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LP2, the first lanthipeptide GPCR agonist in a human pharmacokinetics and safety study

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ABSTRACT

Introduction of a lanthionine into a peptide may enhance target affinity, target specificity and proteolytic resistance. This manuscript reports preclinical safety studies and the first-in-human study with the lanthipeptide AT₂R agonist LP2, a structural analog of cAng-(1–7), whose N-terminus was protected against aminopeptidases by the presence of a D-lysine. None of the preclinical studies, including an in vitro multitarget panel, behavioral, respiratory and cardiovascular measurements, genotoxicity and toxicity studies in rat and dog, posed any safety concern. Due to lack of toxicity the maximum tolerated dose was not reached neither in rat nor in dog. In the human dose escalation study, healthy male volunteers received a single 1 mL subcutaneous injection (0.001 mg, 0.01 mg or 0.1 mg) of LP2 or matching placebo. In contrast to angiotensin II which has a T_{1/2} in plasma of < 1 min, LP2 has a T_{1/2} of approximately 2.1–2.6 hours. The fraction of the dose excreted unchanged in urine ranged from 84.73 ± 10.4 % at a dose of 0.001 mg to 66.4 ± 3.9 % at 0.1 mg. There were no deaths, serious adverse events or subject withdrawals as a result of an adverse event. The incidence of adverse events was 16.7 %; each was mild in severity. One adverse event, peripheral coldness, was considered to be possibly related to LP2 at 0.001 mg LP2. None of the results was considered to pose a clinically relevant safety concern. This study supports the potential for the therapeutic use of lanthipeptides.

1. Introduction

The decapeptide angiotensin I can be cleaved by several peptidases, and in different order, yielding bioactive peptides that can stimulate angiotensin receptors. One of such peptidases is angiotensin converting enzyme (ACE), a dipeptidylcarboxypeptidase which cleaves off two C-terminal amino acids and thus converts angiotensin I into the octapeptide angiotensin II (Ang II). Another peptidase is angiotensin converting enzyme 2 (ACE2) which cleaves off only one C-terminal amino acid converting Angiotensin I into angiotensin-(1–9) and Ang II into angiotensin-(1–7). Amino peptidases convert for instance Ang II into Ang-(2–8) and Ang-(3–8). The main effector peptide, Ang II, stimulates two receptors: the angiotensin II type1 receptor (AT₁R) and angiotensin II type 2 receptor (AT₂R). Another important peptide is angiotensin-(1–7) whose in vivo activity appears to depend on the Mas receptor [23] and AT₂ receptor [30]. The biological activities of angiotensin-(1–7)

generally are opposed to those resulting from the angiotensin II-stimulated AT₁R activity.

Unbalanced (over)stimulation of the AT₁R may lead to adverse effects like increased blood pressure, inflammation and tissue fibrosis. Most of the AT₁R-mediated detrimental effects may be counterbalanced by stimulation of the AT₂R or the Mas receptor. Ang II has similar affinity for AT₁R and AT₂R but AT₂R is usually expressed at a low level. However, in the foetus AT₂R expression is relatively high [11]. Furthermore in case of tissue injury AT₂R expression is upregulated [21]. Interestingly an AT₂R specific ligand might also induce upregulation of AT₂R [25]. Upregulated AT₂R can subsequently be stimulated by endogenous agonists, or may initiate intracellular pathway activation in the absence of ligand due to its constitutive activity [19]. In many diseases, including myocardial infarction, vascular remodeling, neuronal and metabolic diseases, inflammation and cancer, stimulation of the AT₂R leads to therapeutic effects [26]. However, the natural

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ligand of AT₂R, Ang II, lacks specificity as it also stimulates AT₁R, and lacks resistance to further breakdown by peptidases.

The introduction of a conformational constraint, such as a lanthionine, may confer enhanced resistance to breakdown by peptidases and even more importantly may enhance the target affinity and target specificity. Previously we generated a 4,7 D,L lanthionine-stabilized variant of Ang-(1–7) which was completely resistant to ACE and had a prolonged *in vivo* $t_{1/2}$ and potential for pulmonary delivery [14,27]. Its *ex vivo* vasodilating activity on rat aorta rings could be abolished or reduced by the MasR antagonists D-Pro7-Ang-(1–7) and D-Ala7-Ang-(1–7), respectively [14]. This cyclic Ang-(1–7) exerted significant therapeutic efficacy in a rat model of LPS and ventilation-induced acute respiratory distress syndrome [32], in a hyperoxia rat pup model of acute lung injury [28], in the monocrotaline rat model of pulmonary arterial hypertension [3], in a coronary ligation rat model of myocardial infarction [8], in the transient middle cerebral artery occlusion rat model of cerebral stroke [16], in the ob/ob mouse model of diabetic nephropathy [5] and in a multiple low dose streptozotocin-induced model of type 1 diabetes [15] and in a db/db model of type 2 diabetes [15].

