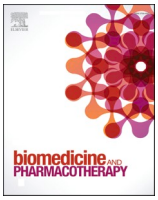
Biomedicine & Pharmacotherapy 171 (2024) 116068

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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



Subcutaneous therapy for portal hypertension: PHIN-214, a partial vasopressin receptor 1A agonist☆

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ARTICLE INFO

Keywords:
Portal hypertension
Cirrhosis
Hepatorenal syndrome
Ascites
Vasopressor
V1a agonist

ABSTRACT

Cirrhosis is a liver disease that leads to increased intrahepatic resistance, portal hypertension (PH), and splanchnic hyperemia resulting in ascites, variceal bleeding, and hepatorenal syndrome. Terlipressin, a prodrug that converts to a short half-life vasopressin receptor 1 A (V1a) full agonist [8-Lys]-Vasopressin (LVP), is an intravenous treatment for PH complications, but hyponatremia and ischemic side effects require close monitoring. We developed PHIN-214 which converts into PHIN-156, a more biologically stable V1a partial agonist. PHIN-214 enables once-daily subcutaneous administration without causing ischemia or tissue necrosis and has a 10-fold higher therapeutic index than terlipressin in healthy rats. As V1a partial agonists, PHIN-214 and PHIN-156 exhibited maximum activities of 28 % and 42 % of Arginine vasopressin (AVP), respectively. The potency of PHIN-156 and LVP relative to AVP is comparable for V1a (5.20 and 1.65 nM, respectively) and V1b (102 and 115 nM, respectively) receptors. However, the EC_{50} of PHIN-156 to the V2 receptor was 26-fold higher than that of LVP, indicating reduced potential for dilutional hyponatremia via V2 agonism compared to terlipressin/LVP. No significant off-target binding to 87 toxicologically relevant receptors were observed when evaluated in vitro at 10 μM concentration. In bile duct ligated rats with PH, subcutaneous PHIN-214 reduced portal pressure by 13.4 % ± 3.4 in 4 h. These collective findings suggest that PHIN-214 could be a novel pharmacological treatment for patients with PH, potentially administered outside of hospital settings, providing a safe and convenient alternative for managing PH and its complications.

1. Introduction

Cirrhosis affects 2.2 million people in the US [1]. The primary causes are viral hepatitis, alcohol-associated liver disease, or, increasingly, metabolic dysfunction-associated steatotic liver disease (MASLD) [2]. As cirrhosis progresses, it disrupts the hepatic sinusoid, leading to increased intrahepatic resistance to blood flow and splanchnic artery dilation, which increases blood flow to the splanchnic area. This results in an increase in portal vein pressure, known as portal hypertension (PH), which is characterized as hepatic venous pressure gradient (HVPG) that is ≥6 mmHg, where normal is considered ≤5 mmHg. HVPG is defined as the difference between the wedged hepatic venous pressure (WHVP) and the free hepatic venous pressure. WHVP is equivalent to hepatic sinusoidal pressure and an estimate of pressure within the portal venous

Abbreviations: PH, portal hypertension; HVPG, hepatic venous pressure gradient; WHVG, wedged hepatic venous pressure; HRS, hepatorenal syndrome; V1a, vasopressin receptor 1A; LVP, Lysine Vasopressin/[8-Lys]-Vasopressin; AVP, arginine vasopressin; IV, intravenous/ly; MPVP, mean portal veinous pressure; BDL, bile duct ligation/ligated; IACUC, Institutional Animal Care and Use Committees; SPPS, solid phase peptide synthesis; PK, pharmacokinetic; SD, Sprague Dawley; SC, subcutaneous/ly; EC50, half maximal effective concentration; V1b, vasopressin receptor 1B; V2, vasopressin receptor 2; TI, therapeutic index; NOAEL, no observable adverse effect level; NOEL, no observable effect level; LOAEL, lowest dose with lethargy and paleness; AEL, lowest dose with ataxia; HED, human equivalent dose; NO, nitric oxide; MAP, mean arterial pressure.

☆ Trial Registration Number: ClinicalTrials.gov Identifier: NCT05490888.

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https://doi.org/10.1016/j.biopha.2023.116068
Received 27 September 2023; Received in revised form 14 December 2023; Accepted 21 December 2023
Available online 4 January 2024

system. PH causes development of porto-systemic shunts (varices) and fluid accumulation in the peritoneal cavity (ascites). It also reduces the circulating blood volume, which may lead to renal vasoconstriction, reduced glomerular filtration, and renal dysfunction (hepatorenal syndrome or HRS), which can eventually result in renal failure and death. Patients with cirrhosis are frequently hospitalized due to complications of PH.

Current pharmacologic treatments include the use of splanchnic vasoconstrictors such as vasopressin receptor 1A (V1a) agonists (terlipressin and vasopressin), or somatostatin receptor agonists (e.g., octreotide). Another approach is to lower splanchnic inflow by using non-selective beta receptor blockers (carvedilol, propranolol, or nadolol). Terlipressin, a synthetic peptide prodrug, effectively reduces PH-driven ascites by vasoconstricting splanchnic arteries and restoring hemodynamic balance [3–7]. It converts to the vasoactive full V1a agonist, lysine vasopressin (LVP), through various tissue peptidases, with a short release delay that improves tolerability and safety compared to native arginine vasopressin (AVP). Terlipressin has been widely used in Europe and other countries for 20 years, demonstrating cost-effective treatment and improved patient survival rates [8–11]. However, its short distribution half-life of only 8-50 min [10] and need for continuous intravenous (IV) infusion or frequent IV administration limit its use to acute care and hospital settings. Side effects include arterial hypertension, nausea, diarrhea, abdominal pain, peripheral ischemia, skin necrosis, and headache [12–16]. To overcome these limitations, we developed a novel partial V1a agonist that can potentially be administered subcutaneously inside or outside of hospital settings. This study evaluates its properties, activity, safety profile, and efficacy in reducing mean portal veinous pressure (MPVP) in a bile duct ligated (BDL) rat model of PH. Compared to a full V1a agonist such as LVP, our findings suggest that this new treatment may provide a safer, more convenient, and cost-effective alternative to current therapies for managing PH and its associated complications.

2. Materials and methods

All animal studies were carried out in accordance with relevant ethical guidelines and approved by Institutional Animal Care and Use Committees (IACUC) of PharmaIN Corp (Bothell, WA, Animal Welfare Assurance number: D20–01079), Pharmaron TSP Beijing Testing Facility (Animal Welfare Assurance number: F16–00202*),* and CorDynamics (Chicago, IL, Animal Welfare Assurance number: D16–00290). All in-vivo studies were considered exploratory.

2.1. Peptide synthesis

Peptides were synthesized by solid phase peptide synthesis (SPPS) with Rink Amide Resin – ProTide (0.59 mmol/g) as the starting solid support (CEM, Matthews, NC) in an Automated Microwave Peptide Synthesizer (LibertyBlue HT12, CEM, Matthews, NC), see S1 Figure in supplement for detailed methods. Each amino acid was sequentially anchored onto the peptide resin using Fluorenyl methoxycarbonyl chemistry to achieve the cyclized protected peptide on resin. The cyclized crude peptide was obtained through acidolysis with 95 % trifluoroacetic acid in the presence of carbocation scavengers and ether precipitation. Finally, the peptide was purified and characterized by reversed phase HPLC (1260 Infinity II Preparative LC Systems, Agilent Technologies, Santa Clara, CA) and the mass is confirmed using an LCMS system (6100 Series Single Quadrupole LC/MS, Agilent Technologies, Santa Clara, CA).

