenous injection of 100 $\mu\text{g}/\text{kg}$ of LMN-NKA (study 2). Levels of LMN-NKA were highest at the first sampling time point and fell rapidly after 5 minutes. The calculated pharmacokinetic parameters were as follows: $C_{\text{max}} = 1040 \pm 185$ ng/ml, half-life ($t_{1/2}$) = 2.8 ± 1.1 minutes, and $\text{AUC}_{0-\text{last}} = 1849 \pm 218$ ng/ml·min (mean $\pm$ S.D. from $N = 4$; Fig. 3), where AUC denotes the area under the curve. Analysis of six plasma samples per dog collected from 2 to 60 minutes after subcutaneous administration of the same dose (study 7) indicated a 40-fold lower peak plasma exposure, with a C_{max} of 26.1 ± 12.0 ng/ml, $t_{1/2} = 15.9 \pm 6.4$ minutes,

and $\text{AUC}_{0-\text{last}} = 666 \pm 168$ ng/ml·min ($N = 10$; Fig. 3). Bioavailability, based on the ratio of $\text{AUC}_{0-\text{last}}$ values, was estimated at 64%.

Discussion

The present studies demonstrate that LMN-NKA can elicit highly efficient urinary and fecal voiding a few minutes after intravenous or subcutaneous administration in conscious animals. A single dose of 10, 30, or 100 $\mu\text{g}/\text{kg}$, i.v., elicited urination in >90% and defecation in >35% of dogs within 4 minutes of injection, and a single subcutaneous injection of 100 or 300 $\mu\text{g}/\text{kg}$ caused urination and defecation in all dogs in under 7 minutes. These results are especially encouraging because no attempt was made to ensure that there was urine in the bladder or stools in the rectum prior to dose administration. The voided amounts of urine and feces appeared typical for dogs, and this was confirmed by the measurement of urine volume in one study (~16 ml). The ability of LMN-NKA to reliably elicit urination and defecation in conscious animals is a significant extension of findings using anesthetized preparations that showed elevations in intravesical and colorectal pressure (Kullmann et al., 2017; Marson et al., 2018; Rupniak et al., 2018). In both anesthetized (Rupniak et al., 2018) and conscious dogs (present studies), LMN-NKA-induced micturition was an all-or-none response rather than a graded, dose-related, effect. The high incidence of urination and defecation after administration of LMN-NKA demonstrates that contraction of the bladder and colorectal smooth muscle

TABLE 5

Incidence of urination, defecation, and emesis after single subcutaneous injection of LMN-NKA (3–300 $\mu\text{g}/\text{kg}$)

Values are the number of observations from three to 16 dogs per group from studies 2, 5, and 6. Equal numbers of male and female dogs were examined and the data were pooled since no difference between sexes was apparent. Animals were observed for 30–60 minutes after dosing.

Treatment and Dose $\mu\text{g}/\text{kg}$, s.c.	Response Rate (Number of Responses/Number of Injections) and Percentage (%)		
	Urination	Defecation	Retching and Emesis
Vehicle	0/12 (0)	0/12 (0)	0/12 (0)
3	0/3 (0)	0/3 (0)	0/3 (0)
10	0/9 (0)	0/9 (0)	0/9 (0)
30	11/16 (68.8)*	4/16 (25.0)	8/16 (50.0)*
100	14/14 (100)*	14/14 (100)*	10/14 (71.4)*
300	8/8 (100)*	8/8 (100)*	8/8 (100)*

* $P \leq 0.01$ compared with vehicle, Fisher's exact test.

TABLE 6

Incidence of urination, defecation, and emesis after repeated subcutaneous injection of LMN-NKA alone or after CP-99,994 twice daily for 7 days

Values are the number of observations from six dogs per group from study 6. Equal numbers of male and female dogs were examined and the data were pooled since no difference between sexes was apparent. Separate groups received LMN-NKA alone or LMN-NKA 30 minutes after CP-99,994 (1 mg/kg, s.c.). Animals were observed for at least 30 minutes after each dose.

Dose of LMN-NKA (twice daily)	Response Rate (Number of Responses/Number of Injections) and Percentage (%)					
	Urination		Defecation		Retching and Emesis	
	Alone	After CP-99,994	Alone	After CP-99,994	Alone	After CP-99,994
30 $\mu\text{g/kg}$ (days 2–6)	53/60 (88.3)	58/60 (96.7)	50/60 (83.3)	54/60 (90.0)	35/60 (58.3)	5/60* (8.3)
300 $\mu\text{g/kg}$ (days 1 and 7)	24/24 (100)	23/24 (96)	24/24 (100)	24/24 (100)	23/24 (95.8)	13/24* (54.2)

* $P \leq 0.01$ compared with LMN-NKA alone, Fisher's exact test.

by LMN-NKA is not obstructed by simultaneous contraction of the urethral or anal sphincter smooth muscles such as to cause dyssynergia.

