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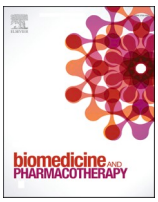

Preclinical pharmacokinetics, pharmacodynamics, and toxicity of novel small-molecule GPR119 agonists to treat type-2 diabetes and obesity

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ABSTRACT

The escalating global prevalence of type-2 diabetes (T2D) and obesity necessitates the development of novel oral medications. Agonism at G-protein coupled receptor-119 (GPR119) has been recognized for modulation of metabolic homeostasis in T2D, obesity, and fatty liver disease. However, off-target effects have impeded the advancement of synthetic GPR119 agonist drug candidates. Non-systemic, gut-restricted GPR119 agonism is suggested as an alternative strategy that may locally stimulate intestinal enteroendocrine cells (EEC) for incretin secretion, without the need for systemic drug availability, consequently alleviating conventional class-related side effects. Herein, we report the preclinical acute safety, efficacy, and pharmacokinetics (PK) of novel GPR119 agonist compounds ps297 and ps318 that potentially target gut EEC for incretin secretion. In a proof-of-efficacy study, both compounds demonstrated glucagon-like peptide-1 (GLP-1) secretion capability during glucose and mixed-meal tolerance tests in healthy mice. Furthermore, co-administration of sitagliptin with investigational compounds in diabetic db/db mice resulted in synergism, with GLP-1 concentrations rising by three-fold. Both ps297 and ps318 exhibited low gut permeability assessed in the *in-vitro* Caco-2 cell model. A single oral dose PK study conducted on healthy mice demonstrated poor systemic bioavailability of both agents. PK measures (mean ± SD) for compound ps297 (C_{max} 23 ± 19 ng/mL, T_{max} range 0.5 – 1 h, $AUC_{0-24\ h}$ 19.6 ± 21 h*ng/mL) and ps318 (C_{max} 75 ± 22 ng/mL, T_{max} range 0.25 – 0.5 h, $AUC_{0-24\ h}$ 35 ± 23 h*ng/mL) suggest poor oral absorption. Additionally, examinations of drug excretion patterns in mice revealed that around 25 % (ps297) and 4 % (ps318) of the drugs were excreted through faeces as an unchanged form, while negligible drug concentrations (<0.005 %) were excreted in the urine. These acute PK/PD assessments suggest the gut is a primary site of action for both agents. Toxicity assessments conducted in the zebrafish and healthy mice models confirmed the safety and tolerability of both compounds. Future chronic *in-vivo* studies in relevant disease models will be essential to confirm the long-term safety and efficacy of these novel compounds.

Abbreviations: ACN, acetonitrile; ALT, alanine aminotransferase; ANOVA, analysis of variance; AUC, area under the curve; AST, aspartate transferase; BPM, beats per minute; BUN, blood urea nitrogen; CMC, carboxymethyl cellulose; Caco-2, colorectal adenocarcinoma-2; DMSO, dimethyl sulfoxide; DPP-IV, dipeptidyl peptidase-IV; DMEM, Dulbecco's modified eagle medium; EEC, enteroendocrine cells; FBS, fetal bovine serum; GPCRs, G protein-coupled receptors; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide; GPR119, G-protein coupled receptor-119; HBSS, Hank balanced salt solution; H&E, hematoxylin and eosin; Hr, hours; Hpf, hours post-fertilization; IpGTT, intraperitoneal glucose tolerance test; Kg, kilogram; LC-MS/MS, liquid chromatography and tandem mass spectrometry; LY, lucifer yellow; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume, mg, milligram; MMTT, mixed-meal tolerance test; OEA, oleoyl ethanolamide; O-LPI, oleoyl-lysophosphatidylinositol; PEA, palmitoylethanolamide; PYY, Peptide YY; Papp, apparent permeability; P-gp, P-glycoprotein; PD, pharmacodynamics; PK, pharmacokinetics; RBC, red blood cells; RA, retinoic acid; SD, standard deviation; SEM, standard error of the mean; SAR, structural-activity relationship; TPs, time points; TEER, transepithelial electrical resistance; T2D, type-2 diabetes; W/v, weight/volume; WBC, white blood cells.

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1. Introduction

Gastrointestinal research continues to elucidate the clinical significance of gut hormones in regulating glucose and energy homeostasis [1], laying the foundation for gut-peptide-based anti-diabetic and weight-loss pharmacotherapies [2,3]. Research progress in this domain is exemplified by the recent regulatory approval of Terzepatide (Mounjaro), a dual incretin receptor agonist for glucagon-like-peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) [4].