Next, we generated a structural analog of cAng-(1–7), LP2, whose N-terminus was protected against aminopeptidases by the presence of a D-lysine. A CEREP multi-target *in vitro* binding study using competition assays with radiolabeled ligands demonstrated selective binding of LP2 to AT₂R. LP2 exerted cardiopulmonary protective effects in a rat pup model of chronic lung injury [28], which were fully abolished by the concomitant administration of AT₂R antagonist PD123319 [29]. In the following, we report the results of preclinical safety assessments as well as human safety and pharmacokinetics data of a single ascending dose study with LP2 outlined in Fig. 1. Two lanthipeptide antibiotics, so-called lantibiotics, have previously been tested in clinical safety studies. To our understanding, LP2 is the first lanthipeptide GPCR agonist tested in man.

2. Materials and methods

2.1. GMP-grade and non-GMP grade LP2

LP2 occurs as a white to off-white amorphous powder. GMP-grade and non-GMP LP2, dKDRV[dAIHA], in which dK is a D-lysine and in which dA..A is a D,L lanthionine, have been produced by Corden Pharma, Brussels, Belgium. For the clinical study, both the vehicle, 0.9 % w/v sodium chloride, which served as placebo, and the GMP-grade LP2 solution, i.e. LP2 dissolved in this vehicle, were manufactured by Quotient Clinical. For the 28 day toxicity studies in Wistar rat and Beagle dogs, GMP-grade LP2 was weighed, dissolved in PBS (Sigma-Aldrich, St Louis, USA) and filtered through a 0.2 µm filter prior to administration.

In the *in vitro* and *in vivo* micronucleus assays, in the Ames test (test for the ability to induce specific bacterial reverse mutations), and in analytical method validations GMP-grade material was used dissolved in water. In all other assays, non-GMP-grade LP2 was used.

2.2. Preclinical safety tests

2.2.1. Off-target interactions

Studies to assess the specificity of LP2 to AT₂R comprised a 71-target panel for *in vitro* binding studies and a 25-enzyme panel to evaluate potential *in vitro* enzyme activity modulation. Measurements were performed in duplicate using a 10^{−5} molar solution of LP2. The studies were performed at Eurofins Cerep SA, Le Bois L'évêque, 86600 Celle L'Evescault, FRANCE.

2.2.2. Use of rat and dog species for assessments of non-clinical toxicity

AT₂R is highly conserved in mammals. Rats and dogs were selected for assessing non-clinical safety based on high protein sequence similarity between the species specific protein sequence (BLASTP on NCBI website) in comparison to the human sequence (accession number XP_000677.2). As a result, percent sequence identity was calculated at 95.59 % for the dog (XP_022271898.1) and at 92.29 % for the rat (NP_036626.1).

2.2.3. Irwin screen

The potential effects of LP2 on the central nervous system were investigated in male Han Wistar rats. A single intravenous bolus dose of LP2 at 0, 20, 65 or 200 µg/kg, was administered and the animals, 6 animals per dose-group, 24 animals in total, were assessed for a standard battery of behavioral changes pre-dose and at 0.5, 1, 2, 5 and 24 h after dosing. The measurements were performed with the animals in a cage and in an open field and included: spontaneous activity (Central Nervous System excitability: 13 parameters), autonomous functions (8 parameters), gait and muscle tone, motor and sensory reflexes, and temperature. Inter-group comparisons were performed as follows: rank order, count and quantal data were compared at each observation time-point, using Kruskal-Wallis test followed, if statistically significant, by Wilcoxon rank sum test. The tests were performed at WIL Research Europe B.V. Hambakenwetering 7, 5231 DD 's-Hertogenbosch, The Netherlands.

2.2.4. Respiratory function

The effects of LP2 on respiratory function including, respiration rate, inspiratory time, expiratory time, relaxation time, tidal volume, minute volume, peak inspiratory flow, peak expiratory flow, enhanced pause, were investigated in conscious, unrestrained male Han Wistar rats, 8 animals per group, 32 animals in total, following a single intravenous

Fig. 1. Schedule of studies.

bolus administration at dose levels of 0 (vehicle), 20, 65 or 200 µg/kg using whole body barometric plethysmography. Before dosing (30-minute baseline condition), the values of the parameters were continuously collected and averaged for 5 min, at 5-min interval. After dosing with vehicle or LP2, the values of the parameters were continuously collected and averaged at 15-minute intervals, from 30 min up to 240 min. The tests were performed at WIL Research Europe-Lyon, 329 Impasse du Domaine Rozier Les Oncins, 69210 Saint-Germain-Nuelles, France.

2.2.5. Cardiovascular effects in dogs

This study assessed the potential cardiovascular effects of an intravenous (bolus) dose of LP2 at 0 (vehicle), 20, 65 and 200 µg/kg in conscious, telemetered beagle dogs, 4 animals per group, 2 males and 2 females. Each animal served as its own control and received vehicle and the three doses of the test item with a minimal wash-out period of two days between each dose. Parameters measured were body temperature, blood pressure, heart rate and ECGs. The tests were performed at WIL Research Europe-Lyon, 329 Impasse du Domaine Rozier Les Oncins, 69210 Saint-Germain-Nuelles, France.