2.2. Pharmacokinetic studies of terlipressin and PHIN-214 and their active metabolites

For the PK study of PHIN-214 and PHIN-156, Male (280–420 g) and Female (200–280 g) Sprague Dawley (SD) rats (Vital River Laboratory Animal Technology, Co. Ltd, Beijing, China) were randomly assigned to groups and treated subcutaneously (SC) between the scapular regions with PHIN-214, (n = 18, 9 F + 9 M; 0.1 mg/kg) dissolved in 15 mM Histidine with 0.9 % NaCl at pH 5.5. Subgroups of n = 6 (3 F + 3 M) were assigned to specific time points used to determine plasma concentrations. Blood samples (0.6 mL) were collected via puncture of retro-orbital plexus in tubes containing K_2EDTA at several time points (0.25-, 0.5-, 0.75-, 1-, 2-, 4-, 6-, and 8-hours post dose), processed into plasma within 60 min of collection, and stored frozen at –75 ± 15 °C until LC-MS/MS analysis (text in S2 Protocol). The calibration curve standards consisted of rat plasma samples from naïve animals spiked with various concentrations of known amount of analyte (PHIN-214 and PHIN-156) and processed similarly by adding internal standards before analysis. Weighted linearity (1/X2) calibration curves were constructed from calibration standard of multiple reaction monitoring results where analyte peak area ratio to IS was plotted as a function of analyte concentration.

For the PK study of terlipressin and LVP, Male SD rats (250–300 g; Charles River, Hollister, CA) pre-cannulated at the jugular vein, were randomly assigned to groups and treated SC between the scapular regions with terlipressin (n = 6; 0.2 mg/kg) dissolved in saline. SC administration was chosen over IV delivery to utilize the skin depot effect. This approach provides a more accurate comparison of the pharmacokinetic properties between PHIN-214 and terlipressin, reflecting their respective absorption and distribution profiles more realistically. Due to vasoconstriction, jugular vein cannulated rats were used to easily withdraw blood. Another cannula was used to dose intravenously. To avoid exceeding the allowed maximum blood volume collection per animal, subgroups of n = 3 were assigned to specific time points. Blood samples (0.5 mL) were collected from the cannula in tubes containing K_2EDTA at several time points (0- (pre-dose), 0.25-, 0.50-, 0.75-, 1-, 1.5-, 2-, and 4-hours post dose), processed into plasma within 30 min of collection, acidified with (1:10, v/v) of 15 % O-phosphoric acid in water, inverted and frozen –75 ± 15 °C until LC-MS/MS analysis (text in S2 Protocol). For calibration curve, rat plasma samples from naïve animals were spiked with various concentrations of known amount of analyte (terlipressin and LVP) and processed similarly by adding internal standards before LC-MS/MS analysis. Data acquisition and processing determined by Analyst 1.6.2 software. Analyte concentrations are obtained from a calibration curve constructed by plotting peak area ratio versus concentration. Concentrations were calculated using linear regression according to the following equation (with $1/x^2$ weighting): $y = ax + b$, where y = peak area ratio of analyte/internal standard, a = slope of the corresponding standard curve, x = concentration of analyte (ng/mL) and b = intercept of the corresponding standard curve.

2.3. Cell based assay for the determination of EC50 and maximum activity relative to AVP

Vasopressin receptor agonist calcium flux assays were performed at Eurofins Cerep lab (Celle-Levescault, France). Chinese Hamster Ovary, CHO, cells expressing human vasopressin receptor 1 A (V1a), 2 (V2), and 1B (V1b) were plated in microplates. Intracellular calcium levels were measured using fluorimetry before and after adding known test article concentrations (0.0128, 0.064, 0.32, 1.6, 8, 40, 200, and 1000 nM). Changes in fluorescence intensity reflected cytosolic calcium concentration. The results were quantified as a percentage of the response to 100 nM AVP for V1b and 1000 nM for V1a and V2, with duplicate assays performed. AVP served as the reference agonist, and the maximum activity of other agonists was expressed as a percentage of the maximum AVP response. EC_{50} values (calculated as half maximum concentration) were determined by non-linear regression analysis of concentration-response curves using GraphPad Prism 9.5.1.

2.4. Effects of PHIN-214 on plasma sodium levels in the rat

Given the affinity for V1a and V2 receptors, we evaluated several concentrations of PHIN-214 in healthy rats to evaluate changes in sodium levels present in the blood. 30 male SD rats (250–300 g; Charles River, Hollister, California; n = 5 per group) were randomized into groups and dosed SC in the scapular region once a day for 2 days with vehicle (15 mM Histidine with 0.09 % NaCl, 5.5–6 pH), or PHIN-214 at 0.05, 0.1, 0.2, 0.3, or 0.4 mg/kg dissolved in vehicle. To avoid masking drug effects, a uniform dose volume of 1.0 mL/kg was administered. The choice of 2 doses was implemented to decrease animal variability and reach a steady state of sodium levels based on concentration. Blood was collected into micro-green top plasma separator tube with gel and lithium heparin at 1.5- and 3-hours post 2nd dose through lateral tail vein puncture. Tubes were transferred directly onto ice packs, following pick up and testing by Antech Diagnostics (Kent, WA). Statistics were calculated using Student's t test analysis against vehicle group on GraphPad Prism 9.5.1.

2.5. Assays for binding activity against 87 toxicologically relevant receptors and enzymes to evaluate specificity and safety

The inhibition assays for receptor binding, channel uptake activity, and enzyme activity (Safetyscreen87) were conducted by Eurofins Cerep (Celle-Levescault, France). The experiment was accepted in accordance with Eurofins validation Standard Operating Procedure, see text in S3 Protocol. This assay package consisted of 87 primary molecular targets including 13 enzymes and 74 receptors binding assays, which are tabulated in S4 Table. In each experiment and if applicable, the respective reference compound was evaluated concurrently with PHIN-214, and the data were compared with historical values of reference compound determined at Eurofins. LVP, PHIN-156, and PHIN-214 were evaluated in duplicates and the mean value was reported. LVP, PHIN-156, and PHIN-214 binding were calculated as a % inhibition of the binding of a radioactively labeled ligand specific for each target. LVP, PHIN-156, and PHIN-214 enzyme inhibition effect were calculated as a % inhibition of control enzyme activity (each receptor result is not shown here). Results showing an inhibition or stimulation higher than 50 % represent significant effects. Fifty percent is the most common cut-off value, and any test articles that do not exceed 50 % threshold in any of the 87 off-target proteins that are critical for drug safety are considered safe.

2.6. Determination of relative therapeutic index between terlipressin and PHIN-214

In this study we used the surrogate therapeutic index (TI) to measure the safety of V1a agonists, defined as the ratio of the highest dose that does not cause lethargy or any other adverse effects (no observable adverse effect level or NOAEL) to the highest dose before observable peripheral vasoconstriction caused by V1a activation (no observable effect level or NOEL), represented as NOAEL/NOEL. This study defines the following symptoms relative to untreated control: peripheral vasoconstriction caused by V1a activation as paleness of the extremities (e.g., feet and ears); lethargy as half-closed eyes, deep breathing, and lack of activity. The NOAEL/NOEL ratio, being a quantitative measure of relative safety, compares the amount of the agent that causes the desired therapeutic effect (peripheral vasoconstriction in this study) to the amount that causes toxicity or lethargy. A V1a agonist that causes severe vasoconstriction can affect not only the peripheral vessels, but also vessels present in other organs, leading to lethargy and ataxia. Therefore, V1a agonists with a high therapeutic index have a wider margin of safety for dosing during treatment. Partial agonists are expected to have a higher therapeutic index than full agonists, which can cause severe vasoconstriction.