Intestinal enteroendocrine cells (EECs), specifically K and L-cells are the principal endogenous source for the incretins GIP and GLP-1, respectively [5]. Abundant evidence suggests the involvement of gut-located G protein-coupled receptors (GPCRs) signalling in the physiological secretion of incretins in response to nutrients or endogenous/synthetic ligands [6,7].

G-protein coupled receptor-119 (GPR119) is a class-A, cannabinoid, Gs-coupled receptor activated by endogenous ligands such as oleoyl ethanolamide (OEA), palmitoylethanolamide (PEA), lysophosphatidylinositol (LPI), lysophosphatidylcholine (LPC), and retinoic acid (RA) [7]. The high expression of GPR119 receptors in pancreatic β-cells and EECs suggests its role in insulin and incretin secretion [8–10]. This dual metabolic regulatory mechanism of GPR119 agonism has garnered research attention to tackle T2D and obesity. Indeed, pharmacological activation of GPR119 signalling by synthetic small molecules is reported to leverage GLP-1 and GIP concentrations in healthy and diabetic populations [11,12]. However, despite enormous scientific efforts, drug development progress in this field is hindered by suboptimal physiochemical/physiological properties of drug candidates and off-target cardiac and central nervous system-related safety concerns that pose a significant challenge. Designing a nutrient-like non-systemic agonist is suggested to address obstacles by targeting intestinal GPR119 receptors for incretin secretion through stimulating EECs from the luminal side [13]. Although the lack of a direct pancreatic β-cell action could limit the desired efficacy, this design presents a safer approach for leveraging physiological incretin secretion that may prove beneficial in chronic settings.

Our previous research revealed that the food-derived lysophospholipid oleoyl-lysophosphatidylinositol (O-LPI) acts as a GPR119 biological ligand that potently stimulates L-cells for GLP-1 release, and pancreatic β-cells for insulin release, by activating the ERK1/2 pathway and the cAMP/PKA/CREB signalling [14]. Furthermore, metabolically stable synthetic small-molecule agents developed as O-LPI-mimetics are capable of leveraging physiological GLP-1 secretion from EECs by interacting with the orthosteric site of GPR119 receptors in the gut [15]. The current study presents acute pharmacodynamics (PD), pharmacokinetics (PK) and toxicity of lead O-LPI-mimetics compounds, ps297 and ps318 in preclinical models that can assist in navigating the future development of these novel GPR119 agonist entities to treat T2D and obesity.

2. Materials and methods

2.1. Drugs, chemicals, reagents, and cell lines

Carboxymethyl cellulose (CMC), D-glucose, Tween-80®, dimethyl sulfoxide (DMSO), atenolol (A7655), propranolol (PO884), digoxin (D6003), and tolbutamide (T0891) were purchased from Sigma-Aldrich, St Louis, MO. Investigated compounds ps297 and ps318 were synthesised as previously described [15]. The human colorectal adenocarcinoma (Caco-2) cell line was purchased commercially (ATCC-HTB-37) and cultured according to standard instructions. Sitagliptin (Januvia®) was purchased from the local pharmacy.

2.2. Experimental animals, housing conditions, and ethics statement

Healthy C57BL6/J and diabetic db/db adult (8–10 weeks) male mice were purchased from the Ozgene (previously Animal Resources Centre) Perth, Western Australia. Animals were acclimatized at Curtin University's animal facility for 1 week. During the acclimatization, all animals received *ad libitum* normal pellet diet and drinking water. On the day of the experiment, animals had *ad libitum* access to drinking water only. All animals were housed in individually ventilated cages at 22 ± 2°C temperature and 55 ± 5 % humidity with 12:12 h light/dark cycle. Animal use was in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes. The study protocols were approved by the institutional animal ethics committee (Approval number: ARE2022-12). Experiments involving zebrafish were conducted at the Western Australian Zebrafish Experimental Research Centre (Biomedical Research Facility, Shenton Park, Perth, Western Australia) under the institutional set protocols. Pharmacokinetic studies were performed commercially at Syngene International Limited (Bangalore, India).