2.2.6. Toxicity in rat and dog

2.2.6.1. Dose range finding. Dose range finding studies were performed in rats. In Han Wistar rats, LP2 was administered subcutaneously for three days at dose levels of 300 or 1000 mg/kg/day. Each group consisted of 3 females. The following observations and examinations were evaluated: mortality (daily), clinical signs (daily) and body weight (days 1 and 4). In Beagle dogs, LP2 was administered by twice daily intravenous bolus injection for 7 consecutive days at 10 (2 × 5) and 50 (2 × 25) mg/kg/day. Here, each group consisted of 1 male and 1 female. The following observations and examinations were evaluated: mortality, clinical signs (daily), body weight (day 1 of pretest and on days 1, 4 and 7 of treatment), food consumption (daily), blood pressure (days 2 and 4), clinical pathology (at pretest and at the end of the treatment period), macroscopy at termination. Blood was sampled for bioanalysis on days 1 and day 7. Haematologic analyses comprised: white blood cells (differential leukocyte count), red blood cells, reticulocytes, red blood cell distribution width, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets. As clotting parameters, prothrombin time and activated partial thromboplastin time, were measured. Clinical biochemistry parameters were determined in plasma samples, using the AU400® Chemistry System: alkaline phosphatase, γ-glutamyl transferase, lactate dehydrogenase, glutamate dehydrogenase, total protein, albumin, total globulin, albumin globulin ratio, total bilirubin, urea, creatinine, glucose, cholesterol, triglycerides, phospholipids, sodium, potassium, chloride, calcium, inorganic phosphate. The studies were performed at WIL Research Europe B.V., Hambakenwetering 7, 5231 DD 's-Hertogenbosch, The Netherlands.

2.2.6.2. Maximum tolerated dose. LP2 was administered subcutaneously for 7 days to Han Wistar rats and Beagle dogs at dose levels of 5 and 20 mg/kg/day. Each group of rats consisted of 3 males and 3 females; each group of dogs consisted of one male and one female. Viability was tested twice a day; clinical signs were checked three times a day with special attention to the injection sites; body weights were measured on days 1, 4 and 7. For dogs, 2 groups were used, each consisting of 1 male and 1 female. In dogs, but not in rats, food consumption was tested daily during pretest and during treatment. No histology or histopathology was performed. The study was performed at WIL Research Europe B.V. (presently: Charles River Laboratories), Hambakenwetering 7, 5231 DD 's-Hertogenbosch, The Netherlands.

2.2.6.3. Repeated dose toxicity. Four week repeated dose toxicity studies were performed in rats and dogs in compliance with GLP regulations. To Han Wistar rats LP2 was administered subcutaneously for at least 28 days

at dose levels of 0, 0.1, 1.0 and 10 mg/kg/day. In total 4 groups were used, each consisting of 10 animals/sex/group. The following observations and examinations were evaluated: clinical signs (daily), body weight (twice weekly), food consumption (twice weekly), ophthalmoscopic examination (during pretest and week 4), clinical pathology (at the end of the treatment and recovery period), macroscopy at termination, organ weights and histopathology on a selection of tissues. Blood was sampled for toxicokinetic evaluation and formulations were analyzed on day 1 and week 4. Haematologic analyses comprised those mentioned in 2.2.6.2. Clinical biochemistry parameters comprised: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total protein, albumin, total globulin, albumin globulin ratio, total bilirubin, urea, creatinine, glucose, cholesterol, triglycerides, phospholipids, sodium, potassium, chloride, calcium, inorganic phosphate. Urinalysis comprised: volume, specific gravity, color, pH, blood, white blood cells, bilirubin, urobilinogen, protein, ketones, glucose, nitrite, sedimental cells and crystals. Organ samples were collected and subjected to histopathologic analyses. Organ weights were measured. To Beagle dogs LP2 was administered subcutaneously for at least 28 days at dose levels of 0, 0.1, 1.0 and 10 mg/kg/day. Each group consisted of 3 males and 3 females. In addition to the parameters measured for rats as mentioned above in this same section, also ECG was analyzed, and in the blood plasma γ-glutamyl transferase, lactate dehydrogenase, and glutamate dehydrogenase were additionally measured. The studies were performed at WIL Research Europe B.V. Hambakenwetering 7, 5231 DD 's-Hertogenbosch, The Netherlands.

2.2.7. Genotoxicity

2.2.7.1. Bacterial reverse mutation assay. Genotoxicity studies comprised a bacterial reverse mutation assay with 33.3, 100, 333, 1000, 3333 and 5000 µg of LP2 per plate to test the ability of LP2 to induce reverse mutations at selected loci of several tester strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 uvrA in the presence and absence of an exogenous metabolic activation system. This assay was conducted by BioReliance Corporation, 9630 Medical Center Drive, Rockville, MD 20850.