In this acute exploratory study, male SD rats (250–300 g; n = 3–6 per group; Charles River, Hollister, CA) were randomized into groups prior to dosing and used to determine NOEL, NOAEL, Low adverse effect level (LOAEL; lowest dose with lethargy and paleness), and adverse effect level (AEL; lowest dose with ataxia) of a single SC dose of PHIN-214 (0.001 to 3.84 mg/kg) or a single IV dose of terlipressin (0.005 to 1.5 mg/kg). Animals were monitored for 8 h post dose and then reassessed after 24 h; all clinical observations were based on direct comparison with untreated control animals. The start and end time of any effects (paleness, lethargy, and ataxia) were recorded, and subsequent doses were adjusted based on observations.

2.7. Local tolerance study

Given that PHIN-214 is a vasoconstrictor administered subcutaneously, an evaluation of the risk of skin necrosis at the injection site was conducted. In a local tolerance 7-Day repeat injection study, male SD rats (250–300 g; n = 6 per group) were randomized into groups and dosed SC in the scapular region once daily for 3 or 7 days with vehicle, or at 0.32, 0.5, or 1 mg/kg (dose concentrations of 3.2, 5, and 10 mg/mL, respectively) to assess local tolerance of PHIN-214. To avoid masking drug effects, a uniform dose volume of 0.1 mL/kg was administered using the Neuros 1700 Series Gas Tight Syringe, 50 μL with removable needle (Cat. #65460–16, Hamilton, Reno, NV). All dose groups were divided into subgroups of n = 3 animals, one group allowed for interim sacrifice to assess any adverse effects of initial injection sites before any potential recovery. Injection sites were observed for erythema, edema, or eschar formation prior to dosing and 1 to 2 h post-dose. The first and last skin injection sites for each animal were harvested and sent to StageBio (Mason, OH) for histopathology.

2.8. Diuretic study in rats

In this study (results represented in Fig. 6), male Wistar Rats (240–350 g; Envigo; Somerset, NJ) were fasted overnight (18 h; free access to water), then to achieve uniform hydration between all animals, rats were administered saline at 20 mL/kg via oral gavage ~45 min prior to dosing. The animals (n = 4 per group) were then treated SC with vehicle (15 mM histidine, pH 5.5 in 0.9 % NaCl for injection), 0.05 mg/kg IV bolus terlipressin (Bachem, Torrance CA), or SC with 0.003, 0.01, 0.03, and 0.08 mg/kg PHIN-214. After being dosed, animals were placed in metabolic cages (Tecniplast; Province of Varese, Italy) and urine was collected and measured at 2-, 4-, and 8-hours post administration of the test articles. Urine volumes were divided by the body weight (pre-dose) times 100 to express in mL/100 g body weight.

In this same study (results represented in Fig. 6), Bile Duct Ligation (BDL) surgery was performed on one group of male Wistar Rats (n = 4; 240–350 g; Envigo; Somerset, NJ), see text in S5 Protocol. On Day 14 following surgery animals were treated as above by saline gavage. Animals were then administered vehicle (15 mM histidine, pH 5.5 in 0.9 % NaCl for injection) SC, and urine was collected to determine the effect of vehicle alone on urine volume and sodium. Following a washout period, on Day 16 these same animals were treated with saline gavage as above and administered 0.08 mg/kg of PHIN-214 SC. Urine was collected to determine the effect of PHIN-214 on urine volume and sodium. Statistics were calculated using Student's t test analysis against vehicle group on GraphPad Prism 9.5.1.

2.9. Efficacy in reducing portal pressure in bile duct ligated portal hypertensive rats

In this study, male SD Rats (240–350 g; Charles River, Hollister, CA) underwent telemeter probe implantation (PhysioTel transmitter, DSI System) and Bile Duct Ligation (BDL) surgery to assess dynamics of mean portal vein pressure, see text in S6 Protocol. A sham BDL group was also included to serve as control (n = 3). The day of surgery was denoted Study Day 1. After recovery from surgery, all animals had their

portal vein pressure monitored continuously via telemetry until Day 22. Due to 2 animals requiring exclusion from the study (one recovered poorly from surgery, and the other had a telemetry malfunction), the final group numbers reflected in the data are n = 4 for BDL groups and n = 3 for sham group. When rats were fully recovered by Day 14 post-surgery, they were dosed with Vehicle (SC route; 15 mM histidine, pH 5.5, 0.9 % NaCl); on Days 16 and 17, rats were administered PHIN-214 SC at 0.012 mg/kg and 0.031 mg/kg, respectively; on Day 21 rats were administered terlipressin IV at 0.041 mg/kg. The MPVP was analyzed by averaging 5-minute intervals during the first 4 h after the dose, and thereafter averaging 1-hour intervals until 24 h. To establish the baseline, we calculated the MPVP at time zero (T0) using the average of 45 to 15 min prior to dosing. The data is presented in absolute value format as well as percentage change from T0 for each day for both the sham and BDL groups.

3. Results

In these studies, we developed a novel chemical entity, PHIN-214, that enhanced the beneficial V1a activity of terlipressin and AVP while decreasing toxicity. Like terlipressin, PHIN-214 is a prodrug that gets converted to a more potent metabolite, PHIN-156 (Fig. 1).

PHIN-156 exhibits a 5-fold higher maximum concentration (Cmax) than LVP at half the concentration of its respective prodrug and 3-fold longer detectable presence in the blood per dose compared to LVP (Fig. 2). When comparing area under the curve (AUC), the calculated AUC for LVP after terlipressin dosed SC at 0.2 mg/kg was divided in half to compare directly to PHIN-156 dosed SC at 0.1 mg/kg. After this adjustment, PHIN-156 exhibits a 14-fold greater blood AUC than LVP. The half-life of PHIN-156, 0.82 h, also surpasses vasopressin which has a very short half-life (T1/2 ~10 min)).

Stimulating potencies of LVP and PHIN-156 on human V1a receptor were similar, with EC50 values at 1.65 and 5.20 nM, respectively. However, at maximal saturation, LVP and PHIN-156 stimulated the V1a receptor at 93 % and 42 % of AVP's maximal saturation activity, respectively (Fig. 3A). The parent molecule, PHIN-214, achieved a maximal saturation activity of 28 % compared to AVP (Fig. 3A and D). PHIN-156, with its longer half-life than PHIN-214, exhibits an EC50 9-fold lower (9-fold more potent) than PHIN-214 (Fig. 3A), requiring lower concentrations of PHIN-156 for V1a receptor activation than PHIN-214. Additionally, exposure to PHIN-156 is 26-fold greater than PHIN-214 (AUC 34.8 and 1.34, respectively; Fig. 2B). Considering exposure/dose and EC50 difference, the biological activity relevance of PHIN-156 is 234-fold greater than PHIN-214, making PHIN-214 a prodrug of PHIN-156. This establishes PHIN-156 as the pharmacologically relevant drug species for future toxicokinetic evaluation of PHIN-214 in animal and human studies.