2.3. Quantification of metabolic biomarkers and drug concentrations

Plasmatic total GLP-1 (EZGLP1T-36 K) and insulin (EZRMI-13 K) levels were determined using commercial ELISA kits (EMD Millipore, Germany) according to the manufacturer's instructions. Readings were obtained on an automated multimode 96-well plate reader (PerkinElmer). Blood glucose levels were measured using a commercial blood glucometer (Accu-chek Performa®, Roche Ltd). Blood haematology (BC-2800, Mindray) and plasma biochemistry (Element DC, Heska) were performed on automated instruments. Test drug concentrations in plasma, urine, faeces, and cell culture matrix samples were quantified using fit-for-purpose discovery grade liquid chromatography and tandem mass spectrometry (LC-MS/MS) method (Triple Quad 4500-(LC-MS/MS) system with analyst 1.7.0 & QTRAP 5500- LC-MS/MS system with analyst 1.7.2).

2.4. Drug formulation preparations

A vehicle combination of 1 % Tween80® with 99 % of 0.25 % CMC (w/v) was used to prepare suspension formulations of drugs (ps297 ps318, and sitagliptin). For the experiments employing zebrafish and Caco-2 cells drug stock solutions were prepared in DMSO.

2.5. Statistical methods

Statistical analyses were performed using GraphPad Prism© version 8 (San Diego, USA) through one-way ANOVA and subsequent Tukey's post hoc test. The level of significance ($p < 0.05$) at the 95 % confidence interval was considered statistically significant. Pharmacodynamic data are presented as Mean ± SEM. Pharmacokinetic parameters, including Cmax, Tmax, and AUC0-t, were derived using Phoenix WinNonlin software version 8.2 (Pharsight, Mountain View, CA, USA) via non-compartmental analysis and are expressed as Mean ± SD.

2.6. Pharmacodynamics assessments

In-vivo efficacy of synthetic O-LPI mimetic agents (ps297 and ps318) was evaluated in acute experimental mice models after single-oral dose administration. Glucose lowering and GLP-1 secretion activities were evaluated by an intraperitoneal glucose tolerance test (ipGTT) [16] and a mixed-meal tolerance test (MMTT) [17] on healthy mice. Additionally, the diabetic db/db mouse model was utilized to confirm GLP-1 secretion potential in a disease state [15].

2.6.1. Intraperitoneal glucose tolerance test

Healthy mice were morning fasted for 6 h (hr) and randomized into three treatment arms consisting of 10 animals in each group (n=10). Randomization was done based on basal (-1 hr) tail-vein blood glucose levels measured on a glucometer. Treatment groups received respective

investigational drug formulations (ps297 and ps318) by oral gavage at a 100 mg/kg dosage. The placebo group was given an equal volume (10 mL/kg) of vehicle orally. Post-1 hr of test drug administration, blood glucose levels were measured (0 hr) on a glucometer to rule out the possibility of the development of hypoglycaemia. Immediately, a single bolus injection of glucose (2 g/kg) was intraperitoneally injected into all animals at 10 mL/kg volume. Thereafter, blood glucose levels were monitored in all animals at pre-determined time points (TPs) (0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 hrs). Glucose excursion was calculated by measuring the area under the curve (AUC) from the blood glucose-time profile. For GLP-1 and insulin measurement blood samples (approximately 100 μL) were collected in K_2EDTA-coated tubes containing a dipeptidyl peptidase-IV (DPP-IV) inhibitor cocktail (EMD Millipore, DPP4–010) at 0, 0.25, 0.5, and 1.0 hr TPs by submandibular vein (cheek pouch) puncture method using a sterile lancet (5.0 mm). Plasma was collected from blood samples by centrifugation (at 13,000 rpm for 10 mins at 4°C) and stored at −80°C until further bioanalysis.

2.6.2. Mixed-meal tolerance test

To evaluate the post-prandial GLP-1 secretion capability of test drugs, a mixed-meal tolerance test (MMTT) was conducted on 6 hrs fasted healthy mice (n=5/TP/group). All animals were orally challenged with a mixed meal containing an equal volume of glucose solution (2 g/kg) and olive oil after 1 hr oral pre-treatment with test drugs (100 mg/kg). A placebo control group was orally gavaged with an equal volume (10 mL/kg) of vehicle. Blood glucose levels were measured at −1.0, 0.0, 0.08, 0.16, 0.33, 0.75, 1.0, and 2.0 hrs using a blood glucometer. For GLP-1 estimation, blood samples were collected (at 0.0, 0.08, 0.16, 0.33, 1.0, and 2.0 hrs) and processed as described in the ipGTT protocol.