2.2.7.2. In vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes. LP2 was furthermore evaluated in an in vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes obtained from a healthy non-smoking 25 year old female individual using LP2 at 240, 350 and 500 µg/mL, both the absence (4 h and 24 h) and presence (4 h) of an exogenous metabolic activation system. This assay was conducted by BioReliance Corporation, 9630 Medical Center Drive, Rockville, MD 20850.

2.2.7.3. In vivo erythrocyte micronucleus assay in Sprague Dawley rats. The objective of this study was to evaluate LP2 for in vivo clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocyte cells in rat bone marrow. This micronucleus assay is a short-term in vivo cytogenetic assay for detecting chromosomal breakage or spindle malfunction. Vehicle and LP2 at 125, 250 and 500 mg/kg were i.v. administered to male rats, 5 male rats per group. After sacrifice, 24 h after treatment, femoral bone marrow was collected and from the cells slides were prepared. Femoral bone marrow was evaluated with a fluorescence microscope comparing duplicate samples for the presence of polychromatic erythrocytes containing micronuclei. If a clastogenic effect occurs, this may be detected by micronuclei formation during cell division as a result of chromosome breakage or malfunctioning of the mitotic spindle. No LP2-induced effect was measured. This assay was conducted by BioReliance Corporation, 9630 Medical Center Drive, Rockville, MD 20850.

2.3. Clinical study

2.3.1. Study design

The clinical study was conducted at a single center, Quotient Clinical, Mere Way, Ruddington Fields, Nottingham, NG11 6JS, UK, and was conducted in accordance with the guidelines of current Good Clinical Practice including the archiving of essential documents. Approvals from the Ethics Committee and Regulatory Authority (MHRA) were obtained before the study was initiated. This was a, double-blind, randomized, placebo-controlled, single ascending s.c. dose study in healthy male subjects. Randomization and double-blinding was used to enhance the validity of comparisons across dose groups and to reduce bias. A placebo control was used to establish the frequency and magnitude of changes in clinical endpoints that may have occurred in the absence of active treatment. Enrolled subjects were screened over a 4-week period prior to admission on day -1. Eligible subjects were randomized on day 1 to receive a single 1 mL subcutaneous injection of LP2 of 0.001 mg, 0.01 mg and 0.1 mg, or placebo, and remained in-house until discharge 48 h post-dose. Subjects completed the study with a follow-up visit 5–10 days after dosing. Subjects were randomly assigned to receive a single s.c. dose of LP2 or placebo (6 subjects receiving LP2, and 2 subjects receiving placebo for each group) in a sequential dose escalating manner, with each dose studied in a separate group of subjects, and with approximately 2 weeks between dose groups. After each group had been studied, the available blinded PK (i.e. plasma concentrations and interim PK parameter estimations) and safety data, clinical laboratory test results, physical examination findings, injection site assessments, vital signs and 12-lead ECGs, from the preceding groups were reviewed before dose escalation proceeded.

2.3.2. Study objectives

The objectives of the study were to assess the safety, tolerability and PK of LP2 after single s.c. doses in healthy male subjects.

2.3.3. Inclusion and exclusion criteria

Subjects were admitted to the clinical unit on the evening before dosing (day -1), and were dosed on the morning of day 1. Subjects were allowed water up to 1 h before the scheduled dosing time and were provided with 240 mL of water at 1 h post dose. Subjects were in the age range of 18–45 years old with body mass index of 18.0–32.0 kg/m². In order to avoid any interaction with other medication, no co-medication was allowed. The following restrictions were applied: no alcohol during the 24 h prior to screening and 24 h prior to admission until discharge, no food or drinks containing grapefruit, cranberry, caffeine or other xanthines from 24 h prior to admission until discharge, no food containing poppy seeds for 48 h prior to screening and 48 h before admission until discharge, no unaccustomed strenuous exercise from 72 h before the screening visit and then from 72 h prior to admission until discharge.

Subjects were excluded from the study if one or more of the following statements was applicable: subject had received any IMP in a clinical research study within the previous 3 months, subject was a study site employee, or immediate family member of a study site or sponsor employee, history of any drug or alcohol abuse in the past 2 years, regular alcohol consumption >21 units per week, current smoker and those who had smoked within the last 12 months. Further exclusion criteria were: a breath carbon monoxide reading of greater than 10 ppm at screening/admission, current user of e-cigarettes and nicotine replacement products and those who had used these products within the last 12 months, clinically significant abnormal biochemistry, haematology or urinalysis, positive drugs of abuse test result, positive hepatitis B surface antigen, hepatitis C virus antibody or human immunodeficiency virus results, history of psychiatric disorder, cardiovascular, renal, hepatic, chronic respiratory or gastrointestinal disease, serious adverse reaction or serious hypersensitivity to any drug or the formulation excipients, presence or history of clinically significant allergy requiring treatment (hay fever was

allowed unless it was active), donation or loss of greater than 400 mL of blood within the previous 3 months, subjects who were taking, or had taken, any prescribed or over-the-counter drug or herbal remedies in the 14 days before IMP administration.

