Neuropeptides 77 (2019) 101956

Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/npep

Prokinetic effects of the neurokinin NK2 receptor agonist [Lys^5,$MeLeu^9$, Nle^{10}]-$NKA_{(4–10)}$ on bladder and colorectal activity in minipigs

Nadia M.J. Rupniak*, Mary A. Katofiasc, Lesley Marson, Daniel J. Ricca, Karl B. Thor, Edward C. Burgard

Dignify Therapeutics LLC, 2 Davis Drive, P.O. Box 13169, Research Triangle Park, NC 27709, USA

ARTICLE INFO

Keywords:
NK2 receptor agonist
Urination
Defecation
Intranasal
Sublingual
Minipig

ABSTRACT

The effects of the neurokinin NK2 receptor agonist [Lys^5,$MeLeu^9$,Nle^{10}]-$NKA_{(4–10)}$ (LMN-NKA) on bladder and colorectal function were examined in minipigs. In anesthetized animals, subcutaneous (SC) administration of 30–100 μg/kg increased peak bladder and colorectal pressures. Increases in bladder and colorectal pressure were inhibited by a 15 min pretreatment with the NK2 receptor antagonist GR 159897 (1 mg/kg intravenously (IV)). Bladder and colorectal pressures were also increased after IV (0.3 μg/kg), intranasal (IN; 100 μg/kg) and sublingual administration (SL; 5 mg/kg). There was a nonsignificant trend for hypotension (16 or 12% decrease in mean arterial pressure) after 100 μg/kg SC and 0.3 μg/kg IV, respectively, but not after 100 μg/kg IN or 5 mg/kg SL. In conscious minipigs, 30–300 μg/kg SC caused a dose-related increase in defecation that was accompanied by emesis in 38% of subjects receiving 300 μg/kg. Urination was increased after 100 μg/kg SC but not lower or higher doses. The peak plasma exposure (Cmax) after 100 μg/kg SC was 123 ng/mL, and area under the curve (AUC) was 1790 min * ng/mL. Defecation response rates (~82%) were maintained after SC administration of LMN-NKA (30 μg/kg) given 3 times daily over 5 consecutive days. Defecation rates were higher after a single dose of 100 μg/kg IN compared with vehicle, but this did not reach significance. After 7–10 mg/kg SL, 83% of animals urinated and defecated, and none had emesis. The data support the feasibility of developing a convenient and well-tolerated route of administration of LMN-NKA for human use. Minipigs may be a suitable species for toxicology studies with LMN-NKA due to the relatively low rate of emesis in this species.

1. Introduction

Neurokinin A (NKA) and related peptides are able to activate neurokinin 2 receptors (NK2Rs) on visceral smooth muscle and cause contraction of isolated strips of colon and urinary bladder (Warner et al., 2003; Burcher et al., 2008). This contractile activity is conserved across species including rats (Hall et al., 1992), dogs (Mussap et al., 1996; Rizzo and Hey, 2000), pigs (Bolle et al., 2000; Templeman et al., 2003), and humans (Palea et al., 1996; Templeman et al., 2003). It may be possible to harness this property for pharmacotherapy of clinical conditions in which there is an inability to properly initiate and complete evacuation of the bladder and bowel. Recently, in vivo studies have confirmed the ability of NK2R agonists to cause dose-dependent increases in colorectal and bladder pressure and urinary voiding after systemic administration to anesthetized rats (Kullmann et al., 2017; Marson et al., 2018), dogs (Rupniak et al., 2018a), and primates (Rupniak et al., 2018b). Moreover, in conscious rats (unpublished observations) and dogs (Rupniak et al., 2018c), NK2R agonists cause urination and defecation with a rapid onset and short duration of effects that would be appropriate for convenient on-demand administration by people.

A peptide of particular interest is the highly selective NK2R agonist [Lys^5,$MeLeu^9$,Nle^{10}]-$NKA_{(4–10)}$ (LMN-NKA; Chassaing et al., 1991; Warner et al., 2002; Rupniak et al., 2018d). LMN-NKA exhibited 674-fold selectivity for NK2 over NK1 receptors in radioligand binding assays, and ≥74-fold selectivity in functional assays in vitro (Rupniak et al., 2018d). Consistent with these findings, there was ~100-fold separation between NK2R-mediated increases in colorectal and bladder pressure and NK1R mediated hypotension after intravenous (IV) administration of LMN-NKA in rats (Marson et al., 2018). However, surprisingly, there was no separation between the NK2 and NK1 receptor-mediated effects of LMN-NKA in anesthetized dogs and primates when the drug was administered IV. In these species, doses of 0.1 μg/kg IV and above increased colorectal pressure and, as expected, this was blocked by pretreatment with the NK2R antagonist GR 159897. The same doses also elicited hypotension that was blocked by pretreatment

* Corresponding author.
E-mail address: nrupniak@dignifytherapeutics.com (N.M.J. Rupniak).

https://doi.org/10.1016/j.npep.2019.101956
Received 4 April 2019; Received in revised form 17 June 2019; Accepted 10 July 2019
Available online 11 July 2019
0143-4179/

with the NK1R antagonist CP-99,994 (Rupniak et al., 2018a,b). Moreover, after subcutaneous (SC) administration to conscious dogs, the same doses of LMN-NKA that were required to elicit urination and defecation were associated with a high rate of emesis that was reduced by pretreatment with the NK1R antagonist, CP-99,994. Since emesis might limit dose escalation with LMN-NKA in dogs, it was important to determine whether alternate non-rodent species are less sensitive to emesis, allowing higher drug exposures to be evaluated for toxicology studies.

A number of studies have employed minipigs to study bladder pharmacology and function in vivo (Dalmose et al., 2004; Scheepe et al., 2007; Huppertz et al., 2015; Li et al., 2017), and in vitro studies have shown a similar potency of NKA to contract pig and human bladder strips (EC_{50} ~40 nM; Templeman et al., 2003). The present studies examined the pharmacodynamic effects of LMN-NKA in anesthetized and conscious minipigs. In addition to SC and IV injection, intranasal (IN) and sublingual (SL) administration were also examined as these are potentially more convenient and tolerable methods of drug delivery for human subjects.

2. Materials and methods

2.1. Animals

Animals were socially housed 2–3 per pen and provided with environmental enrichment. Experiments conformed with Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Synchrony Labs, Durham NC (pharmacodynamic studies), or Charles River Laboratories Inc., Spencerville, OH (pharmacokinetic study). The number of animals was the minimum needed to establish the pharmacodynamic and pharmacokinetic effects of LMN-NKA. The subjects weighed 6.7–16 kg at the start of the studies and included naïve and non-naïve, male and female, Gottingen (Marshall BioResources, North Rose, NY, USA) and Sinclair (Sinclair Bio-Resources, Auxvasse, MO) minipigs. The non-naïve animals had participated in a previous 10 day study that included isoflurane anesthesia; they were drug-free for 2 months prior to the present study. Animals were acclimated to the testing facility for ~1 month prior to observation of voiding behavior. Terminal procedures under anesthesia were performed on the same subjects that were used for conscious observational studies to conserve animals, with a drug washout period of at least 5 days between studies.

