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[Lys^{5},MeLeu^{9},Nle^{10}]-NKA$_{(4–10)}$ Elicits NK2 Receptor Mediated Micturition and Defecation, and NK1 Receptor Mediated Emesis and Hypotension, in Conscious Dogs

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Running Title: Voiding Elicited by [Lys^5,MeLeu^9,Nle^{10}]-NKA$_{(4-10)}$ in Dogs

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Nonstandard abbreviations: [Lys^5,MeLeu^9,Nle^{10}]-NKA$_{(4–10)}$ (LMN-NKA)

(2S,3S)-N-(2-methoxybenzyl)-2-phenylpiperidin-3-amine; (2S,3S)-N-[(2-methoxyphenyl)methyl]-2-phenylpiperidin-3-amine (CP-99,994)

Neurokinin 2 receptor (NK2R)

Neurokinin 1 receptor (NK1R)

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Abstract

Tachykinin NK2 receptor agonists may have potential to alleviate clinical conditions associated with bladder and gastrointestinal under-activity by stimulating contraction of visceral smooth muscle. The ability of [Lys^5,$MeLeu^9$,Nle^{10}]-$NKA_{(4–10)}$ (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., 4 times daily for 6 consecutive days, reliably elicited micturition after ≥90% of doses and defecation after ≥50% of doses. Voiding occurred <4 min after dosing and was short-lasting (<10 min). 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 7 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 min) but was more prolonged than after IV administration (30-60 min). 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 min pretreatment with the NK1 receptor antagonist, 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.

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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 neurological 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; Warner et al, 2003; Burcher et al, 2008), the potenial to exploit this prokinetic activity therapeutically has not been explored until recently. [Lys^5,$MeLeu^9$,Nle^{10}]-$NKA_{(4-10)}$ (LMN-NKA) was employed as a prototype NK2R agonist based on its >700-fold higher affinity for human cloned NK2 over NK1 receptors in radioligand binding assays, and >100-fold selectivity for NK2Rs than NK1Rs using intracellular calcium mobilization as an in vitro functional assay (unpublished observations). 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 min) and short in duration (3 – 10 min) at efficacious doses, depending on the route of administration (i.v. or s.c). Furthermore, the prokinetic effects were blocked by pretreatment with the NK2R antagonist, GR 159897, but not by the NK1R antagonists, spantide I or 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 NKA, the endogenous agonist for the NK2R, undergoes tachyphylaxis in some in vitro preparations (Daniel et al, 1989; Patacchini et al, 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 i.v. 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 s.c. administration while maintaining the prokinetic effect. The presence of hypotension after such low i.v. doses of LMN-NKA in anesthetized dogs was surprising since it was not seen after i.v. injection of prokinetic doses of LMN-NKA in anesthetized rats (Kullmann et al, 2017; Marson et al, 2018) or i.v. infusion of prokinetic doses of NKA in conscious humans (Evans et al, 1988; Lordal 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 i.v. 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.

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Materials and Methods

Animals

Unless otherwise stated, naïve dogs were housed in pairs in runs of up to 28 ft^2 that were divided during test sessions to create individual cages measuring 8.1-9.1 ft^2. Experiments conformed to IACUC regulations, the Guidelines for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of Calvert Laboratories Inc. (Scott Township, PA), Synchrony Labs (Durham, NC), and MPI Research (Mattawan, MI). For studies conducted at Calvert (Studies 1-4) and MPI (Study 7), water and food were available ad libitum. For studies conducted at Synchrony (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 N. The total number of animals was based on regulatory and IACUC 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, USA).