2.3.4. Safety assessments (incl. Safety lab assessments)

Evaluations included adverse events, haematology and clinical chemistry, urinalysis, electrocardiograms, vital signs (supine and standing systolic and diastolic blood pressure and heart rate), physical findings and other observations related to safety, and pharmacokinetics. Blood samples were analyzed with respect to: creatinine, urea, aspartate, aminotransferase, alanine aminotransferase, gamma glutamyl transferase, alkaline phosphatase, total bilirubin, direct bilirubin (only if total was elevated), albumin, total protein, sodium, potassium, phosphate, bicarbonate, glucose, chloride, creatine kinase, and calcium. Urinalysis comprised: pH, specific gravity, glucose, ketones, nitrites, blood, leukocytes, protein, urobilinogen, and bilirubin. Urine samples were tested for drugs, including drugs of abuse, as follows: amphetamines, barbiturates, benzodiazepines, cocaine, marijuana/cannabis, methadone, methamphetamine, ecstasy, morphine/opiates, phencyclidine, tricyclic and antidepressants.

Screening for HBsAg, HCV Ab and HIV antibody was performed using the clinical chemistry blood sample. A 12-lead ECG was performed after the subject had rested in a supine position for a minimum of 5 min. Subjects underwent a physical examination and the subject's body weight and height was measured. The presence of erythema, swelling, pain and tenderness at the injection site were assessed.

2.3.5. Pharmacokinetics

The plasma and urinary pharmacokinetic behavior of LP2 was studied. Plasma samples were taken pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 36 and 48 h post-dose, and urine samples were collected pre-dose and between 0–12, 12–24 and 24–48 hours post-dose. The analytical method employed for the determination of LP2 in human plasma was validated at York Bioanalytical Solutions Ltd, York, UK. The method employed solid phase extraction followed by liquid chromatography with tandem mass spectrometric detection. The lower limit of quantification of the method was determined as 10.0 pg/mL, using a sample aliquot volume of 500 µL and [LP2 + 10 Da] as the internal standard. Reference [LP2 + 10 Da] of >98 % HPLC purity was synthesized by Almac Sciences, 5 the Fleming Building, Edinburgh Technopole, Milton Bridge, Nr Penicuik, EH26 OBE, UK. The estimation of PK parameters by noncompartmental analysis methods was performed using Phoenix WinNonlin software and trapezoidal method.

2.3.6. Data Quality

All study data recorded in the source workbook were transcribed into a validated database. Quality control, data validation and query resolution were carried out in accordance with Quotient Clinical standard operation procedures.

3. Results

3.1. Preclinical safety studies

Preclinical studies are summarized in [Table 1](#).

3.1.1. Off-target effects

No off-target interactions were observed in the multi-target/multi-enzyme panels.

3.1.2. Irwin screen

There were no observable effects and so the no-observed-effect-level (NOEL) in this study is considered to be 200 µg/kg, i.e. the highest dose tested.

Table 1
Preclinical safety studies on LP2.

Study	Description	Results
Multi-target safety	In vitro binding to 71 targets In vitro activity of 25 enzymes	Selective binding to AT ₂ R No activity modulation
Irwin Screen	Behavioral measurements in Han Wistar rats (s.c.)	No observable effects
Respiratory test	Multiple respiratory parameters in conscious Han Wistar rats including respiration rate, inspiratory time, expiratory time, relaxation time, tidal volume, minute volume, peak inspiratory flow, peak expiratory flow, and enhanced pause (s.c.)	No relevant effects on any of the respiratory parameters
Cardiovascular effects	Body temperature, blood pressure, heart rate and ECGs of conscious, telemetered beagle dogs (s.c.)	No relevant changes observed
Toxicity in rat (multiple parameters)	Dose range finding (s.c.) Maximum tolerated dose (s.c.) 28 day repeated dose (up to 10 mg/kg/day s.c.)	> 1000 mg/kg/day for 3 days > 20 mg/kg for 7 days well tolerated
Toxicity in dog (multiple parameters)	Dose range finding (s.c.) Maximum tolerated dose (s.c.) Maximum tolerated dose (i.v.) 28 day repeated dose (up to 10 mg/kg/day s.c.)	> 1000 mg/kg/day for 3 days > 20 mg/kg/day for 3 days > 50 mg/kg/d for 7 days well tolerated
Genotoxicity	In vitro: mammalian cell micronucleus assay in human peripheral blood lymphocytes In vivo: erythrocyte micronucleus assay in Sprague Dawley rats (i.v.) Ability to induce specific bacterial reverse mutations	No evidence of mutagenicity or clastogenicity was obtained

3.1.3. Respiratory function

No relevant effects on respiratory parameters were observed after administration of LP2 at any of the dose levels tested thus demonstrating that LP2 had no effect on the respiratory parameters.

3.1.4. Telemetry

In an in vivo telemetry study in conscious dogs with single intravenous administration of LP2 at dose levels up to 200 µg/mL, no relevant changes in electrocardiogram (ECG) parameters and blood pressure were observed, including the duration of the QT interval.