2.2. Colorectal manometry and cystometry in anesthetized minipigs

Two studies examined the effects of LMN-NKA on colorectal and bladder pressure in anesthetized minipigs after various doses and routes of administration (Table 1). Animals were fasted for 12 h prior to surgery. Telazol (Zoetis, Parsippany, NJ; 3–5 mg/kg IM) and buprenorphine (Reckitt Benckiser, Parsippany, NJ; 0.005–0.3 mg/kg IM) were used to induce anesthesia and analgesia. Animals were intubated with a 6–8 FR endotracheal tube and anesthesia was maintained with 2–3% isoflurane via a ventilator (15–25 breaths/min; tidal volume 120–140 mmHg). A 20–22 gauge IV catheter (Surflo, Terumo Medical Products, Somerset, NJ) was inserted into the ear vein for delivery of sterile saline (5–10 mL/kg/h). A 4–5 FR catheter (PreludeEase™, Merit Medical, Jordan, UT or Fast-Cath™, St Jude Medical, St. Paul, MN) was inserted into the femoral artery to monitor cardiovascular parameters, and an 8 FR catheter was inserted into the femoral vein to permit blood sample collection for pharmacokinetic (PK) analysis. For colorectal manometry, minipigs received a warm water enema to clear the rectum and lower colon of feces before a 4″ balloon catheter was inserted into the rectum and taped to the base of the tail. In the same animals, the urinary bladder was exposed via a midline incision and a modified 5 FR catheter was inserted through the dome of the bladder and sutured in place. The arterial catheter, colorectal balloon, and bladder catheter

Table 1
Anesthetized and conscious minipig studies.

Study	Description	Doses and routes	Subjects	Design
Anesthetized				
DT-063	SC dose-response of colorectal and urodynamic effects	Vehicle, 1, 3, 10, 30 or 100 μg/kg SC	3 non-naïve Gottingen minipigs (1 male, 2 females)	All subjects received ascending doses 40–60 min apart
DT-105	Colorectal and urodynamic study comparing routes of administration	0.3 μg/kg IV 30 μg/kg SC 100 μg/kg IN 5 mg/kg SL	4 non-naïve Sinclair minipigs (2 of each sex)	2–4 minipigs received each treatment with 40–60 min between doses
Conscious				
DT-050	A. SC dose-response: micturition, defecation, AEs	10, 30, 100 or 300 μg/kg SC	2 naïve Gottingen minipigs (1 of each sex)	Both minipigs received each dose separated by ≥3 days
	B. SC pharmacokinetics	100 μg/kg SC	10 naïve Gottingen minipigs (5 of each sex)	Blood samples were collected at 2, 5, 10, 20, 40, 60 and 240 min post-dose
DT-061	Single dose (SC) pharmacodynamics	Vehicle, 30 or 100 μg/kg SC	6 non-naïve Gottingen minipigs (3 of each sex)	Minipigs received each treatment on 2 separate occasions with 2–5 days between doses.
DT-105	A. Single dose pharmacodynamics after different routes of administration	Vehicle, 10, 30 or 100 μg/kg SC Vehicle or 100 μg/kg IN Vehicle, 3–5, 5.1–7 or 7.1–10 mg/kg SL	8 naïve Sinclair minipigs (4 of each sex)	Minipigs were dosed over a 5-week period, alternating between SC, IN and SL routes of administration. Some animals received the same dose on 2 separate occasions.
	B. Repeated dose (SC) pharmacodynamics	Vehicle or 30 μg/kg SC	5 non-naïve Sinclair minipigs (3 females, 2 males)	Minipigs were dosed 3 times per day (4 h apart) for 5 days with vehicle or LMN-NKA. After a 2-day washout period, animals were crossed over to receive the opposite treatment.

were connected via a 3-way stopcock to DelTran II pressure transducers (Utah Medical Products Inc., Midvale, UT). Data were captured using a PowerLab/8SP acquisition system and LabChart software (version 7.3.7; ADInstruments, NSW, Australia).

During the post-surgery stabilization period (30–60 min), the colorectal balloon and bladder catheters were gradually filled with saline and the volume adjusted to maintain a baseline pressure of 8–15 mmHg. Once physiological parameters were stable, the effect of vehicle or LMN-NKA following various routes of administration on colorectal and bladder pressure amplitude, onset, time to peak, duration, and AUC (0–20 min) was determined. In study DTI-063, the effects of increasing subcutaneous (SC) doses were examined. At least 30 min after giving the 100 μg/kg dose, the NK2R antagonist GR 159897 (1 mg/kg IV) was administered and 15 min later, 100 μg/kg SC of LMN-NKA was repeated. Study DTI-105 compared the effects of SC, intravenous (IV), intranasal (IN) and sublingual (SL) administration of LMN-NKA (Table 1). At the end of the experiments, animals were euthanized with an IV overdose (100 mL) of potassium chloride (40 mEq/100 mL; Hospira, Lake Forest IL) under isoflurane anesthesia.

2.3. Urination and defecation in conscious animals

During a preliminary study, urination and defecation were observed in almost every animal within a few minutes after administration of vehicle, so that no effect of LMN-NKA could be discerned; data from this study are not included. In subsequent studies, it was found that habituation of minipigs to the housing and testing environment for approximately 1 month led to a marked reduction in the rates of urination and defecation after injection of vehicle, so that the pharmacodynamic effects of LMN-NKA could be more readily detected. All animals received oral fluids (200–400 mL of diluted Ensure, Abbott Nutrition, Lake Forest, IL) 30 min before dosing. Three studies examined various doses and routes of administration of LMN-NKA as summarized in Table 1. Minipigs were continuously observed for 30 min either in their home cage (36 × 36″; study DT-050), or in individual metabolism cages (37.5 × 40″; study DT-061 and single dose studies in DT-105); for the repeat SC dose study, animals were observed in their home cages (48 × 84″; study DT-105). The observers were blind to treatment in study DT-105, and the same animals were examined after SC, IN and SL administration of LMN-NKA. These minipigs were supplied with vascular access ports for intravenous (IV) injection of propofol to facilitate IN and SL dose administration. Pharmacodynamic responses were also recorded after repeated SC administration of LMN-NKA or vehicle 3 times per day for 5 consecutive days to examine possible development of tachyphylaxis or tolerance. The incidence and latency to the first urination and defecation events were the primary pharmacodynamic endpoints. Other signs, including emesis, diarrhea, hypoactivity, restlessness, and dermal flushing, were recorded when present.

2.4. Pharmacokinetics

Seven blood samples (~2 mL) were collected at 2, 5, 10, 20, 40, 60 and 240 min after SC injection of 100 μg/kg LMN-NKA by venipuncture of the cranial vena cava in 10 conscious Gottingen minipigs (study DT-050). Blood samples were collected in EDTA tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing ascorbic acid (1% final concentration). The solution was gently mixed by inversion and kept on ice until centrifugation within 40 min of collection at 2–3000 r.p.m. for ~10 min at 4 °C. Plasma was flash frozen in liquid nitrogen and stored at −70 °C until assay. Aliquots (100 μL) were assayed for LMN-NKA using a qualified analytical method that employed 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 concentration range of 1 to 100 ng/mL using 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.

2.5. Formulation and administration of test compounds

Stock solutions (10 mg/mL) of LMN-NKA (Asp-Lys-Phe-Val-Gly-Leu (NMe)-Nle-NH_2; synthesized by Bachem, Torrance, CA, or GenScript, Piscataway, NJ) and GR 159897 (5-Fluoro-3-[2-[4-methoxy-4-[[(*R*)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole; Tocris, Minneapolis, MN) were prepared in sterile saline, filtered, and stored in aliquots at –20 °C until use. Thawed stock solutions were diluted with saline to achieve the required injection volume. Doses of LMN-NKA were based on previous studies conducted in dogs (IV and SC route, Rupniak et al., 2018a) or rats (IN route, Marson et al., 2018; SL route, Bae et al., 2018).