2.6.3. GLP-1 secretion in diabetic db/db mice

GLP-1 secretion capability of investigational agents was studied in a disease state by utilising a diabetic db/db mice model. In this protocol, drugs under scrutiny were co-administered with a DPP-IV inhibitory mechanism (sitagliptin) to study the combination effect on circulating GLP-1 levels by prolonging the half-life of endogenous GLP-1 peptide [18]. On the day of the experiment, adult (8–10 weeks) male diabetic db/db mice (n=4–5) were morning fasted for 3 hrs. Plasmatic total GLP-1 concentrations were estimated from the blood samples collected at basal (Predose/0 hr), 0.5, and 1.0 hr post-treatment with investigational agents alone (100 mg/kg, oral) and in combination with sitagliptin (20 mg/kg, oral). The difference in GLP-1 levels was represented by subtracting respective basal (Predose) values.

2.7. Pharmacokinetics assessments

PK studies have been performed at Syngene International Limited (Bangalore, India), as described below. The *in vitro* Caco-2 cell monolayer model was used to study the unidirectional gut permeability of test compounds [19]. Single-oral dose (10 mg/kg) PK study was conducted on healthy mice to investigate the drug's systemic bioavailability and excretion. Bioanalytical estimation of the test compounds in biological samples (plasma, urine, faeces, and the aqueous cell culture matrix) was done using the LC-MS/MS method (Supplementary data 1).

2.7.1. Caco-2 cell permeability assay

Test drug transport in the gut absorptive phase (apical to basolateral; A-B) was calculated as apparent permeability (A-B P_{app}) in Caco-2 cells. Briefly, cells (passage no. 30) were seeded onto Corning® 96-well cell culture inserts plates (polycarbonate, 0.4 μm) at a density of 14,300 cells/well in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % foetal bovine serum (FBS) and maintained in an incubator (37°C, 5 %CO_2, 95 %RH) for 21 days. An experiment was conducted using Hank balanced salt solution (HBSS) with donor solution spiked with the test compound (1 μM). Test drug working solutions were prepared in HBSS (pH 7.4) from the DMSO stock solution (1 mM). Atenolol and propranolol (both 10 μM) were tested as references for low and high permeability markers, respectively. Digoxin (10 μM) was used as a substrate for the P-glycoprotein (P-gp) transport pathway. Post-120 mins of assay initiation, samples were collected from the receiver (0.2 mL) and donor (0.06 mL) compartments. Samples (60 μL) were processed by addition and mixing of 200 μL acetonitrile (ACN) with internal standard (Tolbutamide, 500 ng/mL). 100 μL supernatant was obtained by centrifugation at 4000 rpm for 10 mins. The compound flux was determined as drug concentrations employing the bioanalytical (LC-MS/MS) method. The mean apparent permeability of the test compounds (ps297 and ps318) was calculated as -

$$Papp = \frac{dq}{dt} X \frac{1}{Co} X \frac{1}{A}$$

Where dq/dt=rate of transport of compound into the receiver compartment, C_0 =initial concentration of a compound in the donor compartment, and A=surface area of the effective filter membrane.

The percentage recovery was determined at the end of the assay to rule out non-specific loss of the test compounds due to instability or surface adsorption to plastic material using the formula-

$$Recovery\ (\%) = \frac{Total\ compound\ in\ donor\ \&\ receiver\ chamber\ at\ the\ end\ of\ the\ experiment}{The\ initial\ amount\ of\ the\ compound\ loaded\ in\ the\ donor\ chamber} X\ 100$$

Pre- and post-assay, the integrity of the monolayers was monitored by transepithelial electrical resistance (TEER) readings. Additionally, the transport of lucifer yellow (LY) across the Caco-2 cell monolayers was monitored after the incubation period.

2.7.2. Single-oral dose PK study

To study the drug's systemic bioavailability, compound formulations (ps297 & ps318) were orally administered (10 mg/kg) to fasted mice (n=3). For each mouse, approximately 25–30 μL blood sample was collected from the saphenous vein in a tube containing anticoagulant (K_2EDTA) at designated TPs (0.25, 0.5, 1, 2, 4, 6, 8 & 24 h) post-dose. Plasma was separated from blood samples by centrifugation at 13,000 rpm for 10 mins at 4°C and immediately stored at −80°C until further analysis.

To study drug excretion patterns, a separate group of animals (n=3) were individually kept in metabolic cages. Post-dose, urine and faeces samples were collected from each mouse at time intervals of 0–8 h and 8–24 h. Collected samples were immediately stored at −80°C until bioanalysis.

Test drug concentrations were quantified for each plasma, urine, and faeces sample using the LC-MS/MS method. The PK parameters were determined based on the plasma concentration-time profile.