Cardiovascular Parameters

Arterial blood pressure and heart rate were monitored in 4 non-naïve male dogs that had previously been implanted with telemetry probes following a drug washout period of at least 30 days since the last test compound. At least 24 h prior to surgery, a fentanyl transdermal patch (Duragesic, Janssen Pharmaceutica, Beerse, Belgium; 2.5 mg for dogs under 10 kg, 5 mg for dogs over 10 kg) was applied for continuous analgesia. On the day of surgery, dogs received atropine (Vedco Inc., St Joseph MO; 0.02-0.3 mg/kg

i.m.) and induction anesthesia with telazol (Zoetis, Parsippany, NJ; 2.2-4.4 mg/kg i.v.). Following endotracheal intubation, anesthesia was maintained by inhalation of isoflurane. The antibiotic cefazolin (Kefzol, Pfizer, NY; 20-25 mg/kg) was administered by i.v. infusion. An incision was made extending ~4 in from the dorsal scapula to the 5th rib, and another incision was made in the abdomen just below the diaphragm through which the radiotelemetry 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 the pressure and ECG sensors were routed subcutaneously to the thorax, and the transmission antenna was routed subcutaneously towards 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 6th 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, Zoetis; 4.4 mg/kg s.c.) and an antibiotic (Baytril, Bayer, Leverkusen, Germany; 2.5 mg/kg s.c.). Rimadyl was continued at 2.2 mg/kg p.o. twice daily for 7 days, and cephalexin continued twice daily (20-25 mg/kg p.o.) 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 h after i.v. administration of LMN-NKA (1, 10 or 100 µg/kg) or vehicle (Study 1).

Observation of Pharmacodynamic Effects

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Pharmacodynamic data were captured in 6 studies 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 1 or 2 observers monitoring 2 or 3 dogs simultaneously. On test days, dogs were gently restrained for i.v. injection of test compounds into a cephalic vein, alternating between injection sites and veins in repeat dose studies; s.c. 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 below.

The pharmacodynamic effects of a single administration of vehicle or LMN-NKA (1, 10 and 100 µg/kg i.v.) were recorded in 4 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 min, and the number of animals voiding and the latency to the first void were recorded. Events were counted only once after each dose in order to estimate incidence (i.e. responder rate). There were 2 repeat dose i.v. studies. In Study 3, 4 daily injections were given 4 h apart, for 5 consecutive days. In Study 4, injections were given 30 min apart on day 1, and 4 h apart on days 2-7. Dogs were observed for at least 20 min after each injection.

The incidence of pharmacodynamic effects after a single s.c. dose of vehicle or LMN-NKA (3, 10, 30 or 100 µg/kg) was determined by combining data from 3 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 ft^2) for at least 3 days prior to study. In this study, dogs were observed in the metabolism cage for up to 60 min, and the

volume of voided urine was recorded. However, since water was available ad libitum, the pre-dose 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 6 naïve dogs (3 of each sex) received twice daily doses of 300 µg/kg s.c. LMN-NKA on days 1 and 7, and twice daily doses of 30 µg/kg s.c. on days 2-6. A separate group of 6 dogs received the same treatment but were pretreated with the selective NK1R antagonist CP-99,994 (1 mg/kg s.c.) 30 min before each dose of LMN-NKA (30 or 300 µg/kg s.c.) on days 1 through 7. Animals were observed for 30 min after each dose of LMN-NKA.

Pharmacokinetics

In Studies 2 and 7, 6 or 7 blood samples, respectively, (~2 ml) were collected from the jugular vein of 4 non-naïve dogs prior to, and at 0.5, 1, 2, 4, 6, 10 and 20 min after i.v., and at 2, 5, 10, 20, 40, and 60 min after s.c., administration of 100 µg/kg of LMN-NKA. Samples were collected in tubes containing EDTA anticoagulant (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), 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 min of collection at 2-3,000 r.p.m. for ~10 min 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 (LC/MS/MS). Concentrations of LMN-NKA were calculated with a $1/x^2$ linear regression over a qualified concentration range of 1-1,000 ng/mL using [^{13}C,^{15}N]-substituted LMN-NKA as an internal standard. An API 5000 platform was operated under optimized conditions for

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detection of LMN-NKA positive ions formed by electrospray ionization.