In studies using conscious minipigs, animals were manually restrained or placed in a sling for SC injections using a volume of ≤0.1 mL. SC injections alternated between the left and right sides; Gottingen minipigs were injected in the lateral neck caudal to the ear, and Sinclair minipigs were injected in the inguinal region in order to avoid the vascular access port. For IN administration, LMN-NKA was delivered into the left or right nares using an atomizer (MAD Nasal™, Teleflex, Morrisville, NC) in a volume of < 250 μL. Initially, animals tolerated IN dosing with gentle manual restraint for dosing but resisted on subsequent attempts so that mild sedation with propofol (3–8 mg IV) was needed for approximately half of all dose administrations. For SL administration, LMN-NKA was formulated as a lyophilized tablet as described by Bae et al. (2018). All minipigs were briefly anesthetized with propofol (3–8 mg IV) followed by isoflurane (2–3.5%). Tablets were placed under the tongue just dorsal to the frenulum, and water (0.3–1 mL) was added to aid dissolution. The tongue was held down over the tablets to ensure contact with the mucosa for 1–2 min.

2.6. Data analysis and statistics

Colorectal, bladder, and cardiovascular parameters from anesthetized animal experiments, and the latency to onset of pharmacodynamic events in conscious animals, were analyzed using Excel (Microsoft, Redmond, WA) and Prism 6 (GraphPad Software Inc., San Diego, CA). If no urination or defecation was observed during the 30 min observation period, a latency of 30 min was used for statistical analysis. Data were subjected to one-way ANOVA with Dunnett's multiple comparison *t*-tests to compare the effect of LMN-NKA with vehicle; $p \leq .05$ was considered statistically significant. Values are expressed as the mean + 1 standard deviation (SD). For conscious animal studies, the responder rate (number of animals responding after an injection of vehicle or LMN-NKA) for observed pharmacodynamic events was expressed as a fraction of the total number of treatments. Nonparametric incidence data were compared using logistic regression. Since some analyses contained levels of zero responses, models were estimated using Fisher's exact method. Pairwise comparisons of doses versus control group were performed using an adjusted alpha level (0.01) to account for multiple comparisons.

PK data were collected using Analyst (MDS Sciex, Framingham, MA, USA) and parameters were calculated using Phoenix WinNonlin 6.3 (Certara, St Louis, MO, USA). Concentrations that were below the level of quantification (BLQ) were assigned a value of zero if they occurred before the first measurable concentration. If a BLQ value occurred after a measurable concentration and was followed by a measurable value, or at the end of the collection interval, it was treated as missing.

3. Results

3.1. Effect of LMN-NKA on bladder and colorectal pressure in anesthetized animals

LMN-NKA produced a statistically significant, non-linear, dose-

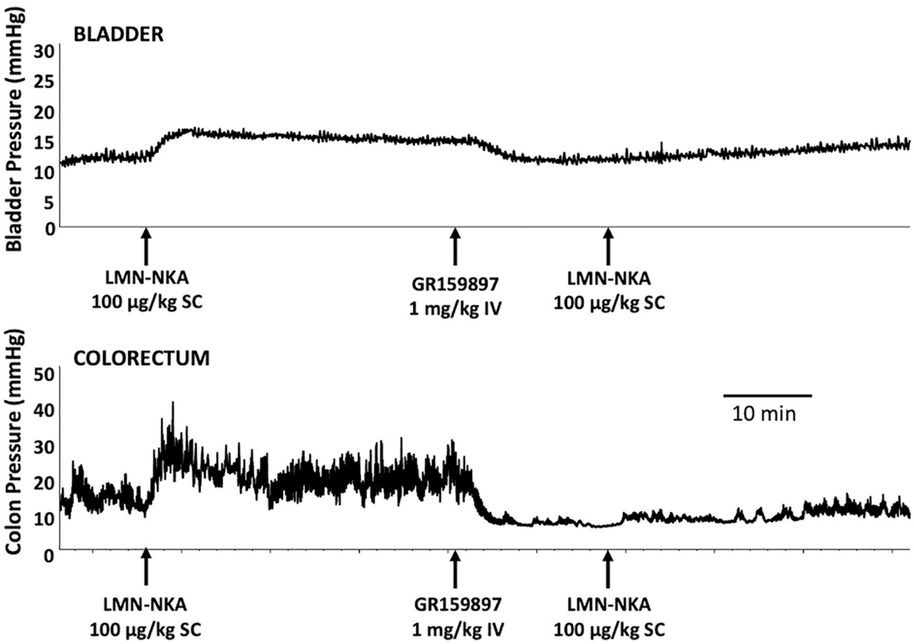

Fig. 1. Inhibition of LMN-NKA Induced Increase in Bladder and Colorectal Pressure by GR 159897. Representative physiographs showing the effect of pretreatment with GR 159897 on bladder and colorectal pressure responses to LMN-NKA in study DT-063. Compounds were administered at the times and doses indicated by arrows.

Table 2
Effect of different routes of administration of LMN-NKA on bladder and colorectal pressure in anesthetized minipigs.

Study number	Dose and route	Peak pressure (change from baseline, mmHg)	AUC (mmHg.s)	Time to onset (min)
Bladder				
DT-063	Vehicle SC (*N* = 3)	0 ± 0	0 ± 0	NA
	1 µg/kg SC (*N* = 3)	0 ± 0	0 ± 0	NA
	3 µg/kg SC (*N* = 3)	8.2 ± 3.87	6942 ± 4931	3.78 ± 1.50
	10 µg/kg SC (*N* = 3)	11.02 ± 3.77	15,652 ± 5521	2.58 ± 0.46
	30 µg/kg SC (*N* = 3)	12.62 ± 2.15*	15,033 ± 9038*	2.81 ± 0.64
	100 µg/kg SC (*N* = 3)	9.58 ± 3.67	10,246 ± 6701*	2.28 ± 0.79
DT-105	Baseline (*N* = 4)	0.25 ± 0.25	0 ± 1	NA
	30 µg/kg SC (*N* = 2)	11.37 ± 2.62	5545 ± 2011	1.82 ± 0.07
	0.3 µg/kg IV (*N* = 2)	9.48 ± 0.61	4519 ± 916	0.38 ± 0.07
	100 µg/kg IN (*N* = 4)	4.07 ± 1.21*	1216 ± 362*	3.13 ± 0.50
	5 mg/kg SL (*N* = 4)	2.75 ± 0.69*	371.5 ± 289	8.89 ± 1.67
Colorectum				
DT-063	Vehicle SC (*N* = 3)	0 ± 0	0 ± 0	NA
	1 µg/kg SC (*N* = 3)	0 ± 0	0 ± 0	NA
	3 µg/kg SC (*N* = 3)	16.09 ± 8.29	14,106 ± 12,071	4.29 ± 4.37
	10 µg/kg SC (*N* = 3)	26.35 ± 9.11	45,434 ± 37,129	1.73 ± 0.24
	30 µg/kg SC (*N* = 3)	28.26 ± 4.49*	29,005 ± 7847	2.48 ± 0.83
	100 µg/kg SC (*N* = 3)	24.11 ± 5.53*	23,673 ± 4417	1.73 ± 027
DT-105	Baseline (*N* = 4)	0.50 ± 0.34	6 ± 10	NA
	30 µg/kg SC (*N* = 2)	31.28 ± 5.03	14,138 ± 7584	1.67 ± 0.24
	0.3 µg/kg IV (*N* = 2)	35.69 ± 10.16	21,624 ± 4036	0.13 ± 0.07
	100 µg/kg IN (*N* = 4)	28.87 ± 9.55*	17,265 ± 6928	1.80 ± 0.49
	5 mg/kg SL (*N* = 4)	12.97 ± 5.39*	4375 ± 3047	8.67 ± 2.79