LVP exhibited 26 times stimulating potency on human V2 receptor compared to PHIN-156, with EC50 values of 0.01 nM and 0.26 nM, respectively (Fig. 3B and D). Yet, at maximal saturation, both compounds exhibited similar stimulatory activity at the V2 receptor (96 vs. 99 %, Fig. 3B and D). The maximum stimulating activities of LVP, PHIN-156, and PHIN-214 ligands on the V1b receptor were also similar, with percentages of 115 %, 102 %, and 99 %, respectively (Fig. 3C and D).

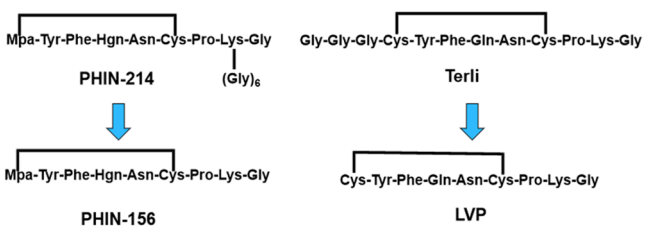


Fig. 1. Conversion of PHIN-214 to PHIN-156 and terlipressin to LVP in vivo. Shown is a novel chemical entity, PHIN-214 prodrug conversion to a more active metabolite, PHIN-156 and conversion to terlipressin prodrug to its active metabolite, LVP.

Based on affinity data for V1a and V2 receptors, we explored the relationship between drug dose and corresponding levels of sodium concentration measured in normal rat plasma (Fig. 4). PHIN-214 caused a decrease in plasma sodium levels at 0.05, 0.1, 0.3, and 0.4 mg/kg compared to vehicle animals. However, at doses ≥ 0.2 mg/kg these effects are less pronounced compared to lower doses.

To ensure preclinical safety, the Safety87 screen was performed to evaluate the adverse effects of PHIN-214, PHIN-156, and LVP on 87 relevant human enzymes and receptors, in order to identify potential off-target binding that could cause unintended adverse effects and toxicity (tabulated in S4 Table). This thorough assessment, commonly performed for regulatory submissions, revealed significant binding of PHIN-214, PHIN-156, and LVP exclusively to the V1a receptor at 10 μM (~13 μg/mL) (Fig. 5), several hundred-fold higher than their respective EC_{50} values for V1a (Fig. 3A). No significant interactions were observed with other targets, indicating a favorable level of specificity (Fig. 5).

Next, we investigated the relative TI of PHIN-214, defined as the ratio of the highest dose that exhibits peripheral vasoconstriction, but with no adverse effects, to the highest dose with no observable effects (i.e., NOAEL / NOEL). As outlined in Table 1, PHIN-214 has an approximately ten times higher relative therapeutic index, compared to terlipressin. Moreover, in a dermatological tolerance study, we confirmed that PHIN-214 does not cause injection site necrosis in rats, even after repeated SC administration for 7 days at up to 1 mg/kg. This translates to a Human Equivalent Dose (HED) of 0.166 mg/kg (based on the ratio of the body areas, a dose 12.5-fold higher than the estimated therapeutic dose for humans (0.0133 mg/kg).

Terlipressin and PHIN-214 activate V2 receptors, inducing antidiuretic effects via their active metabolites LVP and PHIN-156. LVP is 26 times more potent than PHIN-156 in activating V2, as seen in EC_{50} values (Fig. 3B). To assess the impact of terlipressin and PHIN-214 on water and sodium elimination, we conducted in vivo studies in healthy and cirrhotic rats with PH. In healthy rats, low doses of terlipressin (0.05 mg/kg, IV) and PHIN-214 (≤0.01 mg/kg, SC) reduced urine volume compared to vehicle controls, indicating antidiuretic effects. However, higher doses of PHIN-214 (≥ 0.03 mg/kg, SC) led to increased urine output and significantly more absolute sodium excretion compared to vehicle controls (Fig. 6). This pattern persisted in BDL rats treated with PHIN-214 at 0.08 mg/kg, where sodium elimination significantly increased compared to vehicle-treated BDL rats.

Based on receptor activity, we compared the efficacy of PHIN-214 and terlipressin in reducing portal vein pressure in BDL rats with PH in a small exploratory study. To evaluate the success of BDL surgery, we measured mean portal venous pressure (MPVP) in sham and BDL groups (Fig. 7A). BDL rats showed elevated MPVP compared to sham, but with high variability between animals. To account for the high inter-animal variability in MPVP, we compared the relative MPVP change from baseline (T0) up to 4 h post drug administration for each animal; Fig. 7B highlights Day 16 post-surgery where sham and BDL rats were dosed with PHIN-214 SC at 0.012 mg/kg. The percent change of MPVP post treatment from baseline was evaluated for day 16 (PHIN-214 SC, 0.012 mg/kg; sham and BDL), day 17 (PHIN-214 SC, 0.031 mg/kg; BDL), and day 21 (terlipressin IV, 0.041 mg/kg; BDL) post-surgery (Fig. 7C). Indeed, a single SC dose of PHIN-214 (0.012 mg/kg) resulted in a 4-hour average MPVP reduction of 13.4 % (±3.4 %). This effect appeared dose-dependent as PHIN-214 at dose of 13 % of NOAEL (0.031 mg/kg) decreased MPVP by 17.5 % (± 3.64 %). IV dose terlipressin at 82 % of NOAEL (0.041 mg/kg) had less impact on MPVP (–9.38 % ± 3.88 %) (Fig. 7C). While the nadir of the MPVP decrease was observed after 4 h post PHIN-214 administration, MPVP remained lower than baseline after treatment within 23 h (see S7 Figure & S8 Figure).

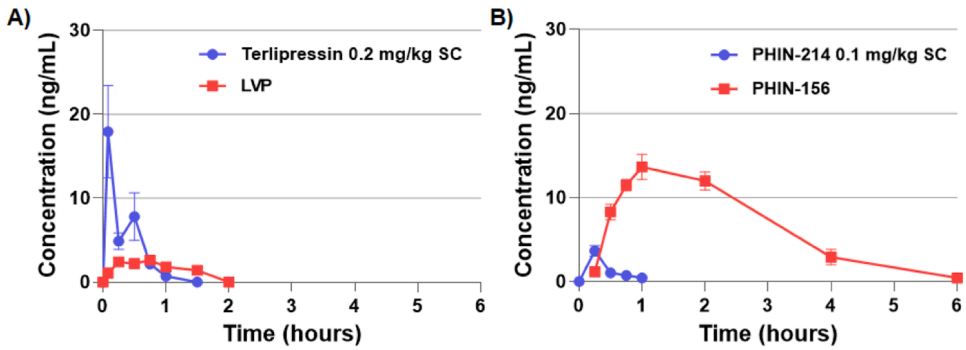
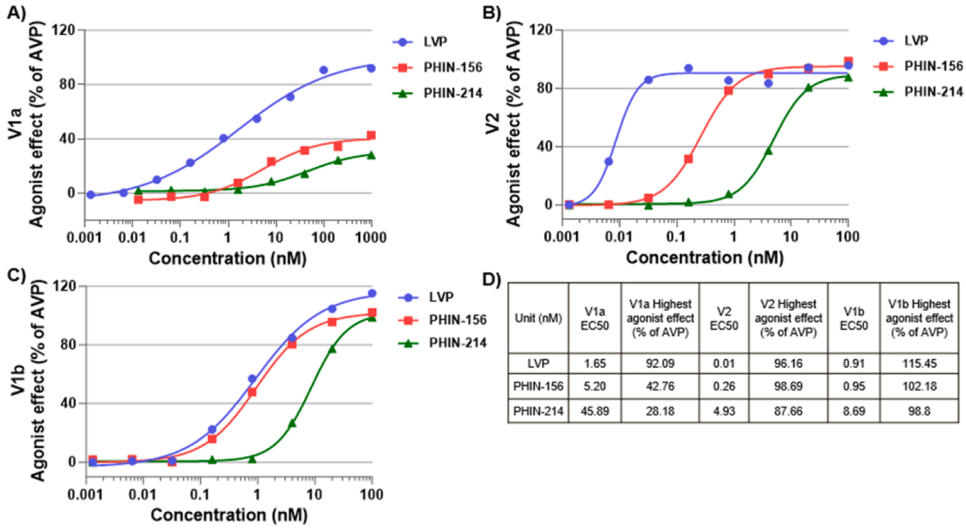