Preparation of Test Compounds

LMN-NKA (Asp-Lys-Phe-Val-Gly-Leu(NMe)-Nle-NH_2) was manufactured using solid phase synthesis (Bachem, Torrance, CA, USA) to a purity ≥95%. Stock solutions (10 mg/ml) of LMN-NKA and the NK1R antagonist CP-99,994 ((2S,3S)-N-(2-methoxybenzyl)-2-phenylpiperidin-3-amine; (2S,3S)-N-[(2-methoxyphenyl)methyl]-2-phenylpiperidin-3-amine; Monomerchem, Durham, NC, USA) 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 min time bins and expressed as a percentage of the mean baseline value (recorded for 15 min prior to dosing) using Excel (Microsoft, Redmond, WA, USA) and Prism 6 (GraphPad Software Inc., San Diego, CA). Means were compared to vehicle control values using a 2-way ANOVA followed by a Bonferroni Multiple Comparison Test (Prism 7, GraphPad Software, La Jolla, CA). Differences with p values ≤0.05 were considered statistically significant. Values are expressed as the mean + 1 standard deviation (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 or doses versus control group were performed using Fisher's exact test using an adjusted

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alpha level (0.01) to account for multiple comparisons. PK data were collected using Analyst (MDS Sciex, Framingham, MA, USA) and parameters calculated using Phoenix WinNonlin 6.3 (Certara, Princeton, NJ, USA).

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Results

Cardiovascular Parameters

The cardiovascular effects of single i.v. injections of LMN-NKA were monitored in 4 male dogs with implanted radio telemetry probes. During the 15 min pretreatment baseline periods, mean arterial blood pressure (MAP) was 132.13 ± 11.15 mmHg (mean ± S.D.). MAP remained stable during the first 10 min after i.v. injection of vehicle or 1 μg/kg LMN-NKA. Blood pressure was highly variable after 10 μg/kg, with no consistent change across animals. After 100 μg/kg of LMN-NKA, hypotension was reliably seen in all dogs compared to administration of vehicle. Mean MAP fell up to 32 mmHg below baseline 3-5 min after dosing (Figure 1). Blood pressure returned to baseline levels within 10 min.

During the 15 min pretreatment baseline periods, mean heart rate was 102.53 ± 10.13 b.p.m (mean ± S.D.). Heart rate was generally stable during the 10 min post-injection period and there was no consistent change after administration of vehicle or any dose of LMN-NKA (Figure 2).

Urination, Defecation and Other Pharmacodynamic Effects

i.v. Administration. Urination and defecation were the most consistent and prominent effects of a single i.v. 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 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 i.v.

injection of LMN-NKA (10 or 100 µg/kg), the mean latency to the first urination and defecation was 3.5 ± 8.4 and 3.8 ± 5.1 min, respectively. Other pharmacodynamic effects also had a rapid onset (2-5 min after dosing), and all were short lasting, with behavior returning to normal within 10 min of dosing.

In an exploratory, multiple dose study in which 4 groups of 2 dogs (male and female) received vehicle, 1, 10 or 30 µg/kg i.v. of LMN-NKA 4 times per day with 4 hours between doses for 5 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 through day 5.

In a larger, repeated dose study to determine the maximum tolerated dose for chronic administration, vehicle or LMN-NKA (1, 10 or 100 µg/kg i.v.) was administered 4 times daily for 7 consecutive days to groups of 10 dogs (5 of each sex). On day 1, injections were initially given 30 min apart, but this dosing interval was poorly tolerated in dogs receiving 10 or 100 µg/kg; on the second to fourth dose, these animals often strained and vocalized but did not urinate or defecate. Accordingly, on days 2-7, injections were given 4 h apart, for a total of 240 doses (4/day per dog X 10 dogs X 6 days). As in the previous study, urination and defecation were elicited after almost every injection of 10 µg/kg in both sexes; this dose was generally well tolerated, with occasional episodes of emesis (Table 4). 100 µg/kg was also tolerable when given 4

times daily, and again elicited urination and defecation after almost every dose, but the incidence of adverse effects was higher, with emesis or salivation occurring after ~23% of injections. Urination and defecation response rates remained consistent from day 2 through day 7. Straining to defecate and vocalization was seen on 8% of doses, and reddening of the skin on the ears and muzzle was noted in 2 dogs after one injection. These events were distributed throughout the course of the study.