Values are the mean ± SD obtained using Gottingen (study DT-063) or Sinclair (study DT-105) minipigs. Data were subjected to 1-way ANOVA with repeated measures. Study DT-063: bladder pressure, F (1.4, 2.8) = 23.5, $p = .019$; AUC, F (1.5, 2.9) = 8.6, $p = .061$. Colorectal pressure, F (1.3, 2.7) = 19.5, $p = .026$; AUC, F (1.0, 2.1) = 4.7, $p = .160$. Study DT-105: bladder pressure, F (1.3, 4) = 20.8, $p = .009$; AUC, F (1.8, 5.3) = 32.1, $p = .001$. Colorectal pressure, F (1.2, 3.7) = 18.4, $p = .014$; AUC, F (1.1, 2.1) = 12.5, $p = .066$. *$p \leq .05$ compared with baseline values, Dunnett's multiple comparison test. NA: not applicable.

dependent increase in peak bladder and colorectal pressure after doses of 30 to 100 µg/kg SC (Fig. 1, Table 2). The peak effect was observed after 30 µg/kg, and the dose-response appeared bell-shaped. The onset of bladder and colorectal contractions was rapid (2–4 min), peak pressure was reached within 11 min, and pressure remained elevated up to 38 min (bladder) or 60 min (colorectal). The bladder and colorectal pressure AUCs appeared maximal after 10–30 µg/kg SC, although this did not reach statistical significance (Table 2). Colorectal AUC could not be determined accurately as pressures did not fully return to baseline before escalating to the next dose of LMN-NKA. Bladder leakage or voiding was not detected after any dose. At the end of the experiment, manual compression of the bladder was applied to determine the leak point pressure (LPP). The mean pressure required to induce leakage was 93.5 mmHg (*n* = 6, range 50–120 mmHg), with 1 animal reaching a pressure of 130 mmHg without evoking release.

Pretreatment with GR 159897 (1 mg/kg IV, 15 min prior to LMN-NKA; *N* = 2) blocked the increases in bladder and colorectal pressure induced by 100 µg/kg SC of LMN-NKA (Fig. 1).

The ability of LMN-NKA to increase bladder and colorectal pressure was next examined after IN and SL administration (Table 2; Fig. 2). In this series of experiments, a small number of animals (*N* = 2) also received single SC or IV doses to confirm assay sensitivity. As seen

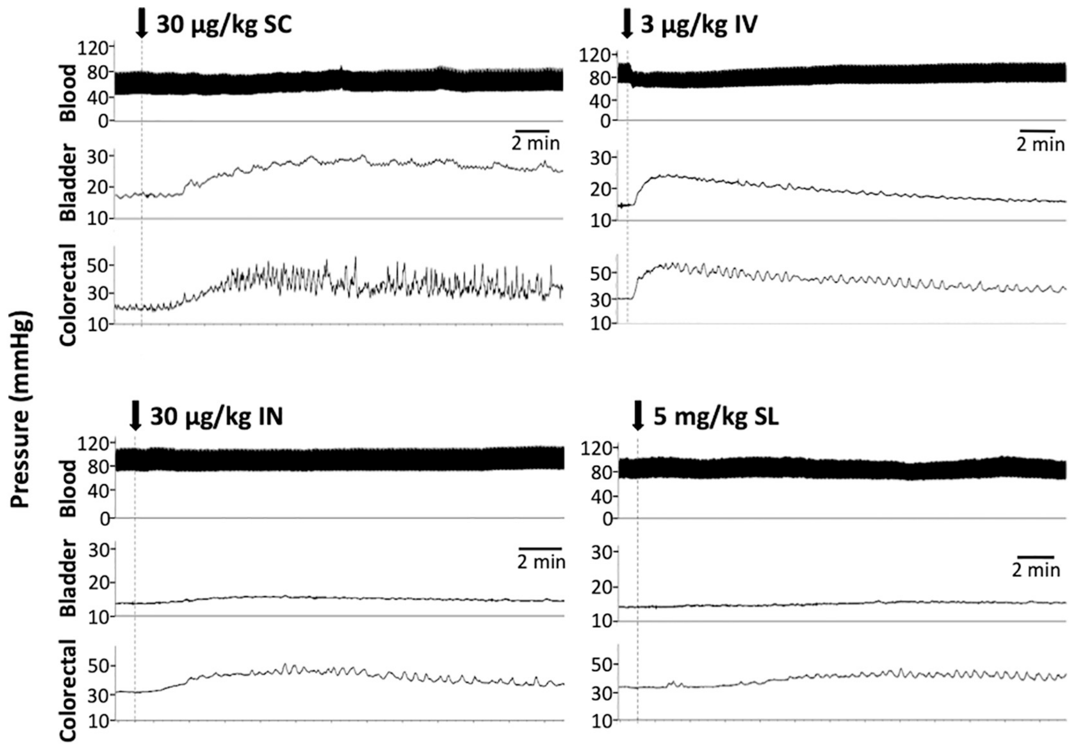

Fig. 2. Effects of SC, IV, IN or SL administration of LMN-NKA on blood, bladder and colorectal pressure in anesthetized minipigs. Representative physiographs showing the effect of SC, IV, IN or SL administration of LMN-NKA (plotted at the same pressure and time scales to allow comparison) on blood, bladder and colorectal pressure in Sinclair minipigs from study DT-105. LMN-NKA was administered at the times indicated by arrows.

previously, administration of 30 μg/kg SC increased peak bladder and colorectal pressure and AUC, and IV administration of 0.3 μg/kg caused similar increases in peak pressure and AUC ($N = 2$). Intranasal application of 30 or 100 μg/kg produced significant increases in peak bladder pressure and AUC ($N = 4$), and in peak colorectal pressure (Table 2; Fig. 2); there was a nonsignificant increase in colorectal AUC. Assessment of SL administration was complicated by incomplete dissolution of the tablet formulation and uncertainty over the dose delivered to each animal; nonetheless, significant increases in peak bladder and colorectal pressure (but not AUC) were detected after SL application of 5 mg/kg LMN-NKA ($N = 4$; Table 2; Fig. 2). The onset of bladder and colorectal contraction was rapid after IN dosing (≤3 min) but slower (8–9 min) after SL dosing (Table 2). Bladder leaking or voiding did not occur after any dose of LMN-NKA.

3.2. *Effect of LMN-NKA on cardiovascular parameters in anesthetized minipigs*

After SC injection of 1–100 μg/kg LMN-NKA, there was a linear trend for a dose-related decrease in blood pressure of up to 16% (slope = −3.14, $p = .0008$), but individual doses were not statistically significant (Table 3). Intravenous administration of 0.3 or 3 μg/kg LMN-NKA in 2 minipigs caused a transient decrease in mean arterial pressure (MAP) of ~12%, whereas no effect was detected after 30 or 100 μg/kg IN or 5 mg/kg SL (Table 3; Fig. 2). There was no consistent effect on heart rate after any dose or route of administration of LMN-NKA.