Fig. 2. Based on PK in rats, PHIN-156 is more stable than terlipressin and its metabolite, LVP. Error bars indicate standard error of the mean. **(A)** Plasma concentration of terlipressin and its metabolite, LVP, in SD rats [n = 6] after SC administration of 0.2 mg/kg terlipressin (higher dose used for improved detection). Cmax are 17.93 (± 9.51) and 2.63 (± 0.77) for terlipressin and LVP, respectively. The AUC (hr*ng/mL), adjusted to the 0.1 mg/kg dose by dividing 0.2 mg/kg dose AUC by 2, for terlipressin is 4.4 and for LVP is 2.5. **(B)** Plasma concentration of PHIN-214 and its metabolite, PHIN-156, in SD rats [n = 6] after SC administration of 0.1 mg/kg PHIN-214. Cmax are 13.64 (± 3.64) and 3.65 (± 1.54) for PHIN-156 and PHIN-214, respectively. Based on AUC (hr*ng/mL) at 0.1 mg/kg dose, PHIN-214 is 1.34 and PHIN-156 is 34.8. At half the dose of terlipressin, PHIN-214 metabolite, PHIN-156, exhibits a Cmax that is 5 times higher compared to LVP, blood AUC that is 14 times greater, and detectable presence in the blood of 6 h as opposed to 2 h (3x longer).



Unit (nM)	V1a EC50	V1a Highest agonist effect (% of AVP)	V2 EC50	V2 Highest agonist effect (% of AVP)	V1b EC50	V1b Highest agonist effect (% of AVP)
LVP	1.65	92.09	0.01	96.16	0.91	115.45
PHIN-156	5.20	42.76	0.26	98.69	0.95	102.18
PHIN-214	45.89	28.18	4.93	87.66	8.69	98.8

Fig. 3. PHIN-214 and PHIN-156 showed partial agonism at V1a receptor and higher EC_{50} to V2 receptor. Compound affinity for human vasopressin receptors (V1a, V2, and V1b) was assessed using radioligand binding assays in transfected CHO cells. For the purpose of this study, EC_{50} was calculated as half maximum concentration. **(A)** LVP and PHIN-156 had similar potency in stimulating the human V1a receptor, with V1a EC_{50} values of 1.65 and 5.20 nM, respectively. Yet, PHIN-156, the active metabolite of PHIN-214, has a 9-fold higher potency for V1a than PHIN-214. LVP maximally stimulated the V1a receptor at 93 % of AVP's saturation, while PHIN-156 and PHIN-214 functioned as partial agonists, stimulating the V1a receptor at 42 % and 28 % of AVP's saturation, respectively. **(B)** LVP exhibited 26 times greater potency than PHIN-156 in activating the human V2 receptor, with V2 EC_{50} values of 0.01 nM vs. 0.26 nM, respectively. **(C)** LVP, PHIN-156, and PHIN-214 had similar maximum stimulating activities (as % of AVP) on human V1b, with percentages of 115 %, 102 %, and 99 %, respectively. **(D)** A summary table presents the V1a EC_{50} values and agonist effect (% of AVP) for the human V1a, V2, and V1b figures.

4. Discussion

In PH, reduced effective circulating blood volume [17–20] triggers baroreceptors, leading to increased sympathetic signaling to the kidney and decreased kidney perfusion, which can contribute to kidney failure and HRS [21]. Activation of the V1a receptor helps restore blood pressure sensed by baroreceptors, counteracting this signal, and reducing sympathetic stimulation; this improves compromised kidney perfusion resulting from decreased effective circulating volume. It is important to note that heightened renal sympathetic efferent nerve activity can induce renal ischemia/reperfusion. When combined with angiotensin II (both peripherally and centrally), this leads to reduced nitric oxide (NO) availability, increased production of reactive oxygen species, endothelial dysfunction, and inflammation, ultimately causing renal damage [21].

In managing PH, alternative therapies to V1a agonists (e.g., terlipressin and AVP) have their own limitations. For instance, somatostatin receptor agonists like octreotide, require continuous infusion and offer only temporary efficacy. Beta-blockers (e.g., propranolol, nadolol, and carvedilol), while used to decrease portal pressure, can lead to reduced effective circulating blood volume and hemodynamic imbalances. These effects may adversely impact renal perfusion and function, particularly as the disease progresses. Somatostatin receptor agonists inhibit glucagon and limit the postprandial increase in blood flow to the splanchnic bed, transiently alleviating PH [22]. Beta receptor blockers lower overall systemic pressure or increase NO to reduce PH and improve endothelial health in the intrahepatic circulation [12,23]. As a non-selective beta blocker, carvedilol induces splanchnic arterial vasoconstriction by blocking beta-2 receptors and decreases cardiac output through beta-1 blockade. Moreover, carvedilol's blockade of the alpha-1 receptor results in systemic vasodilation, further lowering overall blood pressure. These combined effects contribute to the

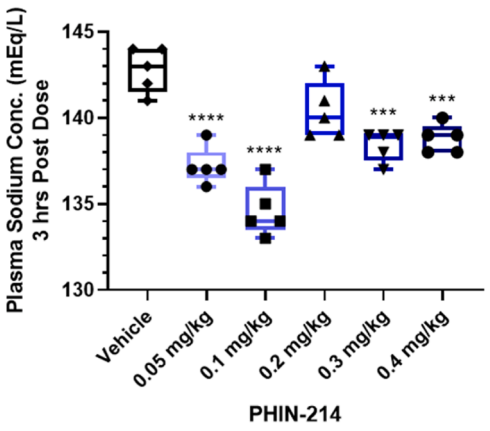


Fig. 4. Sodium levels in rat plasma appear to stabilize at higher concentrations of PHIN-214. Error bars represent minimum and maximum. Plasma sodium levels of male SD rats [n = 5 per group] following 2 SC doses (once a day for 2 days) of vehicle (15 mM Histidine with 0.09 % NaCl, 5.5–6 pH) or PHIN-214 (0.05, 0.1, 0.2, 0.3, 0.4 mg/kg). Blood was collected 3 h post 2nd dose. PHIN-214 caused a significant drop in plasma sodium levels at several dose levels, with the exception of 0.2 mg/kg, compared to vehicle animals. However, at doses $\geq$ 0.2 mg/kg these effects are less pronounced compared to lower doses. Statistical analysis was based on student's t test against vehicle performed by using GraphPad Prism 9.5.1. **** $P < 0.0001$, ***$P < 0.001$.

reduction of PH [13,14], but may worsen kidney injury and HRS under conditions of decreased effective circulating volume. Given these limitations, a new, effective, and self-administered pharmacological treatment that can reduce hospitalizations and healthcare costs would significantly benefit both patients and hospitals.