To be suitable for on-demand use by people with impaired voiding, pharmacotherapy would require the rapid onset of action observed here and in previous studies with LMN-NKA (Kullmann et al., 2017; Marson et al., 2018; Rupniak et al., 2018), and its effects would also need to be complete within ~10 minutes of administration, with no residual carryover. The duration of action of LMN-NKA after intravenous administration in dogs appears to be consistent with the desired profile since voiding was complete by 10 minutes. However, after subcutaneous administration, LMN-NKA-induced voiding was more prolonged and behavioral evidence of continued effects was seen for 30–60 minutes after administration, depending on the dose. The more prolonged duration of action of LMN-NKA after subcutaneous dosing was undesirable for therapeutic application and was also associated with instances of delayed straining, loose stools, or diarrhea after the highest dose. The difference in the time course of pharmacodynamic effects was correlated with the plasma

profiles, since the plasma concentration peaked at 0.5 minutes and declined rapidly 5 minutes after intravenous injection, whereas the peak and decline of plasma concentrations was much lower and slower after subcutaneous administration, presumably due to delayed and prolonged absorption. Preliminary studies using prototype intranasal and sublingual formulations indicate that plasma exposures associated with prokinetic activity can be achieved using alternative routes of delivery that are more convenient for repeated use in a clinical setting (Bae et al., 2018; Marson et al., 2018).

An important extension from previous studies using anesthetized animals was the demonstration that LMN-NKA-induced urinary and fecal voiding is maintained consistently with repeated intravenous and subcutaneous dosing given multiple times daily on consecutive days. After intravenous administration every 4 hours, four times daily over six consecutive days, the effects of LMN-NKA were maximal after 10 $\mu\text{g/kg}$, with urination in 95% and defecation in 76% of doses. At this dose, LMN-NKA was well tolerated with low rates of emesis and no other observable adverse effects. After 100 $\mu\text{g/kg}$, the same high level of voiding was accompanied by emesis and salivation in ~22% of animals, and occasionally by lacrimation.

Despite the overall similarity in the effects produced by intravenous and subcutaneous administration of LMN-NKA, there were also some apparent differences. While single or repeated intravenous administration consistently resulted in a higher rate of urination than defecation, the rates for both were similar after subcutaneous administration. Furthermore, the rate of emesis was higher after subcutaneous than after intravenous dosing. Twice daily subcutaneous administration over 7 days of LMN-NKA (30 or 300 $\mu\text{g/kg}$) reliably elicited urination and defecation after $\geq 83\%$ of injections. While the incidence of voiding was only marginally higher when increasing the dose from 30 to 300 $\mu\text{g/kg}$, the rate of emesis almost doubled (from 58% to 96%). Also, unlike intravenous administration, salivation was not seen after subcutaneous dosing. Salivary glands express predominantly NK1 tachykinin receptors (Buck and Burcher, 1985), and salivation signals activation of the autonomic nervous system and is often a prequel to emesis (Horn et al., 2014). When present, vomitus in the present studies often had the appearance of a clear, frothy discharge, possibly due to sialorrhea and other gastric secretions. Thus, the presence of salivation in animals with low rates of vomiting after intravenous dosing may reflect a shorter duration of activation of the emetic pathway than was apparent after subcutaneous injection of LMN-NKA. It is also possible that vomiting

Fig. 3. Plasma concentrations following intravenous or subcutaneous administration of 100 $\mu\text{g/kg}$ LMN-NKA. Values are the mean $\pm$ S.D. from four (intravenous) and 10 (subcutaneous) dogs per group from studies 2 and 7, respectively. Open circles = intravenous; filled circles = subcutaneous. The lower limit of quantification was 1 ng/mL.

after subcutaneous dosing obscured the presence of salivation. The different pharmacodynamic and plasma profiles indicate that the duration of exposure to LMN-NKA is more prolonged after subcutaneous than intravenous injection, and hence the prolonged activation of NK1Rs, especially in the gut, after subcutaneous administration may cause emesis.