3.3. *Pharmacodynamic effects of LMN-NKA in conscious minipigs*

3.3.1. *Subcutaneous administration*

Data from 3 studies were combined to examine the effect of a single SC administration of LMN-NKA on urination and defecation. After injection of vehicle or 10 μg/kg LMN-NKA, urination and defecation occurred sporadically, at a similar rate, and no other effects were observed. There was a dose-related increase in the rate of defecation and a reduction in the latency to defecate after 30 to 300 μg/kg SC (Table 4). After injection of 30 μg/kg and 100 μg/kg, some animals ($N = 1$ and $N = 4$, respectively) exhibited diarrhea and alternating hypoactivity (standing with the head down, or asleep) and restlessness (repeatedly standing up and lying down). LMN-NKA appeared to exert more pronounced effects on the gastrointestinal tract than on the bladder since the threshold dose for defecation was lower (30 μg/kg) than for urination (100 μg/kg). LMN-NKA-induced urination was only consistent at a dose of 100 μg/kg SC and not after higher or lower doses (Table 4). Emesis was present in 15% and 38% of minipigs at doses of 100 or 300 μg/kg, respectively (Table 4), and usually occurred within 5 min of injection. In two Gottingen minipigs that were observed for changes in skin pigmentation, flushing of the snout and conjunctiva was seen immediately after injection of 100 or 300 μg/kg; due to their dark skin pigmentation, flushing could not be reliably detected in Sinclair minipigs.

Since LMN-NKA was generally well tolerated after single injections of 30 μg/kg SC, this dose was chosen for a repeated dose study. After a 3-day washout period, 5 Sinclair minipigs that had previously received single doses of LMN-NKA in study DT-105 received 3 SC injections per day (4 h apart) for 5 consecutive days. Urination, defecation and emesis were recorded for 30 min after the first and last dose of each day, for a total of 50 observations. Rates of urination and defecation after injection of vehicle were lower in this study than previously. These animals were not fluid loaded prior to dosing, and this may explain the lower rate of urination; the lower rates of voiding may also reflect habituation of the animals to handling and dosing. As was seen after single doses of 30 μg/kg SC, repeated injections of LMN-NKA did not affect the rate or onset time of urination as compared with vehicle, whereas the rate of defecation was almost 7-fold higher after LMN-NKA (Table 5). There was no difference in the latency for urination after vehicle or LMN-NKA, whereas the time to onset of defecation was halved after SC

Table 3
Effects of different routes of administration of LMN-NKA on cardiovascular parameters in anesthetized minipigs.

Study number	Dose and route	MAP (% change from baseline)	Heart rate (% change from baseline)
DT-063	Vehicle SC (*N* = 3)	2.19 ± 0.58	1.76 ± 4.69
	1 μg/kg SC (*N* = 3)	−0.62 ± 0.42	−1.68 ± 2.02
	3 μg/kg SC (*N* = 3)	−1.67 ± 2.95	−1.46 ± 0.46
	10 μg/kg SC (*N* = 3)	−4.60 ± 2.67	3.15 ± 4.88
	30 μg/kg SC (*N* = 3)	−5.98 ± 4.75	0.14 ± 7.12
	100 μg/kg SC (*N* = 3)	−16.01 ± 10.15	0.48 ± 6.14
DT-105	Baseline (*N* = 4)	0.55 ± 2.68	0.17 ± 0.29
	30 μg/kg SC (*N* = 2)	1.97 ± 3.35	−5.21 ± 3.22
	0.3 μg/kg IV (*N* = 2)	−11.94 ± 4.04	4.20 ± 5.16
	100 μg/kg IN (*N* = 4)	−1.50 ± 3.99	5.51 ± 13.92
	5 mg/kg SL (*N* = 4)	−1.25 ± 3.65	−1.56 ± 3.26

Values are the mean ± SD changes from baseline and obtained using Gottingen (study DT-063) or Sinclair (study DT-105) minipigs. Data were subjected to 1-way ANOVA with repeated measures. DT-063: $F(1.3, 2.6) = 5.2$, $p = .122$ for MAP; $F(1.8, 3.6) = 0.4$, $p = .665$ for heart rate. DT-105: $F(1.2, 3.7) = 0.97$, $p = .408$ for MAP; $F(1.0, 3.1) = 0.68$, $p = .472$ for heart rate.

Table 4
Incidence and time to onset of urination, defecation and emesis after single subcutaneous administration of LMN-NKA.

Treatment and dose (μg/kg SC)	Urination		Defecation		Emesis
	Response rate	Latency (min)	Response rate	Latency (min)	Response rate
Vehicle (*N* = 16)	6/16 (38%)	21.88 ± 11.79	7/16 (44%)	22.40 ± 11.26	0/16 (0%)
10 (*N* = 4)	1/4 (25%)	24.84 ± 10.32	2/4 (50%)	22.86 ± 10.94	0/4 (0%)
30 (*N* = 26)	14/26 (54%)	19.51 ± 10.72	23/26 (88%)*	12.12 ± 9.56*	0/26 (0%)
100 (*N* = 20)	17/20 (85%)*	10.47 ± 9.46*	20/20 (100%)*	7.24 ± 7.15*	3/20 (15%)
300 (*N* = 8)	4/8 (50%)	18.63 ± 12.64	8/8 (100%)*	4.80 ± 2.11*	3/8 (38%)*

Values are the number of observations using Gottingen (studies DT-050 and DT-061) and Sinclair (DT-105) minipigs; some animals received vehicle, 30 and 100 μg/kg on 2 separate occasions. N refers to the total number of doses administered. Animals were observed continuously 30 min after SC injection of LMN-NKA. Response Rate = number of responses/number of injections. Response rates were compared using Fisher's exact test; $^*p \le .05$ compared with vehicle. Latencies were subjected to 1-way ANOVA. If no urination of defecation was observed, a latency of 30 min was employed. One-way ANOVA: $F(4, 69) = 3.5$, $p = .012$ for urination; $F(4, 69) = 9.4$, $p < .001$ for defecation. $^*P \le .05$ compared with vehicle, Dunnett's multiple comparison test.

injection of LMN-NKA (Table 5). Defecation was elicited consistently on each day of treatment, without evidence of tolerance to the effects of LMN-NKA. LMN-NKA appeared to be well tolerated at this dose, and emesis and diarrhea were not observed; restlessness was noted after ~30% of injections.

3.3.2. Intranasal and sublingual administration

After a 9-day washout period from the SC study, the effects of IN and SL administration of LMN-NKA were examined in 6 Sinclair minipigs. For IN administration, approximately half of the animals had to be mildly sedated using propofol (3–8 mg IV) to enable use of a nasal spray applicator. With or without sedation, accurate IN dose administration was difficult as animals often pulled away from the atomizer before instillation was complete, or snorted the drug out of the nostril after delivery. There was a higher rate of defecation, and shorter latency to defecate, after 100 μg/kg IN of LMN-NKA compared with the vehicle group (Table 6), but across a large number of dose administrations (*N* = 12), the effects were not consistent and were not statistically significant. Moreover, a higher dose of 300 μg/kg IN had no effect on defecation. Although no statistically significant effect on urination was detected at any dose compared to vehicle, this may be attributable to the high rate of micturition (75%) seen in the vehicle group. Emesis and changes in activity were not observed after IN administration of LMN-NKA.