3.1.5. Toxicity in rat and dog

3.1.5.1. Dose range finding study in rats and dogs. In rats and dogs, as the administered doses were all well tolerated independent of the route of administration, the MTDs were defined > 1000 mg/kg/day after 3 days administration.

3.1.5.2. Maximum tolerated dose (MTD) in rats and dogs. Following once daily s.c. administration of 5 and 20 mg/kg in rats and dogs for 7 days, no treatment related findings were observed in any of the parameters investigated in the MTD studies with either rat or dog. Therefore the MTD was in excess of 20 mg/kg/day. In dogs, directly after the i.v. dose of 50 mg/kg/d (2 × 25 mg/kg/d) lethargy, salivation and watery discharge from the eyes and mouth was observed, which is often seen in animals treated with a high concentration of an alkaline peptide. The dose was well tolerated and therefore the MTD in dogs in this study is > 50 mg/kg/d after 7 days of administration.

3.1.5.3. Four-week repeated dose toxicity study. The toxicity profile of LP2 has been studied up to 28-days of exposure in rats and dogs after repeated s.c. administrations. No toxicologically relevant findings were obtained. No mortality occurred in this study, nor were any clinically relevant signs noted. In rats no effects at the injection site were seen. In dogs LP2-related non-adverse morphologic alterations were seen at the injections sites in males and females after 28 days of treatment. A dose-dependent increase in incidence and severity of subcutaneous inflammatory infiltrates was observed. These findings, which were mild in nature, correlated with macroscopical red discoloration considered to be the cause of the scratching. After a recovery period of 2 weeks the number of affected injection sites was reduced. Overall, the administration of LP2 by once daily subcutaneous administration was well tolerated in rats and dogs at dose levels up to 10 mg/kg/day, i.e. the highest dose tested.

3.1.6. In vitro and in vivo genotoxicity

No evidence of mutagenicity or clastogenicity was obtained in the studies described in the methods section.

3.2. Clinical study

3.2.1. Demographics and subject disposition

All subjects completed the study. Demographic and baseline characteristics are summarized in Table 2.

3.2.2. Safety findings

There were no deaths or serious or severe adverse events (SAEs) and no subject had withdrawn as a result of an adverse event (Table 3A). Overall incidence of adverse events was low. All AEs, peripheral coldness, application site erythema and pain in extremity, were classed as mild in severity (Table 3B).

3.2.3. Pharmacokinetics

Individual LP2 concentrations were used to calculate pharmacokinetic parameters presented in Table 4 and the plasma concentration profiles over time are presented in Fig. 2. Rapid absorption of LP2 from the injection site was measured with a median T_{max} below 2 h (range 1.25–1.75 h). The exposure increased in an apparent dose-proportional manner. A doubling of dose is predicted to increase exposure by approximately 2-fold; however, as only three dose levels were tested the formal statistical analysis could not conclude proportionality. The inter-

Table 2
Demographic and baseline characteristics for the treatment groups.

	Placebo	LP2 0.001 mg	LP2 0.01 mg	LP2 0.1 mg
Subjects (n)	6	6	6	6
Age (years) ^{a,b}	28.3 ± 6.9 (21–37)	29.3 ± 10.0 (18–45)	38.5 ± 6.1 (28–45)	31.3 ± 9.2 (20–42)
Height ^a (cm)	181.3 ± 8.8 (172–196)	180 ± 4.3 (174–186)	174.8 ± 5.7 (166–182)	177.8 ± 4.6 (173–185)
Weight ^a (kg)	83.47 ± 9.56 (72.9–96.2)	83.18 ± 10.70 (67.2–100.2)	78.87 ± 11.07 (58.4–87.2)	83.05 ± 14.27 (63.9–102.6)
BMI (kg/m ²) ^{a,c}	25.32 ± 1.17 (24.4–27.5)	25.62 ± 2.50 (22.2–29.0)	25.70 ± 2.36 (21.2–28.0)	26.22 ± 3.94 (21.4–31.7)
Race, white (n)	6	6	4 ^d	6

^a Data presented as mean ± SD (range).

^b Permitted age range 18–45 years.

^c Permitted BMI range 18.0–32.0 kg/m².

^d One subject was black and another subject was Asian.

Table 3A

Treatment-emergent adverse events.

	Placebo (n = 6)	LP2 0.001 mg (n = 6)	LP2 0.01 mg (n = 6)	LP2 0.1 mg (n = 6)
Subjects with > 1 TEAE	0	0	0	0
Subjects with 1 TEAE	1	1	1	1
IMP-related TEAE	0	1	0	0
Serious TEAEs	0	0	0	0
Death	0	0	0	0

Table 3B

Incidence of adverse events.

	Placebo (n = 6)	LP2 0.001 mg (n = 6)	LP2 0.01 mg (n = 6)	LP2 0.1 mg (n = 6)
Pain in extremity	1	0	1	0
Application site erythema	0	0	0	1
Peripheral coldness	0	1	0	0

Table 4

LP2 pharmacokinetic parameters.