2.8. Toxicological assessments

Compound-related cardiac toxicity and teratogenicity were evaluated in a zebrafish model by exposing embryos to various drug concentrations. An acute repeat dose toxicity was conducted on healthy mice by orally administrating the investigational agents for five consecutive days [20].

2.8.1. Toxicity in zebrafish model

An established experimental protocol was followed for the drug toxicity assessment on zebrafish [21]. Briefly, at 24 hrs post-fertilization (hpf), zebrafish embryos were evenly (10 embryos/well) distributed into each well of the 24-well plate. Subsequently, each well was treated with compound ps297 in an escalating concentration (50, 100, 150, 200, 300, and 500 μM). Drug stock solution was prepared in DMSO while working concentrations were prepared by further dilution in a physiological solution (E2 embryo medium). Thereafter, each embryo was observed at 24 h time intervals and toxicity scores (hatching and mortality) were recorded until 120 hpf. For the heart rate assessment, the embryo was

anaesthetized with tricaine (ethyl 3-aminobenzoate methanesulfonate) at 48 hpf and heartbeats were counted under the stereomicroscope for 1 min. Teratogenicity was assessed by treating embryos with drug treatments starting from 2 hpf and images were captured at 24 hpf. DMSO was used as a vehicle control while Cisplatin was used as a reference control.

2.8.2. Acute toxicity in mice

For acute *in-vivo* toxicological investigations, a repeat oral dose study was conducted on healthy C57BL6/J male mice (n=8). Test drugs (ps297 and ps318) were administered orally (100 mg/kg) for five consecutive days at a *once-a-day* dosing frequency. Animals in a placebo control group were orally gavaged with a drug vehicle at 10 mL/kg volume. During the treatment period, daily body weights, and feed intake were recorded. Animals were observed closely for any signs of drug-related clinical abnormality. Post-treatment period, 6 hrs fasted animals were culled by cervical dislocation under the influence of isoflurane (Piramal Pharma Ltd, India) inhalation anaesthesia. Post-mortem, vital organs (lung, liver, kidneys, heart, and spleen) were isolated and weighed. Immediately, all organs were fixed in a 10 % neutral formalin solution for microscopic investigations. Earlier, terminal blood samples (app 250 μL) were obtained from each mouse by submandibular vein puncture technique. Blood samples collected in K_2EDTA-coated tubes were processed for the biochemical (glucose, triglycerides, cholesterol, alanine aminotransferase (ALT), aspartate transferase (AST), creatinine, albumin, blood urea nitrogen (BUN), total protein, and GLP-1) and haematological (white blood cells (WBC), red blood cells (RBC), lymphocytes, monocytes, granulocytes, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width, platelets, mean platelet volume (MPV), and procalcitonin) assessments. For gross organ histological analysis, samples embedded in paraffin section were cross-cut into slices (4–5 μm), mounted on a glass slide, and stained with hematoxylin and eosin (H&E) for microscopic evaluation (x20) under digital slide scanner Zeiss AxioScan Z.1 (Carl Zeiss GmbH, Germany). Images were captured employing Zeiss Zen software (version 3.8). Organ morphology was blindly evaluated by an experienced veterinary pathologist.

3. Results

3.1. Efficacy of ps297 and ps318

3.1.1. Effect on ipGTT

The impact of synthetic O-LPI mimetic agents, compounds ps297 and ps318 (Fig. 1) on ipGTT was evaluated to assess their effect on glucose homeostasis in healthy mice (Fig. 2A-C). Following a single intraperitoneal glucose injection, a substantial elevation in the blood glucose levels was observed in all animals from their respective basal blood glucose values. Compared to the placebo control arm, mice orally pre-treated with ps297 (100 mg/kg) demonstrated a significant reduction in blood glucose levels at 0.5 hr ($p<0.05$) and 1.0 hr ($p<0.01$) (Fig. 2A), resulting in 30 % reduction of overall glucose excursion, as indicated by lower AUC values (268 ± 20 *vs* 373 ± 41) (Fig. 2B and 2C). Comparatively, compound ps318 exhibited a modest effect, with an overall 18 % reduction in the glucose excursion (Fig. 2A-C). Both ps297 (at 0.25 hr) and ps318 (at 1 hr) significantly ($p<0.05$) stimulated physiological total GLP-1 concentrations (Fig. 2D) during the time course of ipGTT, leading to significant ($p<0.01$; 12.8 ± 0.9 *vs* 21.8 ± 2.6 and $p<0.05$; 12.8 ± 0.9 *vs* 20.3 ± 1.4, respectively) differences in AUC values compared to the placebo control group (Fig. 2E). However, no statistical difference was observed in plasma insulin concentrations (Fig. 2F and 2G).