V1a receptor agonists have shown promise as one of the most effective treatments for PH, with a successful clinical history for over 30 years. They are currently approved in multiple countries, including the European Union, United Kingdom, India, Pakistan, UAE, New Zealand, Australia, and more recently, the United States. Our data supports PHIN-214 as a potential alternative to terlipressin for PH treatment. PHIN-214 acts as a partial agonist of the V1a receptor, reducing the likelihood of severe vasoconstriction observed with terlipressin, which can cause cardiovascular side effects, tissue ischemia, and cutaneous necrosis [24–26]. While V2 activation, along with V1a activation, helps increase systemic blood pressure and address low effective circulating blood volume in PH [19], excessive V2 activation can cause dilutional hyponatremia; this is observed with terlipressin use [15,16]. PHIN-214 has 26-fold lower V2 activity than terlipressin, limiting the potential for V2-induced dilutional hyponatremia. Given the highly similar mechanism of action and the existing approval of terlipressin for human use, it is unlikely that PHIN-214 and PHIN-156 pose any additional safety risks.

Furthermore, we evaluated several dose concentrations in rats with corresponding sodium levels measured in plasma and observed an interesting trend. At higher doses, serum sodium levels tend to stabilize closer to baseline values, exhibiting a less pronounced decline that was observed at lower doses. This dose response of PHIN-214 on plasma sodium levels suggests that at low doses, the drug is more likely to cause hyponatremia, which could be of clinical concern. At dose levels of PHIN-214 reflecting lower plasma sodium concentrations in normal rats, we observed increased urine output and absolute sodium elimination compared to vehicle group in normal and BDL rats. This may be due to pressure-induced resistance to V2-mediated water reabsorption or saturation of V2 receptors at their maximum activity potential, while smooth muscle response to V1a activation and kidney perfusion continued to increase with dose. The observed trends warrant further investigation to understand the underlying mechanisms and safety implications of PHIN-214 effects on sodium homeostasis. The significance of the V1b receptor relative to PH is not yet clear as these receptors are primarily localized in the pituitary and various brain regions [24,25]. Further studies are required to better understand their role. Additionally, increase mean arterial pressure (MAP) in cardiovascular studies done in dogs (S9 Figure A) allow for increased kidney perfusion. The elevation in MAP observed in dogs correlates with enhanced estimated glomerular filtration rate noted after 28 days of daily SC administration of PHIN-214, detailed in S9 Figure B.

This study presents other noteworthy findings, highlighting that PHIN-214 has no off-target effects outside vasopressin receptors at

Table 1
The measure of V1a agonists safety using a surrogate therapeutic index (TI), defined as the ratio represented as NOAEL/NOEL.

Definition	Terlipressin IV*	PHIN-214 SC
NOEL: Highest dose with no observable effects	0.005 mg/kg	0.0023 mg/kg
NOAEL: Highest dose with no observable adverse effect, but with paleness from vasoconstriction.	0.05 mg/kg	0.23 mg/kg
(paleness duration, hour ± SD)	(1.3 ± 0.05)	(6.07 ± 0.08)
LOAEL: Lowest adverse effect level. Lowest dose that exhibits both paleness & lethargy.	0.15 mg/kg	0.38 mg/kg
(lethargy duration, hour ± SD)	(0.66 ± 0.02)	(0.95 ± 0.23)
AEL: Adverse effect level. Lowest dose that showed ataxia.	1.5 mg/kg	3.84 mg/kg
(ataxia duration, hour ± SD)	(0.58 ± 0.06)	(0.74 ± 0.16)
Therapeutic Index NOAEL/NOEL	**~10**	**~100**

*Terlipressin is administered IV due to local vasoconstriction and necrosis from SC administration.

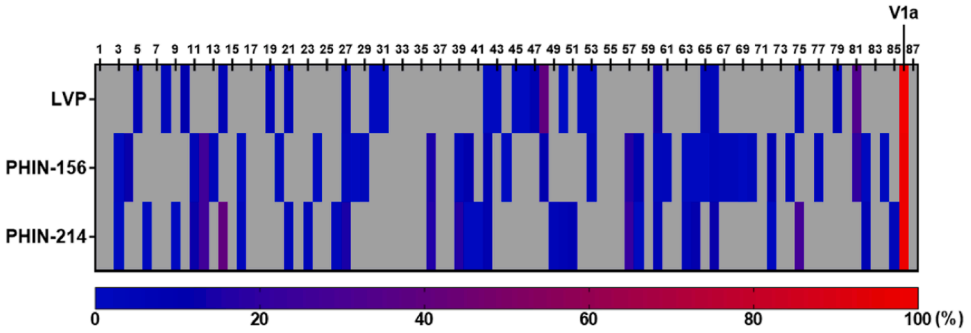


Fig. 5. In the Safetyscreen87, PHIN-214 and its metabolite, PHIN-156, only displayed a significant effect on V1a receptor. The test concentration was chosen at 10 μM (~13 μg/mL), and as expected, LVP, PHIN-156, and PHIN-214 show the highest activity against V1a. The corresponding target number identities are listed in S4 Table. Heat map color denotes red, blue, and grey as high activity, low activity, and negligible activity, respectively. High activity that shows greater than 50 % binding to target indicates a significant effect, which was only observed against V1a receptor with these molecules.