	LP2 0.001 mg (n = 6)	LP2 0.01 mg (n = 6)	LP2 0.1 mg (n = 6)
T _{max} (h)	1.25 (1.00–1.50)	1.75 (1.00–2.00)	1.50 (1.00–2.00)
C _{max} (pg/mL)	38.1 (14.9 %)	335 (15.7 %)	3300 (19.8 %)
AUC _{0–24} (pg.h/mL)	178 (12.7 %)	1550 (15.7 %)	13,800 (9.3 %)
AUC _{0–t} (pg.h/mL)	125 (8.1 %)	1490 (15.1 %)	13,800 (9.1 %)
AUC _{0–∞} (pg.h/mL)	NC ^a	1520 (17.0 %) [n = 5]	13,900 (10.1 %) [n = 5]
T _{1/2} (h)	2.632 (27.5 %)	2.127 (15.9 %) [n = 5]	2.215 (17.1 %) [n = 5]
CLR (mL/min)	76.5 (42.4 %)	73.6 (27.9 %)	77.7 (7.3 %)

Geometric mean (geometric CV %) values are presented above with the exception of T_{max}, where the median (range) is presented.

^a For subjects who received 0.001 mg LP2, there were too few quantifiable LP2 samples for.

AUC_{0–∞} to be reliably determined; no formal statistical analysis of AUC_{0–∞} could be performed.

NC = Not calculated.

Fig. 2. LP2 plasma concentrations following a single subcutaneous injection to men. The presented data are group mean results $\pm$ SEM.

subject variability (geometric CV %) associated with exposure (C_{max} and AUC) was low across the dose range at approximately 8%–20%. Renal clearance remained consistent (range 73.6–77.7 mL/min), independent of dose. Excretion of LP2 in the urine was the main route of elimination. The fraction of the dose excreted unchanged in urine ranged from $84.73 \pm 10.4\%$ at an s.c. bolus dose of 0.001 mg to $66.4 \pm$

Fig. 3. Intact LP2 retrieved from urine.

After s.c. bolus administration to men, intact LP2 was quantified in the collective urine at 12 h interval. Measurements of the collective urine in the 24 h and the 48 h interval did not reveal a further increase larger than 5%, if any at all. The presented data are group mean results $\pm$ SEM.

3.9% at 0.1 mg (Fig. 3). The plasma half-life of LP2 was approximately 2 h (range 2.127–2.632 h) independent of dose.

4. Discussion

The renin-angiotensin system is a key player of cardiovascular homeostasis [26]. Ang II has comparable affinity for the AT₁R and AT₂R [2] and is highly susceptible to peptidase-mediated breakdown. In the past 70 years Ang II has been administered to 31,281 healthy human subjects in 1124 studies of blood pressure regulation and vascular resistance or in patients with cardiovascular, hepatic, renal and pulmonary diseases, traumatic injuries and shock [4]. Adverse drug reactions observed are comprising exacerbation of asthma and congestive heart failure as well as one fatal cerebral hemorrhage. Among 192 pregnant women treated with AngII and monitored for drug sensitivity during pregnancy, 72 developed preeclampsia [4]. In total, in case studies, cohort studies and one placebo-controlled study, refractory hypotensive shock occurred in 55 of 115 patients [4]. Eight studies reported Ang II dose reductions or discontinuations due to excessive pressor responses [4]. In healthy tissue AT₁R is expressed at a much higher level than AT₂R. Unbalanced stimulation of AT₁R may be the basis of Ang II-induced adverse effects. Therefore, an AT₂R-specific agonist devoid of interaction with AT₁R is expected to offer a substantially improved safety profile compared to Ang II. Indeed, preclinical safety studies and first-in-human trial data with LP2 showed no or only mild adverse events.

Besides on-target adverse events (Type A reactions), off-target toxicities related to immune reactions (Type B reactions; such as site reactions or anaphylaxis) or related to the chemical structure of the drug and its metabolites (Type C and D reactions) may occur in subjects treated with biological agents [1]. By introducing a conformational constraint in a peptide the target specificity of the peptide increases and the off-target reactions are limited. Furthermore, a conformational constraint confers resistance to breakdown by peptidases, thereby limiting the generation of metabolites and metabolite-related off-target reactions.

Introduction of a lanthionine-imposed constraint in a peptide is a non-bulky modification to increase its in vivo specificity and stability. Natural lanthipeptides are lanthionine-containing peptides of about 20–40 amino acids with one or several enzymatically installed lanthionine rings of different sizes. They are produced by micro-organisms and can exert different functions, for instance the inhibition of phospholipase A2 by binding to its substrate phosphatidylethanolamine [18], or inhibition of angiotensin converting enzyme [13]. Many lanthipeptides belong to the subclass of lantibiotics, lanthipeptides with antibiotic activity [6].