3.1.2. Effect on MMTT

MMTT was conducted on a cohort of healthy mice to investigate the impact of test drugs on post-prandial glucose homeostasis and incretin secretion capability (Fig. 3). Following an oral mixed meal challenge, vehicle-treated mice exhibited highly elevated blood glucose levels. Conversely, mice pre-treated with synthetic agents demonstrated lower blood glucose values compared to placebo control. Notably, at the 1 hr time-point, blood glucose levels were found to be significantly reduced in mice treated with ps297 ($p<0.05$; 419 ± 16.6 *vs* 289 ± 39.6) and ps318 ($p<0.01$; 419 ± 16.6 *vs* 265 ± 15.6) (Fig. 3A). Although the difference in blood glucose resulted in lower AUC values for these groups (313 ± 38 *vs* 184 ± 60 and 182 ± 36, respectively), the disparity was found to be statically non-significant when compared to the placebo control (Fig. 3B). Furthermore, an increase in plasma GLP-1 concentration was observed in the ps297-treated arm (Fig. 3C-D); however, this difference did not reach statistical significance in the one-way ANOVA test when compared to the placebo control (AUC 33 ± 11 *vs* 70 ± 13). No discernible changes were noted in insulin levels (Fig. 3E-F).

3.1.3. GLP-1 secretion in db/db mice

Fig. 4 depicts the effects of ps297 and ps318 monotherapy (100 mg/kg) and their respective combinations with sitagliptin (20 mg/kg) on circulating GLP-1 levels in diabetic db/db mice. After 1 hr, sitagliptin, ps297, and ps318 alone elevated GLP-1 concentrations in db/db mice from their respective basal values. However, these basal-subtracted GLP-1 concentrations in ps297 and ps318 treated groups were statistically not different from the sitagliptin alone arm (14 ± 6.2 *vs* 30 ± 4.8 and 35.5 ± 8.6, respectively). In contrast, co-administration of sitagliptin and ps297 resulted in significant ($p<0.05$) elevation in GLP-1 levels compared to sitagliptin alone at a 0.5 hr time-point (14.6 ± 6.3 *vs* 60.6 ± 23.7). Similarly, the combined treatment of sitagliptin and ps318 significantly ($p<0.001$ and $p<0.01$) elevated GLP-1 concentrations at 1 hr compared to respective single components (91.6 ± 9 vs 13.9 ± 6.2 and 35.5 ±8.6, respectively) (Fig. 4A). This potentiation effect in combination treatments resulted in a significant ($p<0.01$) increase in the AUC values (39.8 ± 14.7 and 37.7 ± 5.3, respectively) compared to sitagliptin (10.8 ± 5.6), ps297 (13.6 ± 5.5), and ps318 (11 ± 6.7) alone (Fig. 4B).

3.2. PK profiling of investigational compounds ps297 and ps318

3.2.1. In-vitro Caco-2 cell permeability

Table 1 summarises the Caco-2 cell permeability of test compounds. The estimation of gut cell permeability was derived from the permeation rate of compounds ps297 and ps318 across the Caco-2 cells, considering the initial concentration at time zero and the monolayer cell area. Both agents demonstrated low permeability, falling below the limit of quantification (BLOQ; 1 ng/mL) in the absorptive (A to B) direction. The permeability values (P_{app}) for benchmark standards such as atenolol (0.6 $\times 10^{-6}$ cm/sec), propranolol (28.6 $\times 10^{-6}$ cm/sec), and digoxin (1.1 $\times 10^{-6}$ cm/sec) were found to be consistent with established permeability classifications: low (<1); high (>10); moderate (1−10) [22]. The percentage recovery (A to B) for these standard agents was 133.4 %,

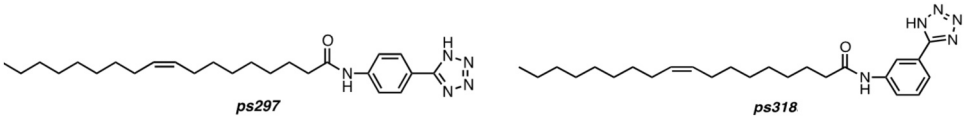

Fig. 1. Chemical structures of synthetic O-LPI mimetic compounds, ps297 and ps318.