s.c. Administration. LMN-NKA also caused a dose-related increase in the incidence of urination and defecation after s.c. administration to male and female dogs (Table 5). After a single s.c. injection of vehicle, 3 or 10 µg/kg of LMN-NKA, no pharmacodynamic effects were observed. At 30 µg/kg, urination was seen after almost 70% of injections, while defecation occurred at a lower rate (25%) and was accompanied by diarrhea in 2 dogs; emesis was observed after 50% of injections. After all injections of 100 µg/kg, urination and defecation were seen, usually with emesis (>70% of occasions). All doses of 300 µg/kg caused urination, defecation, and emesis (Table 5); 2 out of 8 dogs strained to defecate and vocalized, and 1 of these also exhibited flushing of the ears. Lacrimation and salivation were not observed after s.c. administration of LMN-NKA.

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

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A multiple dose tolerability study was initiated with the intention of administering 300 µg/kg s.c. of LMN-NKA twice daily for 7 days, however, after the first day the dose was lowered to 30 µg/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, 2 groups of 6 dogs received twice daily s.c. injections of 300 µg/kg of LMN-NKA on days 1 and 7 (total number of doses = 24), and 30 µg/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 NK1R antagonist, CP-99,994 (1 mg/kg s.c.) 30 min before each administration of LMN-NKA.

After injections of 300 µg/kg s.c., urination and defecation occurred after almost every dose and typically had a rapid onset (within 5 min) and were usually accompanied by emesis. On days 2-6, twice daily administration of 30 µg/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 min after each dose.

In the second group of dogs, on days 1 and 7, pretreatment with CP-99,994 (30 min before 300 µg/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

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Plasma samples were obtained at 7 time points per dog from 0.5 - 20 min after i.v. injection of 100 µg/kg of LMN-NKA (Study 2). Levels of LMN-NKA were highest at the first sampling time point and fell rapidly after 5 min. Calculated PK parameters were as follows: C_{max} = 1040 ± 185 ng/ml, half-life ($t_{1/2}$) = 2.8 ± 1.1 min, and $AUC_{0\text{-last}}$ = 1849 ± 218 ng/ml*min (mean ± S.D. from *N*=4; Figure 3). Analysis of 6 plasma samples per dog collected from 2 - 60 min after s.c. 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 ± 6.4 min, and $AUC_{0\text{-last}}$ = 666 ± 168 ng/ml*min (*N*=10; Figure 3). Bioavailability, based on the ratio of $AUC_{0\text{-last}}$ values, was estimated at 64%.

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Discussion

The present studies demonstrate that LMN-NKA can elicit highly efficient urinary and fecal voiding a few minutes after i.v. or s.c. administration in conscious animals. A single dose of 10, 30 or 100 µg/kg i.v. elicited urination in >90% and defecation in >35% of dogs within 4 min of injection, and a single s.c. injection of 100 or 300 µg/kg caused urination and defecation in all dogs in under 7 min. 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 by LMN-NKA is not obstructed by simultaneous contraction of the urethral or anal sphincter smooth muscles so as to cause dyssynergia.

In order to be suitable for ‘on demand’ use by people with impaired voiding, a 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 min of administration, with no residual carry-over. The duration of action of LMN-NKA after i.v. administration in dogs