For SL administration, animals were anesthetized with propofol (3–8 mg) and isoflurane (2–3.5%) to enable the placement and dissolution of orally disintegrating tablets containing 3–10 mg/kg of LMN-NKA under the tongue. Recovery from anesthesia ranged from 4 to 30 min, and animals were observed continuously for 30 min thereafter. Although the need for anesthesia may have interfered with the effects of LMN-NKA, nonetheless there was a dose-related increase in the incidence of both urination and defecation, and 5 out 6 animals urinated and defecated after 7.1–10 mg/kg SL (Table 6). The latency to void urine and feces after recovery from anesthesia was also shorter after the highest dose of LMN-NKA than vehicle, but this was not significant. Emesis was not seen in any animal, and changes in activity following LMN-NKA could not be accurately assessed because of residual sedation from anesthesia.

Table 5
Incidence of urination, defecation and emesis after repeated subcutaneous administration of LMN-NKA 3 times daily for 5 days.

Treatment and dose (μg/kg SC)	Urination		Defecation		Emesis
	Response rate	Latency (min)	Response rate	Latency (min)	Response rate
Vehicle (*N* = 50)	8/50 (16%)	27.94 ± 5.90	6/50 (12%)	29.24 ± 2.39	0/50 (0%)
30 (*N* = 50)	11/50 (22%)	25.42 ± 8.81	41/50 (82%)*	14.78 ± 9.66*	0/50 (0%)

Data are from 5 Sinclair minipigs receiving either vehicle or 30 μg/kg SC LMN-NKA, 3 times per day for 5 consecutive days, in study DT-105. The same animals were crossed over to receive each treatment. Animals were observed continuously for 30 min after the first and last dose on each day, for a total N of 50 injections. Response rates were subjected to Fisher's exact test; $^*p \le .001$ compared with vehicle. Latencies were subjected to paired *t*-tests; urination, $p = .112$; defecation, $p < .0001$.

Table 6
Incidence and latency to onset of urination, defecation and emesis after single intranasal or sublingual administration of LMN-NKA.

Dose of LMN-NKA and route	Urination		Defecation		Emesis
	Response rate	Latency (min)	Response rate	Latency (min)	Response rate
Vehicle IN (*N* = 4)	3/4 (75%)	18.75 ± 12.42	0/4 (0%)	30.00 ± 0.00	0/4 (0%)
100 μg/kg IN (*N* = 12)	9/12 (75%)	14.74 ± 10.38	8/12 (67%)	21.56 ± 8.98	0/12 (0%)
300 μg/kg IN (*N* = 5)	2/5 (40%)	25.89 ± 5.85	1/5 (20%)	27.60 ± 5.37	0/5 (0%)
Vehicle SL (*N* = 4)	0/4 (0%)	30.00 ± 0.00	0/4 (0%)	30.00 ± 0.00	0/4 (0%)
3–5 mg/kg SL (*N* = 6)	2/6 (33%)	22.58 ± 11.80	2/6 (33%)	26.67 ± 8.16	0/6 (0%)
5.1–7 mg/kg SL (*N* = 3)	1/3 (33%)	22.67 ± 12.70	2/3 (67%)	22.83 ± 11.14	0/3 (0%)
7.1–10 mg/kg SL (*N* = 6)	5/6 (83%)*	12.50 ± 9.24	5/6 (83%)*	18.33 ± 10.21	0/6 (0%)

Data are from 8 Sinclair minipigs (4 of each sex) in study DT-105. Four animals received the 100 μg/kg IN dose on 2 occasions. N refers to the total number of doses administered. *$p \leq .05$ compared with vehicle, Fisher's exact test. If no urination of defecation was observed, a latency of 30 min was used for statistical analysis. Latency data were subjected to 1-way ANOVA. Intranasal administration: F (2, 18) = 2.2, $p = .137$ for urination; F (2, 18) = 2.4, $p = .115$ for defecation. Sublingual administration: F (3, 15) = 2.7, $p = .083$ for urination; F (3, 15) = 1.7, $p = .202$ for defecation.

4. Pharmacokinetics after SC administration

Blood samples were collected from 10 Gottingen minipigs (5 of each sex) after 100 μg/kg SC of LMN-NKA at 2, 5, 10, 20, 40, 60, and 240 min post-dose. Noncompartmental PK parameters were calculated using combined data from males and females. The peak plasma concentration of 123 ng/mL (Cmax) was reached after 10 min (Tmax) (Fig. 3). Plasma concentrations declined thereafter, with an apparent elimination half-life of 9.1 min. AUC_{last} and AUC_{inf} values were 1680 and 1790 min * ng/mL, respectively.

5. Conclusions

The effects of SC administration of 1–100 μg/kg of LMN-NKA on colorectal and bladder pressure in anesthetized minipigs were broadly consistent with previous findings in anesthetized rats (Marson et al., 2018), dogs (Rupniak et al., 2018a), and primates (Rupniak et al., 2018b). Like the effects seen in these other species, LMN-NKA-induced increases in colorectal and bladder pressure in minipigs were completely blocked after pretreatment with GR 159897, confirming that they were mediated by activation of NK2 receptors. However, notable species differences were also apparent. While LMN-NKA caused dose-related increases in peak colorectal and bladder pressure, the effects in minipigs were not linearly dose-related, and the magnitude of the effect was smaller (maximal increases of 29 and 13 mmHg, respectively) than previously reported in other species (increases of up to 50 mmHg for colorectal pressure in dogs and primates, Rupniak et al., 2018a,b; and up to 40 mmHg for bladder pressure in rats and dogs; Marson et al., 2018; Rupniak et al., 2018a). These differences were not attributable to plasma exposures since both the Cmax and AUC were markedly higher after a dose of 100 μg/kg SC in minipigs (123 ng/mL and 1680 ng/mL.min, respectively) than in dogs or primates (26 or 40 ng/mL and 666 or 627 ng/mL.min, respectively; Rupniak et al., 2018a,b). Moreover, the relatively small increase in bladder pressure did not produce leaking or voiding of urine in anesthetized minipigs, whereas voiding was observed in anesthetized rats, dogs, and primates after 3–300 μg/kg SC of LMN-NKA (Marson et al., 2018; Rupniak et al., 2018a, 2018b). While peak colorectal and bladder pressures were lower in minipigs than in other species, AUC values (pressure × time) were higher in minipigs (increase in colorectal AUC up to 43,000 mmHg·s; increase in bladder AUC up to 15,000 mmHg·s) than in dogs (up to 32,000 and 2000 mmHg·s, respectively), or primates (increase in colorectal AUC up to 20,000 mmHg·s), a finding that may be consistent with the greater plasma concentration AUC in minipigs. The marked difference in bladder AUC between minipigs and dogs may also reflect the absence of urine leakage and maintenance of pressure in minipigs, while bladder pressure dropped rapidly in dogs due to voiding.