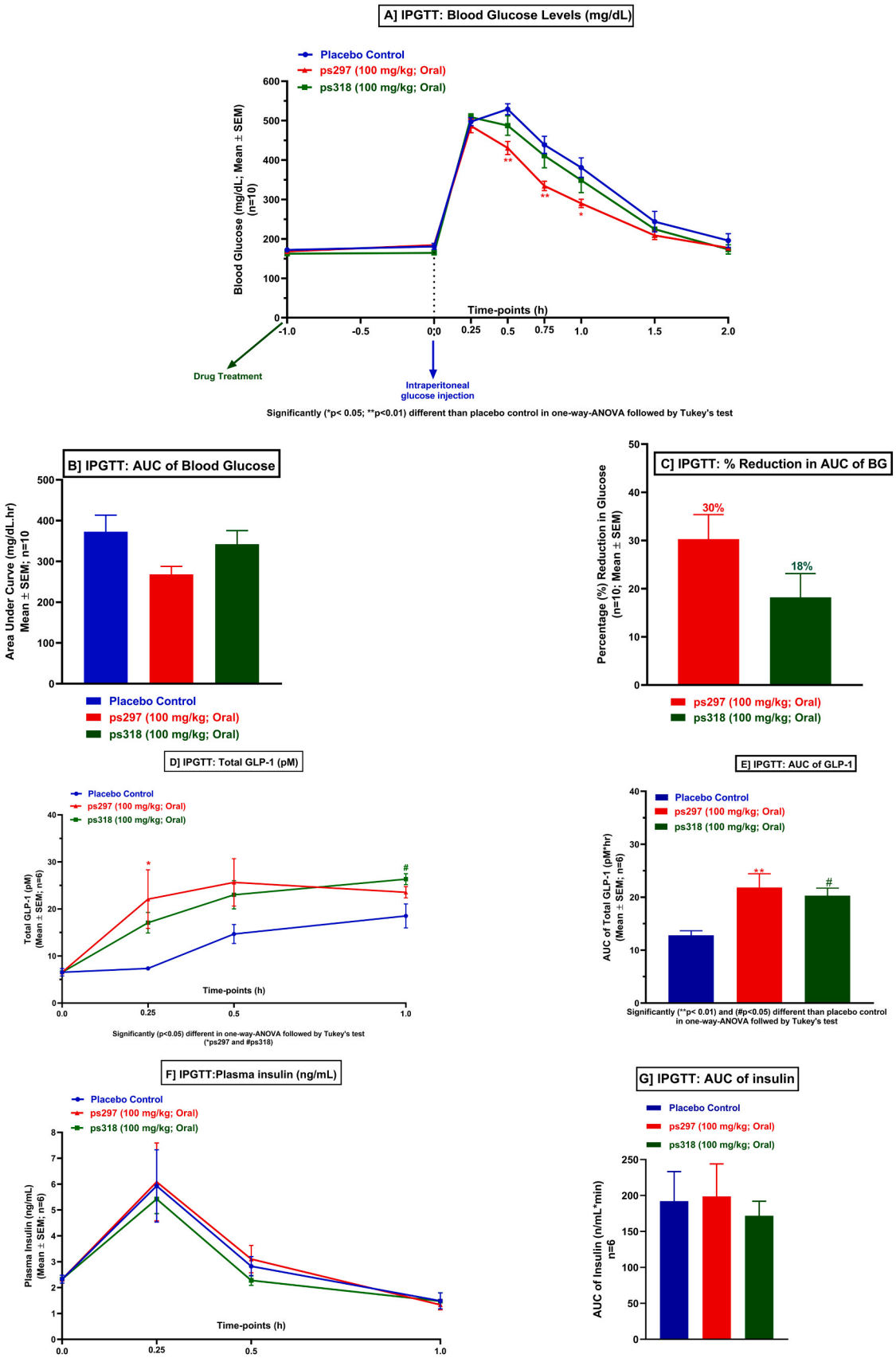

Fig. 2. The effect of synthetic O-LPI mimetics (compounds ps297 and ps318) on ipGTT on healthy mice; A) blood glucose; significantly (*p< 0.05; **p<0.01) different than placebo control in one-way-ANOVA followed by Tukey's test B) AUC of blood glucose, C) percentage reduction in blood glucose, D) plasmatic total GLP-1; Significantly (p<0.05) different in one-way-ANOVA followed by Tukey's test (*ps297 and #ps318), E) AUC of GLP-1, F) plasma insulin, G) AUC of insulin. All data presented as MEAN ± SEM.