Clinical safety of two complex lantibiotics with multiple ring structures, NVB302 [22] and duramycin [34], has previously been evaluated in dedicated phase I trials. A double-blind, placebo-controlled, U.K. Phase I trial in 64 healthy volunteers showed that single and multiple ascending-doses of oral NVB302, an antibiotic for treating *Clostridium difficile*, were well tolerated with negligible systemic absorption of the compound (BioCentury | Aug 13, 2012).

Duramycin interacts with phosphatidylethanolamine and it can uncouple mitochondrial respiration in isolated mitochondria [24]. At concentrations $\geq 0.3 \mu\text{M}$ duramycin affects voltage-gated channels in the hERG assay [33]. However, under therapeutic conditions after administration via inhalation, duramycin plasma concentration stays below 0.5 nM. Duramycin activates an alternative chloride channel in cystic fibrosis nasal and airway epithelia. By this, duramycin bypasses the transmembrane conductance regulator, a chloride channel, which is dysfunctional in cystic fibrosis. A clinical trial with duramycin has been conducted to study whether duramycin is safe, tolerable and induces chloride transport in healthy volunteers and in cystic fibrosis patients. This trial consisted of a placebo-controlled dose escalation study in which duramycin or placebo was administered via intranasal superfusion. Duramycin induced chloride transport in nasal epithelia of healthy volunteers in a dose-dependent manner. In cystic fibrosis patients there was more variability and the duration of the chloride transport might be shorter. No clinically significant changes in toxicity parameters were observed [34].

Lanthionine-ketimine naturally occurs in human brain [10], which might indicate that no generalized toxic effects may be associated to lanthionine-containing peptides. Obviously thioether bridges, which are part of a lanthionine as present in LP2 (dAla-S-Ala), are naturally occurring in man, for instance in the amino acid methionine. The clinical safety of a lanthipeptide receptor agonist has to our knowledge never been studied. LP2 did not show clinically relevant adverse effects in any of the standard preclinical safety tests or in the Phase I clinical trial. Taken together, no data have been obtained that indicate on-target or off-target clinically relevant adverse effects of LP2.

Knowledge about Ang II pharmacokinetics in humans is limited. In healthy men radio-iodinated Ang II was infused intravenously. Based on the radioactivity measurement of the repeatedly collected plasma samples metabolic clearance rate was calculated and subsequently $T_{1/2}$ estimated to be below 1 min [7]. In nonpregnant and pregnant women Ang II was infused for 15 min. Blood samples were collected and Ang II concentration determined with a radioimmunoassay utilizing non-selective anti-serum (cross-reactivity with Ang I, Ang-(2–8), Ang-(3–8) and Ang-(4–8)). The half-life of Ang II, determined by curve stripping method, was similar in nonpregnant and pregnant women averaging 48 and 49 s respectively [17]. In the current study, plasma concentrations of LP2 were quantified using liquid chromatography coupled to tandem mass spectrometry. This analytical setup offered a specific and highly sensitive detection of the drug and its metabolites. We observed a $T_{1/2}$ of about 2 h for LP2 in man. This substantially improved half-life compared to Ang II is most probably related to the D,L lanthionine and N-terminal D-amino acid, leading to an increased resistance of LP2 to peptidase-mediated breakdown. Generally long lasting or continuous exposure of a receptor to an agonist may lead to receptor desensitization. One of the mechanisms of desensitization could be agonist-induced receptor internalization. AT₂R internalization has been reported for a special case following AT₁R/AT₂R heterodimerization in LCK-PK₁ kidney cells [9]. However, likely without heterodimerization, the AT₂ receptor was observed not to internalize after stimulation with Ang II [12,20]. Furthermore, AT₂R stimulation with Ang II induced a concentration-dependent vaso-relaxation without desensitization during the measured interval of 30 min [31]. In cells/tissues, wherein neither agonist-induced receptor internalization nor agonist-induced desensitization takes place, prolonged $T_{1/2}$ of an AT₂R agonist would lead to enhanced pharmacodynamics. The observed $T_{1/2}$ of 2 h could therefore be beneficial from a pharmacodynamic perspective. The here, for the first time, measured pharmacokinetics and safety in man of a lanthipeptide

receptor agonist raise hopes on general opportunities for lanthipeptides as a new class of stable and safe, target-selective receptor agonists.

5. Conclusion

LP2, a selective AT₂R agonist, did not reveal any clinically relevant safety issues in a single ascending dose Phase 1 study in healthy male volunteers. In this study, the terminal half-life of LP2 in plasma was determined at approximately 2 h, which is substantially longer than the previously reported $T_{1/2}$ of Ang II of less than 1 min. The significantly prolonged $T_{1/2}$, along with the fact that LP2 was not associated with any safety concerns, confirms the potential for the therapeutic use of lanthipeptides.

Author contributions

P.N. and G.N.M. wrote the manuscript, A.R. coordinated the clinical study, A.M. supervised the clinical study. All authors have been involved in the study and approved the final manuscript.

Declaration of Competing Interest

Declarations of interest: P.N. and G.N.M. are employees of Lanthio-Pep B.V., which is the owner of a patent on LP2. P.N. and G.N.M. have partial ownership of Lanthio Participatie B.V.

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