appears to be consistent with the desired profile since voiding was complete by 10 min. However, after s.c. administration, LMN-NKA-induced voiding was more prolonged, and behavioral evidence of continued effects were seen for 30-60 min after administration, depending on the dose. The more prolonged duration of action of LMN-NKA after s.c. 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 min and declined rapidly 5 min after i.v. injection, whereas the peak and decline of plasma concentrations was much lower and slower after s.c. 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, 2017; 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 i.v. and s.c. dosing given multiple times daily on consecutive days. After i.v. administration every 4 hours, 4 times daily over 6 consecutive days, the effects of LMN-NKA were maximal after 10 μg/kg, with urination in 95% and defecation in 76% of dogs. At this dose, LMN-NKA was well tolerated with low rates of emesis and no other observable adverse effects. After 100 μg/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 i.v. and s.c. administration of LMN-NKA, there were also some apparent differences. Whereas single or repeated i.v. administration consistently resulted in a higher rate of urination than defecation, the rates for both were similar after s.c. administration. Furthermore, the rate of emesis was higher after s.c. than after i.v. dosing. Twice daily s.c. administration of LMN-NKA (30 or 300 μg/kg) over 7 days reliably elicited urination and defecation after ≥83% of injections. While the incidence of voiding was only marginally higher on increasing the dose from 30 to 300 μg/kg, the rate of emesis almost doubled (from 58% to 96%). Also, unlike i.v. administration, salivation was not seen after s.c. dosing. Salivary glands express predominantly NK1 tachykinin receptors (Buck & 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 i.v. dosing may reflect a shorter duration of activation of the emetic pathway than was apparent after s.c. injection of LMN-NKA. It is also possible that vomiting after s.c. 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 s.c. than i.v. injection, and hence the prolonged activation of NK1Rs, especially in the gut, after s.c. administration may cause emesis.

Emesis, salivation and lacrimation are commonly associated with NK1R activation in vivo (Snider at al, 1991; Rupniak & 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 s.c. 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 for publication). Emesis is a complex reflex involving gastrointestinal, respiratory, cardiovascular, and CNS pathways, and activation of NK1Rs in both the periphery and the CNS can cause emesis (Darmani et al, 2008). The higher incidence of emesis after s.c. than i.v. 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 CNS penetration, was far lower after s.c. than i.v. 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 s.c. 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 s.c.

administration (Rupniak et al, 2018). In the present studies, although hypotension was again observed after i.v. 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, 2017). 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 i.v. infusion of the endogenous agonist NKA, no respiratory effects were reported at doses that elicited gastrointestinal effects (Lordal 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 and Burgard

Conducted experiments: Katofiasc

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

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

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Legends for Figures

Figure 1. Effect of Single i.v. Injection of LMN-NKA (1, 10 or 100 µg/kg) on Arterial Blood Pressure

Values are the mean percentage change in MAP from baseline ± S.D. from 4 dogs in Study 1. Filled circles: vehicle; open circles: LMN-NKA. Data were subjected to two-way ANOVA followed by Bonferroni multiple comparison test: $F(10,132) = 1.0$, $*p \leq 0.01$ compared to vehicle.

Figure 2. Effect of Single i.v. Injection of LMN-NKA (1, 10 or 100 µg/kg) on Heart Rate

Values are the mean percentage change in heart rate from baseline ± S.D. from 4 dogs in Study 1. Filled circles: vehicle; open circles: LMN-NKA. Data were subjected to two-way ANOVA followed by Bonferroni multiple comparison test. $F(10,132) = 2.5$, $*p<0.05$ compared to vehicle.

Figure 3. Plasma Concentrations Following i.v. or s.c. Administration of 100 µg/kg LMN-NKA

Values are the mean ± S.D. from 4 (i.v.) and 10 (s.c.) dogs per group from Studies 2 and 7, respectively. Open circles = i.v.; filled circles = s.c. The lower limit of quantification was 1 ng/ml.

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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 4 non-naïve 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 4 non-naïve 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 2 naïve dogs (1 of each sex)	Separate groups received each treatment up to 4 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 naïve dogs (5 of each sex)	Separate groups received each treatment up to 4 times daily for 7 days
5 (DT-076)	Single dose pharmacodynamics	Vehicle, 3, 10 or 30 μg/kg s.c.	One group of 4 naïve and 6 non-	All dogs received vehicle and 30 μg/kg; 3 dogs also received 3

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			naïve dogs (5 of each sex)	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 6 naïve dogs (3 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-naïve dogs (5 of each sex)	Separate groups received each treatment on 1 day

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Table 2. **Incidence of Urination, Defecation, Emesis, Lacrimation and Salivation after Single i.v. Injection of LMN-NKA (1-100 µg/kg)**

Treatment and Dose (µg/kg i.v.)	Response Rate (Number of responders/N 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)

Values are the number of observations from 16-20 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 min after i.v. injection of LMN-NKA. *$p \leq 0.01$ compared with vehicle, Fisher's exact test.