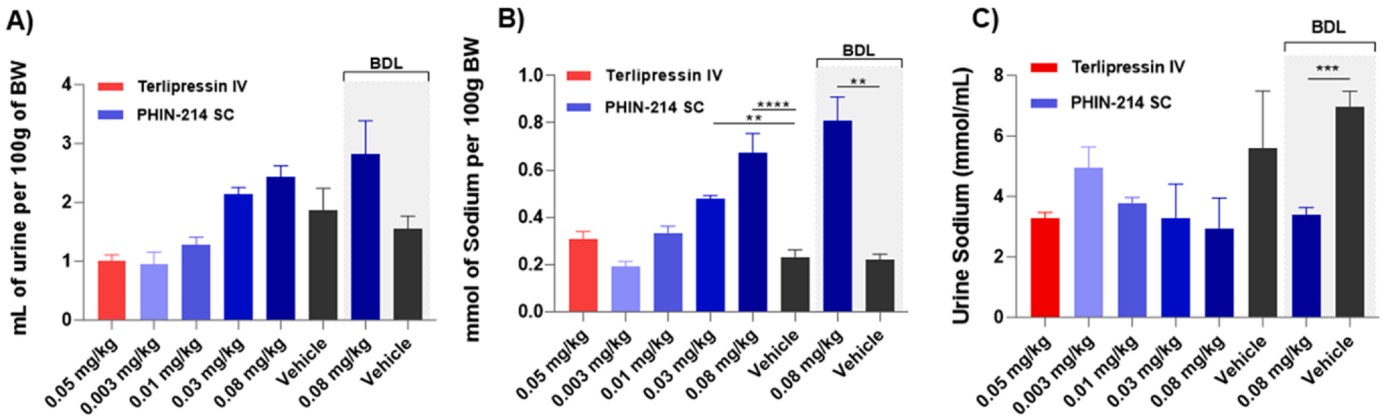


Fig. 6. PHIN-214 showed dose dependent diuretic and sodium excretion effects in rats. Error bars represent standard error of the mean. **(A)** Total volume of urine, **(B)** sodium levels, **(C)** and concentration of urine sodium over 8 h in healthy and cirrhotic (BDL) male Wistar rats [n = 4 per group] after SC administration of vehicle (15 mM histidine, pH 5.5 in 0.9 % NaCl for injection), 0.05 mg/kg IV bolus terlipressin, or SC with 0.003, 0.01, 0.03, or 0.08 mg/kg PHIN-214. Urine volumes were normalized to body weight (pre-dose) and expressed as mL/100 g body weight. In healthy rats, both terlipressin close to NOAEL (0.05 mg/kg) and PHIN-214 below NOAEL (≤ 0.01 mg/kg) have an anti-diuretic effect, as evidenced by a reduction in urine volume relative to the vehicle group. However, we observed that higher doses of PHIN-214 (≥ 0.03 mg/kg) caused an increase in urine output and absolute sodium elimination when compared to vehicle. This finding was consistent in BDL rats treated with PHIN-214 at 0.08 mg/kg, which showed a significant ($P < 0.01$) increase in sodium elimination relative to urine volume compared to vehicle-treated BDL group. Statistical analysis was based on student's t test against vehicle performed by using GraphPad Prism 9.5.1. **** $P < 0.0001$, **$P < 0.01$.

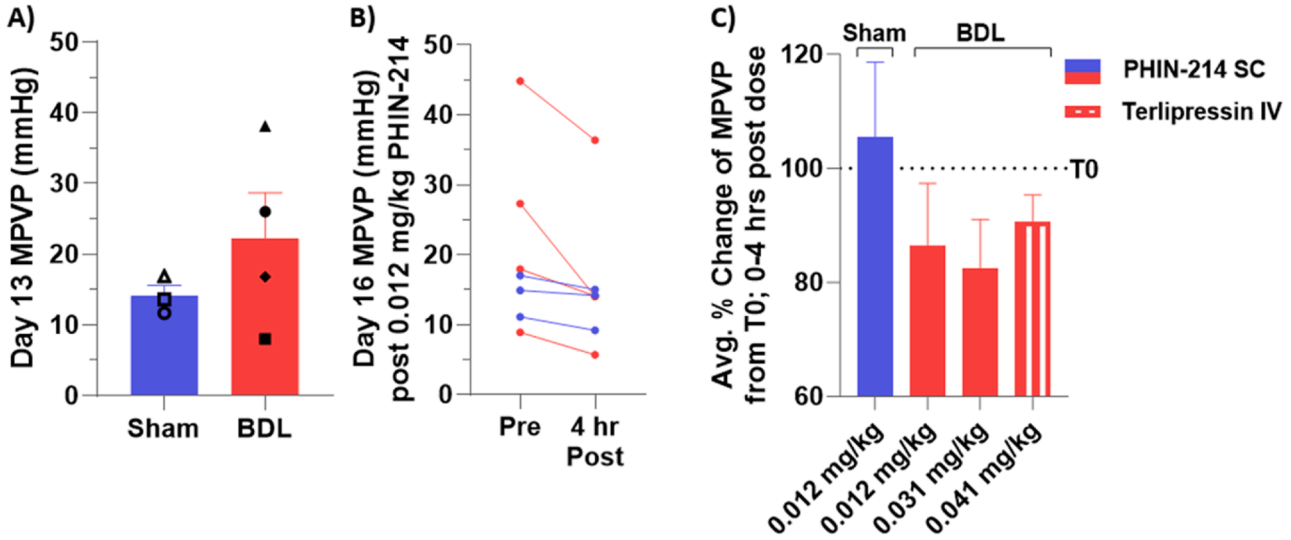


Fig. 7. PHIN-214 is potentially more effective in reducing portal vein pressure than terlipressin at dose below their corresponding NOAEL in rats. Error bars represent standard error of the mean. **(A)** Mean portal veinous pressure (MPVP) of Sham and bile duct ligated (BDL) groups on Day 13 post surgery when rats were fully recovered and prior to dose initiation. MPVP for individual rats between Sham [n = 3] and BDL groups [n = 4] are marked within the figure. **(B)** The measured MPVP of individuals from Sham (in blue) and BDL (in red) groups 4 h post single SC administration of 0.012 mg/kg of PHIN-214, which was scheduled on Day 16 post surgery. Although group means were not statistically different, based on the amount of n per group and the large variability in MPVP, each individual displayed a decrease of MPVP with BDL rats having a more pronounced drop. **(C)** MPVP displayed as a percent change from T0, which was calculated using the average portal venous pressure of 45 to 15 min prior to dosing. Although there was no statistical difference, the drop in MPVP appears to be dose dependent: A single SC dose of PHIN-214 0.012 mg/kg resulted in a 13.4 % ± 3.4 decrease, while a higher dose of 0.031 mg/kg led to a more pronounced drop (17.5 % ± 3.64) in MPVP in BDL rats. Conversely, a single IV bolus dose of 0.041 mg/kg terlipressin had less of an impact on MPVP (–9.38 % ± 3.88). Absolute values are shown on S8 Figure. It is important to note the limitations in this study: 1) A solitary group of BDL animals were used with an incorporated overnight washout period, 2) terlipressin was dosed last in this study (on Day 21), and by this time, the MPVP was similar to the sham group. It is possible that PHIN-214 still had a lasting effect after 24 h despite overnight washout (see S7 Figure & S8 Figure), or that repeat treatment of elevated MPVP using PHIN-214 allowed the condition to improve. In both cases, terlipressin may not have an obvious impact on MPVP that is close to normal.

concentrations several 100-fold higher than the required concentration for maximum V1a saturation. Furthermore, PHIN-214 has a wider therapeutic index than terlipressin, allowing for greater dosing flexibility and safety against severe vasoconstriction. In BDL rats, a single subcutaneous administration of PHIN-214 provided sustained reduction of PH for at least 4 h and displays a greater reduction of MPVP compared to terlipressin, which requires continuous intravenous infusion or more frequent administration. However, it is crucial to acknowledge the limitations of this study. Firstly, only one group of BDL animals were used for all treatments with an incorporated overnight washout between doses. Secondly, terlipressin was administered on the last day of the study, post PHIN-214 treatment. Based on the longer (4-fold) exposure to PHIN-156 compared to LVP, it is possible that repeated doses of PHIN-214 provided a beneficial and lasting effect beyond 24 h despite overnight washout; this trend is supported by tracking MPVP from 4–23 h (see S7 Figure & S8 Figure). It is likely that administering the same, or

higher dose (above NOAEL), of terlipressin to a separate group of BDL portal hypertensive animals without prior exposure to PHIN-214 would result in a similar portal pressure drop as seen with PHIN-214. The lower dose of 82 % of Terlipressin NOAEL was chosen with the fragility of BDL animals in mind. Terlipressin (Terlivaz) dose in human is 0.85 mg every 6 h, so assuming a 70 kg human will require a dose of 0.012 mg/kg, this translates to a rat dose of 0.072 mg/kg. We have not tested 0.072 mg/kg, as it is above the rat NOAEL and can lead to potential side effects that can confound the experimental results. Therefore, while our results are promising and contribute valuable insights into the impact of PHIN-214 compared to terlipressin on MPVP, they should be interpreted with caution and will need to be confirmed in future animal studies or in currently ongoing clinical trials.