Emesis, salivation, and lacrimation are commonly associated with NK1R activation in vivo (Snider et al, 1991; Rupniak and Williams, 1994; Darmani et al., 2008) but were not detected previously with LMN-NKA in anesthetized dogs (Rupniak et al., 2018). In the present studies, the selective NK1R antagonist CP-99,994 markedly inhibited emesis (but not urinary or fecal voiding) induced by subcutaneous injection of LMN-NKA; conversely, the ability of LMN-NKA to increase bladder and colorectal pressure was blocked by the NK2R antagonist GR 159897 in anesthetized dogs (Rupniak et al., 2018), indicating that distinct neurokinin receptors are responsible for mediating the emetic and colorectal effects of LMN-NKA. However, the ability of LMN-NKA to activate NK1Rs to cause emesis in the same dose range that elicits voiding is puzzling given its >100-fold selectivity for NK2 over NK1Rs in vitro (submitted manuscript). Emesis is a complex reflex involving gastrointestinal, respiratory, cardiovascular, and central nervous system pathways, and activation of NK1Rs in both the periphery and the central nervous system can cause emesis (Darmani et al., 2008). The higher incidence of emesis after subcutaneous rather than intravenous dosing of LMN-NKA in dogs suggests that activation of NK1Rs in the periphery is primarily responsible for vomiting since the peak plasma exposure, which is a key determinant of central nervous system penetration, was far lower after subcutaneous than intravenous dosing. A likely mechanism for LMN-NKA induced emesis is activation of gastrointestinal vagal afferent pathways via NK1 receptors on enteric neurons and/or enterochromaffin cells (see Darmani et al., 2008). Since there are marked species differences in susceptibility to different emetogens, with dogs being more sensitive to certain pharmacological classes than primates (Legeza et al., 1982), the present findings may not be predictive of an emetic effect of NK2R agonists in human subjects. Indeed, LMN-NKA was administered subcutaneously to macaques twice daily for 7 days at doses 10-fold higher than those given to dogs, and emesis was almost never observed despite achieving similar plasma concentrations (10–40 ng/ml); blood pressure was not monitored in this study and hypoactivity was not noted (unpublished observations). However, the more subjective sensation of nausea is not readily assessed in animals and should be monitored in future clinical trials with NK2R agonists.

In studies using anesthetized dogs, LMN-NKA caused NK1R-mediated hypotension elicited at doses as low as 0.1 µg/kg, i.v., an effect that was not seen after subcutaneous administration (Rupniak et al., 2018). In the present studies, although hypotension was again observed after intravenous injection of LMN-NKA in conscious dogs, it was significant only after a much higher dose of 100 µg/kg. Similarly, in rats anesthetized with urethane, hypotension was only observed after 100 µg/kg, i.v., of LMN-NKA (Marson et al., 2018). Therefore, there appears to be a pharmacodynamic interaction between LMN-NKA and gaseous (isoflurane) anesthesia in dogs that dramatically alters the threshold for activation of NK1Rs involved in the regulation of blood pressure. The

mechanism responsible for this interaction is unclear. Acute infusion of substance P, the endogenous neuropeptide for NK1R, is known to cause hypotension in conscious dogs, and this effect is more pronounced after ganglionic blockade (Nakamura et al., 1991). It may be speculated that the ability of volatile anesthetics to depress the autonomic nervous system could lower the threshold for hypotension induced by LMN-NKA through a similar mechanism.

The ability of NK2R agonists to elicit on-demand voiding could potentially address a major unmet need in people who lack voluntary control of bladder and bowel function. In dogs, LMN-NKA unexpectedly activated NK1Rs at doses that stimulated voiding, causing emesis and hypotension. It is not known whether similar effects would be seen at therapeutic doses in humans; however, agonists with greater selectivity for NK2 receptors, and a correspondingly improved tolerability profile, should be pursued for future clinical development. There remains a possibility, not examined in the present study, that highly selective NK2R agonists may cause constriction of respiratory smooth muscle that could limit their clinical use in certain populations. In clinical studies employing intravenous infusion of the endogenous agonist NKA, no respiratory effects were reported at doses that elicited gastrointestinal effects (Lördal et al., 1997, 2001; Schmidt et al., 2003). However, with direct pulmonary exposure, some individuals may be susceptible to bronchoconstriction. Whereas in healthy human subjects, inhalation of NKA did not alter respiratory function, it increased airway resistance in asthmatic subjects, and this appeared to be NK2 (rather than NK1) receptor mediated (Joos et al., 1987). Careful assessment of the tolerability of selective NK2R agonists at the doses required for prokinetic activity in human subjects will be required during clinical development.

Authorship Contributions

Participated in research design: Katofiasc, Walz, Thor, Burgard.

Conducted experiments: Katofiasc.

Performed data analysis: Rupniak, Katofiasc, Walz, Thor, Burgard.

Wrote or contributed to the writing of the manuscript: Rupniak, Katofiasc, Walz, Thor, Burgard.

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