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Table 3. **Incidence of Urination, Defecation, Emesis, Lacrimation and Salivation after Repeated i.v. Injection of LMN-NKA (1-30 µg/kg) 4 Times Daily for 5 Days**

Treatment and Dose (µg/kg i.v.)	Response Rate (number of responses/number of injections) and Percentage (%)				
	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)*

Values are the total number of observations from 2 dogs per group (one of each sex) over 5 days of treatment in Study 3. Animals were observed for 20 min after each dose. *$p \leq 0.01$ compared with vehicle, Fisher's exact test.

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Table 4. **Incidence of Urination, Defecation, Emesis, Lacrimation and Salivation after Repeated i.v. Injection of LMN-NKA (1-100 µg/kg) 4 Times Daily for 6 Days**

Treatment and Dose (µg/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)

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 min after each dose.

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

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Table 5. Incidence of Urination, Defecation and Emesis after Single s.c. Injection of LMN-NKA (3-300 µg/kg)

Treatment and Dose (µg/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)*

Values are the number of observations from 3-16 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 min after dosing. *$p \leq 0.01$ compared with vehicle, Fisher's exact test.

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Table 6. Incidence of Urination, Defecation and Emesis after Repeated s.c. Injection of LMN-NKA Alone or After CP-99,994 Twice Daily for 7 Days

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 µg/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 µg/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)

Values are the number of observations from 6 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 min after CP-99,994 (1 mg/kg s.c.). Animals were observed for at least 30 min after each dose. *$p \leq 0.01$ compared with LMN-NKA alone, Fisher's exact test.