Collectively, these findings suggest that PHIN-214 can provide a new pharmacological treatment for human PH that can potentially be administered outside of hospital settings by the patients or their home caregiver, and may provide a safer, more convenient, and hospital cost-saving option for managing PH and its complications.

Funding sources

This work was supported by PharmaIN Corp. and the NIH SBIR Grant # R43DK103553 from National Institute of Diabetes and Digestive and Kidney diseases. There has been no prior publication of the present study.

CRediT authorship contribution statement

Gerardo M. Castillo: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing- original draft, and writing- review & editing. **Yao Yao**: Investigation and Writing- review & editing. **Rebecca E. Guerra**: Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation Visualization, Writing- original draft, and Writing- Review & Editing. **Han Jiang**: Data Curation, Formal analysis, Investigation, Methodology, Project administration, Validation, and Writing- review & Editing. **Akiko Nishimoto-Ashfield:** Data curation and Writing- review & editing. **Alexander V. Lyubimov:** Methodology and Writing- review & editing. **Joshua F. Alfaro:** Validation **Kali A. Striker:** Data curation, Visualization, and Writing- review & editing. **Nikolay Buynov:** Funding acquisition, Resources, and Writing- review & editing. **Philipp Schwabl:** Writing- review & editing **Elijah M. Bolotin:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, and Writing- review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: G.M.C., Y.Y., R.E.G., A.N.A., J.A., K.A.S., and E.M.B. are with financial interest as employees of PharmaIN Corp. A.V.L., N.B., and P.S. are advisors to PharmaIN Corp. with financial interest and/or compensation; H.J. is a former employee of PharmaIN Corp. G.M.C., Y.Y, H.J., A.N.A., and E.M.B. have filed the patents related to composition of PHIN-214 with PharmaIN Corp. (WO2022016064A2).

Data Availability

All relevant data are within the paper. Funding: This research was supported in part by the National Institutes of Health through SBIR grant R43DK103553 to G.M.C. who is an employee of PharmaIN Corp. The funding organization did not play a role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript, and only provided financial support in the form of authors' partial salaries and/or research materials.

Acknowledgments

The authors thank Mr. Tim Strayer and Mr. Jeremy Wik for helping with peptide purification and formulation development. The authors would also like to extend gratitude to Ethan Weinberg, MD for providing his guidance and clinical expertise in hepatology.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.116068.

References

[1] W.R. Caly, E. Strauss, A prospective study of bacterial infections in patients with cirrhosis, J. Hepatol. 18 (3) (1993) 353–358.
[2] W.R. Kim, et al., Burden of liver disease in the United States: summary of a workshop, Hepatology 36 (1) (2002) 227–242.
[3] A. Heinemann, R.E. Stauber, Effect of terlipressin on in vitro vascular hyporeactivity of portal hypertensive rats, J. Hepatol. 24 (6) (1996) 739–746.
[4] F. Vachiery, et al., Hemodynamic and metabolic effects of terlipressin in patients with cirrhosis receiving a nonselective beta-blocker, Dig. Dis. Sci. 41 (9) (1996) 1722–1726.
[5] S.K. Baik, et al., Acute hemodynamic effects of octreotide and terlipressin in patients with cirrhosis: a randomized comparison, Am. J. Gastroenterol. 100 (3) (2005) 631–635.
[6] Y.Y. Huang, et al., Terlipressin resolves ascites of cirrhotic rats through downregulation of aquaporin 2, J. Int. Med. Res. 40 (5) (2012) 1735–1744.
[7] R. Moreau, et al., Comparison of the effect of terlipressin and albumin on arterial blood volume in patients with cirrhosis and tense ascites treated by paracentesis: a randomised pilot study, Gut 50 (1) (2002) 90–94.
[8] A.S. Allegretti, et al., Terlipressin versus placebo or no intervention for people with cirrhosis and hepatorenal syndrome, Cochrane Database Syst. Rev. 6 (6) (2017) p. CD005162.
[9] S.B. Hiremath, L.D. Srinivas, Survival benefits of terlipressin and non-responder state in hepatorenal syndrome: a meta-analysis, Indian J. Pharm. 45 (1) (2013) 54–60.
[10] A.V. Kulkarni, et al., Terlipressin has stood the test of time: clinical overview in 2020 and future perspectives, Liver Int. 40 (12) (2020) 2888–2905.
[11] S.V. Sagi, et al., Terlipressin therapy for reversal of type 1 hepatorenal syndrome: a meta-analysis of randomized controlled trials, J. Gastroenterol. Hepatol. 25 (5) (2010) 880–885.
[12] L. Kalinowski, et al., Third-generation beta-blockers stimulate nitric oxide release from endothelial cells through ATP efflux: a novel mechanism for antihypertensive action, Circulation 107 (21) (2003) 2747–2752.
[13] C. Khouri, et al., Peripheral vasoconstriction induced by beta-adrenoceptor blockers: a systematic review and a network meta-analysis, Br. J. Clin. Pharm. 82 (2) (2016) 549–560.
[14] S.Y. Wong, J. Lee, A.A. Sule, Is carvedilol better than propranolol in portal hypertension? J. Hepatol. (2017).
[15] X. Xu, et al., Development of hyponatremia after terlipressin in cirrhotic patients with acute gastrointestinal bleeding: a retrospective multicenter observational study, Expert Opin. Drug Saf. 19 (5) (2020) 641–647.
[16] A. Poulsen, A. Krag, Severe hyponatraemia to terlipressin treatment, Ugeskr. Laege 175 (39) (2013) 2250–2251.
[17] J. Stange, et al., Industrial stabilizers caprylate and N-acetyltryptophanate reduce the efficacy of albumin in liver patients, Liver Transpl. 17 (6) (2011) 705–709.
[18] B. Barbano, et al., Pathophysiology, diagnosis and clinical management of hepatorenal syndrome: from classic to new drugs, Curr. Vasc. Pharm. 12 (1) (2014) 125–135.
[19] S. Piano, M. Tonon, P. Angeli, Management of ascites and hepatorenal syndrome, Hepatol. Int. 12 (Suppl 1) (2018) 122–134.
[20] A. Mukhtar, H. Dabbous, Modulation of splanchnic circulation: role in perioperative management of liver transplant patients, World J. Gastroenterol. 22 (4) (2016) 1582–1592.
[21] J.W. Osborn, R. Tyshynsky, L. Vulchanova, Function of renal nerves in kidney physiology and pathophysiology, Annu. Rev. Physiol. 83 (2021) 429–450.
[22] A. Escorsell, et al., Desensitization to the effects of intravenous octreotide in cirrhotic patients with portal hypertension, Gastroenterology 120 (1) (2001) 161–169.
[23] R.A. Afonso, et al., Carvedilol action is dependent on endogenous production of nitric oxide, Am. J. Hypertens. 19 (4) (2006) 419–425.
[24] M. Corbani, et al., Neuroanatomical distribution and function of the vasopressin V (1B) receptor in the rat brain deciphered using specific fluorescent ligands, Gen. Comp. Endocrinol. 258 (2018) 15–32.
[25] M. Naganawa, et al., Imaging pituitary vasopressin 1B receptor in humans with the PET radiotracer (11)C-TASP699, J. Nucl. Med. 63 (4) (2022) 609–614.
[26] T.M. Postma, F. Albericio, N-Chlorosuccinimide, an efficient reagent for on-resin disulfide formation in solid-phase peptide synthesis, Org. Lett. 15 (3) (2013) 616–619.