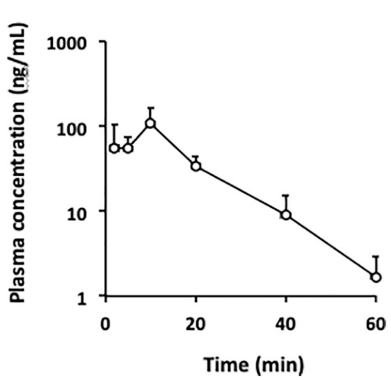

Fig. 3. Plasma concentrations of LMN-NKA following administration of 100 μg/kg SC. Data were obtained using 10 Gottingen minipigs (5 of each sex) in study DT-050. Values are means + SD.

Unlike the species differences in NK2R-mediated colorectal and bladder effects, the transient hypotension caused by LMN-NKA, which was NK1R-mediated in dogs and primates (Rupniak et al., 2018a,b), appeared to be similar in anesthetized minipigs. Although not statistically significant, transient hypotension was present after IV and SC administration of LMN-NKA, similar to findings in isoflurane-anesthetized dogs and primates (Rupniak et al., 2018a,b). These effects differed from those seen in urethane-anesthetized rats, where hypotension only occurred after a high dose of 100 μg/kg IV (Marson et al., 2018). Further studies are needed to clarify if this is a true species difference or due to anesthetic differences.

Other routes of administration of LMN-NKA were explored in order to identify a convenient and tolerable method of drug delivery for human use. Intranasal administration of 100 μg/kg of LMN-NKA elicited a similar increase in peak colorectal pressure to that seen after SC administration. Although the increase in peak bladder pressure was smaller than after SC administration, conclusions are limited by the single IN dose examined. Sublingual administration of 5 mg/kg of LMN-NKA also caused small increases in peak colorectal and bladder pressure. Higher doses may have been more effective since 7–10 mg/kg elicited voiding in conscious minipigs. Since the Cmax and AUC associated with an effective SC dose of LMN-NKA (100 μg/kg) are known, measurement of the plasma exposure to LMN-NKA after IN and SL administration may clarify the appropriate dose range in this species. Further pharmacokinetic studies may also clarify whether the inconsistencies in efficacy seen in the current study with these routes of administration are reflected in variability in achieving effective plasma concentrations. No hypotension was detected after IN or SL administration at the doses tested. An important consideration for future clinical development of NK2 agonists, especially as IN formulations, is the potential risk for bronchoconstriction that has been demonstrated after inhalation of NKA in asthmatic patients (Joos et al., 1987; Schelfhout et al., 2009). However, other clinical studies using IV infusions found that NKA did not alter respiration at doses that stimulated gastrointestinal motility (Lördal et al., 1997, 2001; Schmidt et al., 2003).

In conscious minipigs, there was a dose-related increase in defecation rates following 30–300 μg/kg SC LMN-NKA, similar to the effects previously reported in awake dogs. In contrast, an effect of LMN-NKA on urination was more difficult to detect in minipigs but was obvious in dogs. This may be partly because the rate of urination (and defecation) after vehicle treatment was much higher in minipigs. However, in a subsequent repeated dose study, the rates of urination and defecation in the vehicle group were low (presumably due to habituation to handling and dosing), but the urination rate did not increase after LMN-NKA (30 μg/kg SC), despite a pronounced increase in defecation rate. The lower efficacy for LMN-NKA-induced micturition in minipigs compared to other species is perplexing, since the efficacy of NKA to induce bladder contractions in vitro is fairly similar in rat, dog, and pig (Warner et al., 2003; Templeman et al., 2003; Rizzo and Hey, 2000; Hall et al., 1992). In conscious dogs, the gastrointestinal effects of LMN-NKA were more pronounced after SC than after IV dosing (Rupniak et al., 2018c), suggesting that differences in the absorption and distribution of the peptide can alter the pharmacodynamic profile. There was also evidence of a more pronounced urination response in minipigs after SL (7–10 mg/kg) than SC administration in the present study.

During the repeat dose SC study, LMN-NKA (30 μg/kg SC) consistently elicited defecation after repeated administration 3 times daily for 5 days without tolerance, consistent with previous findings using dogs (Rupniak et al., 2018c). Findings after IN and SL administration were difficult to interpret in this species due to the difficulty of dose administration and need to temporarily sedate the animals. Despite this, 7–10 mg/kg SL was able to increase rates of both urination and defecation, consistent with the feasibility of developing an appropriate method of delivery of this peptide for human use.

Emesis was not seen at all during 5 days of dosing 3 times per day at 30 μg/kg SC, despite consistent drug-induced defecation. Emesis did occur in 38% of minipigs after a single dose of 100 μg/kg SC, a much lower rate than was seen previously after the same dose in dogs (71%; Rupniak et al., 2018c). Since there are marked species differences in susceptibility to emetogens, with dogs being particularly sensitive (Percie du Sert et al., 2012), studies in human subjects are needed to determine whether nausea and emesis would limit dose escalation in the therapeutic range. In the Gottingen minipigs used for pharmacokinetics, dermal flushing and red eyes were observed after 100 μg/kg SC; interestingly, this was also the threshold dose for hypotension in anesthetized minipigs, suggesting that the fall in blood pressure may be secondary to peripheral vasodilation. Although the ability of an NK1R antagonist to inhibit these effects of LMN-NKA was not examined in minipigs, both dermal flushing (Newby et al., 1997) and chromodacryorrhea (Rupniak and Williams, 1994), like emesis, are known effects of NK1R activation in other species. The emergence of emesis after high doses of LMN-NKA in minipigs could be due to off-target NK1 receptor activation, or possibly could reflect increased gastrointestinal activity due to NK2 receptor activation. Given the lower rate of emesis, maximally tolerated doses required for safety studies may be more feasible in minipigs than dogs. Although the lower rate of emesis after acute dosing with LMN-NKA should enable higher exposures in minipigs, whether an adequate margin of safety can be achieved to support human use will depend on clinical and histopathology findings after long term administration.

In summary, the results indicate that minipig bladder and colorectal prokinetic and emetic responses to LMN-NKA are less pronounced in minipigs compared to other species. LMN-NKA-induced defecation could be reliably demonstrated across 5 days, while micturition could not. The anesthetized minipig provides a more reliable means of testing IN and SL routes of administration because of the need to sedate conscious animals for dosing.

Funding

Studies were supported by the National Institute of Diabetes and Digestive and Kidney Diseases under award number R44DK112437, and by Dignify Therapeutics. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

Bae, J., Johnston, T.A., Chaiittianan, R., Sutthanut, K., Jay, M., Marson, L., 2018. Characterization and in vivo efficacy of a heptapeptide ODT formulation for the treatment of neurogenic bladder dysfunction. Int. J. Pharm. 536 (1), 397–404. https://doi.org/10.1016/j.ijpharm.2017.11.036.

Bolle, P., Severini, C., Falconieri-Erspamer, G., Tucci, P., Erspamer, V., 2000. Effects of natural tachykinins on porcine lower urinary tract smooth muscle. J. Auton. Pharmacol. 20 (3), 157–161.

Burcher, E., Shang, F., Warner, F.J., Du, Q., Lubowski, D.Z., King, D.W., Liu, L., 2008. Tachykinin NK2 receptor and functional mechanisms in human colon: changes with indomethacin and in diverticular disease and ulcerative colitis. J. Pharmacol. Exp. Ther. 324 (1), 170–178. https://doi.org/10.1124/jpet.107.130385.

Chassaing, G., Lavielle, S., Loeuillet, D., Robilliard, P., Carruette, A., Garret, C., et al., 1991. Selective agonists of NK-2 binding sites highly active on rat portal vein (NK-3 bioassay). Neuropeptides 19 (2), 91–95.