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Figure 1

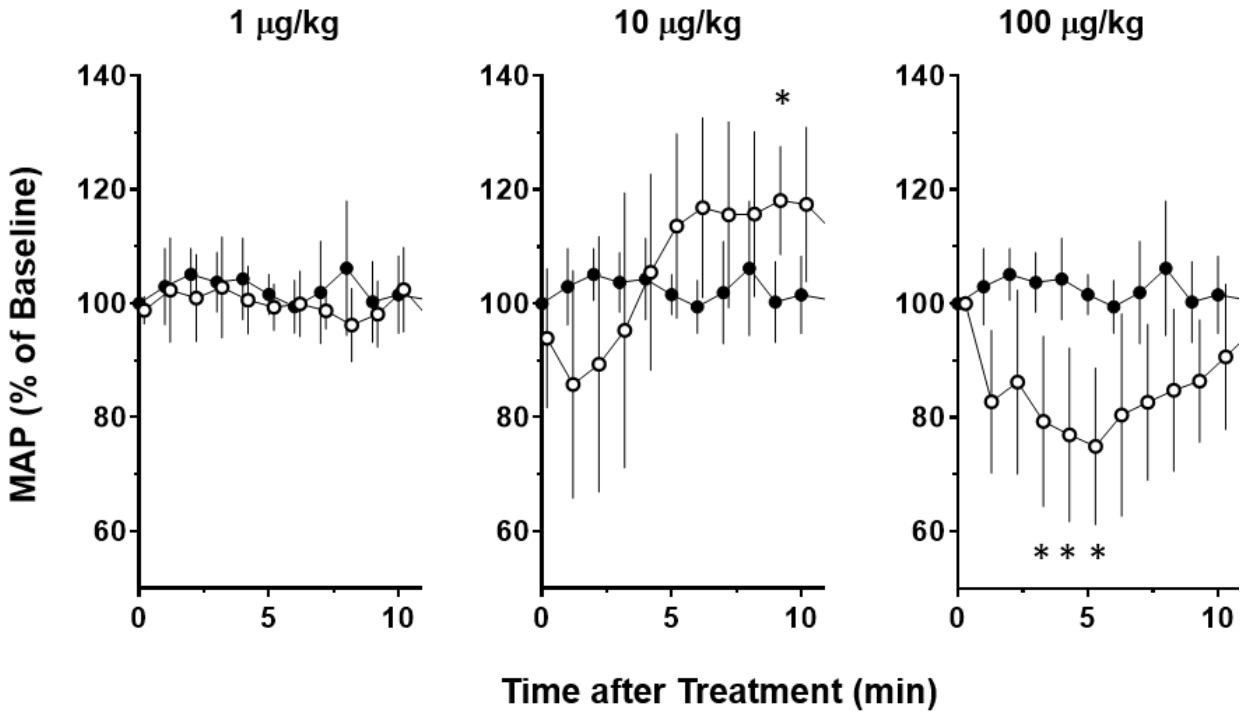

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Figure 2

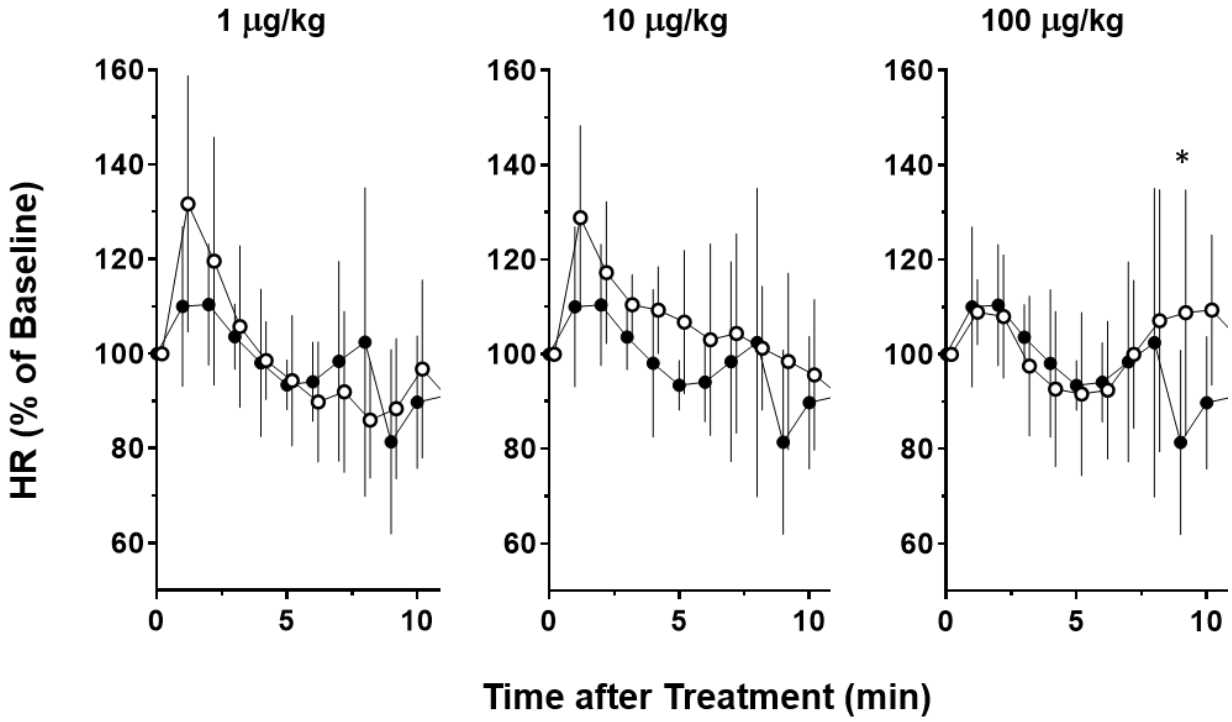

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Figure 3