Dalmose, A.L., Bjarkam, C.R., Sørensen, J.C., Djurhuus, J.C., Jørgensen, T.M., 2004. Effects of high frequency deep brain stimulation on urine storage and voiding function in conscious minipigs. Neurourol. Urodyn. 23 (3), 265–272.

Hall, J.M., Flowers, J.M., Morton, I.K., 1992. A pharmacological study of NK1 and NK2 tachykinin receptor characteristics in the rat isolated urinary bladder. Br. J. Pharmacol. 107 (3), 777–784.

Huppertz, N.D., Tolba, R.H., Grosse, J.O., 2015. Micturition in Göttingen minipigs: first reference in vivo data for urological research and review of literature. Lab. Anim. 49 (4), 336–344. https://doi.org/10.1177/0023677215570993.

Joos, G., Pauwels, R., van der Straeten, M., 1987. Effect of inhaled substance P and neurokinin A on the airways of normal and asthmatic subjects. Thorax. 42 (10), 779–783.

Kullmann, F.A., Katofiasc, M., Thor, K.B., Marson, L., 2017. Pharmacodynamic evaluation of Lys^5, $MeLeu^9$, Nle^{10}-$NKA_{(4-10)}$ prokinetic effects on bladder and colon activity in acute spinal cord transected and spinally intact rats. Naunyn Schmiedeberg's Arch. Pharmacol. 390 (2), 163–173. https://doi.org/10.1007/s00210-016-1317-4.

Li, X., Liao, L., Chen, G., Wang, Z., Deng, H., 2017. Involvement of opioid receptors in inhibition of bladder overactivity induced by sacral neuromodulation in pigs: a possible action mechanism. Neurourol. Urodyn. 36 (7), 1742–1748. https://doi.org/10.1002/nau.23187.

Lördal, M., Theodorsson, E., Hellström, P.M., 1997. Tachykinins influence interdigestive rhythm and contractile strength of human small intestine. Dig. Dis. Sci. 42 (9), 1940–1949.

Lördal, M., Navalesi, G., Theodorsson, E., Maggi, C.A., Hellström, P.M., 2001. A novel tachykinin NK2 receptor antagonist prevents motility-stimulating effects of neurokinin A in small intestine. Br. J. Pharmacol. 134 (1), 215–223.

Marson, L., Thor, K.B., Katofiasc, M., Burgard, E.C., Rupniak, N.M.J., 2018. Prokinetic effects of neurokinin-2 receptor agonists on the bladder and rectum of rats with acute spinal cord transection. Eur. J. Pharmacol. 819, 261–269. https://doi.org/10.1016/j.ejphar.2017.12.017.

Mussap, C.J., Stamatakos, C., Burcher, E., 1996. Radioligand binding, autoradiographic and functional studies demonstrate tachykinin NK-2 receptors in dog urinary bladder. J. Pharmacol. Exp. Ther. 279 (1), 423–434.

Newby, D.E., Sciberras, D.G., Mendel, C.M., Gertz, B.J., Boon, N.A., Webb, D.J., 1997. Intra-arterial substance P mediated vasodilatation in the human forearm: pharmacology, reproducibility and tolerability. Br. J. Clin. Pharmacol. 43 (5), 493–499.

Palea, S., Corsi, M., Artibani, W., Ostardo, E., Pietra, C., 1996. Pharmacological characterization of tachykinin NK2 receptors on isolated human urinary bladder, prostatic urethra and prostate. J. Pharmacol. Exp. Ther. 277 (2), 700–705.

Percie du Sert, N., Holmes, A.M., Wallis, R., Andrews, P.L.R., 2012. Predicting the emetic liability of novel chemical entities: a comparative study. Br. J. Pharmacol. 165 (6), 1848–1867. https://doi.org/10.1111/j.1476-5381.2011.01669.x.

Rizzo, C.A., Hey, J.A., 2000. Activity of nonpeptide tachykinin antagonists on neurokinin a induced contractions in dog urinary bladder. J. Urol. 163 (6), 1971–1974.

Rupniak, N.M., Williams, A.R., 1994. Differential inhibition of foot tapping and chromodacryorrhoea in gerbils by CNS penetrant and non-penetrant tachykinin NK1 receptor antagonists. Eur. J. Pharmacol. 265 (3), 179–183.

Rupniak, N.M.J., Katofiasc, M., Marson, L., Thor, K.B., 2018a. NK2 and NK1 receptor-mediated effects of NKA and analogs on colon, bladder, and arterial pressure in anesthetized dogs. Naunyn Schmiedeberg's Arch. Pharmacol. 391 (3), 299–308. https://doi.org/10.1007/s00210-017-1458-0.

Rupniak, N.M.J., Katofiasc, M., Burgard, E.C., Thor, K.B., 2018b. Colorectal and cardiovascular effects of [Lys^5,$MeLeu^9$,Nle^{10}]-$NKA_{(4-10)}$ in anesthetized macaques. Naunyn Schmiedeberg's Arch. Pharmacol. 391 (9), 907–914. https://doi.org/10.1007/s00210-018-1520-6.

Rupniak, N.M.J., Katofiasc, M., Walz, A., Thor, K.B., Burgard, E.C., 2018c. [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. J. Pharmacol. Exp. Ther. 366 (1), 136–144. https://doi.org/10.1124/jpet.118.248765.

Rupniak, N.M.J., Perdona, E., Griffante, C., Cavallini, P., Sava, A., Ricca, D.J., Thor, K.B., Burgard, E.C., Corsi, M., 2018d. Affinity, potency, efficacy, and selectivity of neurokinin A analogs at human recombinant NK2 and NK1 receptors. PLoS One 13 (10), e0205894. https://doi.org/10.1371/journal.pone.0205894.
Scheepe, J.R., van den Hoek, J., Jünemann, K.P., Alken, P., 2007. A standardised mini pig model for in vivo investigations of anticholinergic effects on bladder function and salivation. Pharmacol. Res. 55 (5), 450–454.
Schelfhout, V., van de Velde, V., Maggi, C., Pauwels, R., Joos, G., 2009. The effect of the tachykinin NK(2) receptor antagonist MEN11420 (nepadutant) on neurokinin A-induced bronchoconstriction in asthmatics. Ther. Adv. Respir. Dis. 3 (5), 219–226.
Schmidt, P.T., Lördal, M., Gazelius, B., Hellström, P.M., 2003. Tachykinins potently stimulate human small bowel blood flow: a laser Doppler flowmetry study in humans. Gut. 52 (1), 53–56.
Templeman, L., Sellers, D.J., Chapple, C.R., Rosario, D.J., Hay, D.P., Chess-Williams, R., 2003. Investigation of neurokinin-2 and -3 receptors in the human and pig bladder. BJU Int. 92 (7), 787–792.
Warner, F.J., Miller, R.C., Burcher, E., 2002. Structure-activity relationship of neurokinin A(4-10) at the human tachykinin NK(2) receptor: the effect of amino acid substitutions on receptor affinity and function. Biochem. Pharmacol. 63 (12), 2181–2186.
Warner, F.J., Miller, R.C., Burcher, E., 2003. Human tachykinin NK2 receptor: a comparative study of the colon and urinary bladder. Clin. Exp. Pharmacol. Physiol. 30 (9), 632–639.