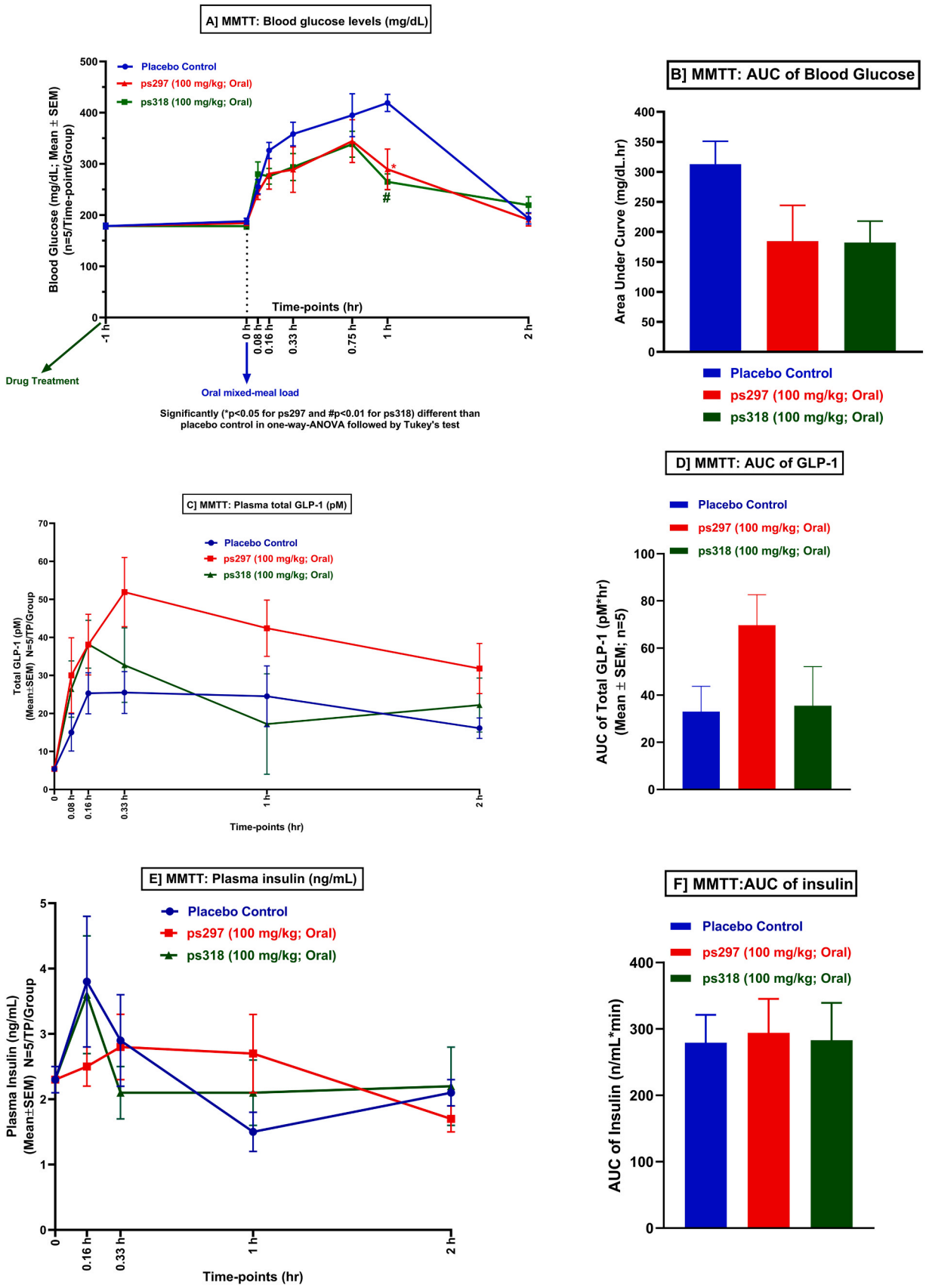

Fig. 3. The effect of compounds ps297 and ps318 on MMTT; A) blood glucose; significantly (*p<0.05 for ps297 and #p<0.01 for ps318) different than placebo control in one-way-ANOVA followed by Tukey's test B) AUC of blood glucose, C) plasma GLP-1, D) AUC of GLP-1, E) plasma insulin, and F) AUC of insulin. Data presented as MEAN ± SEM.

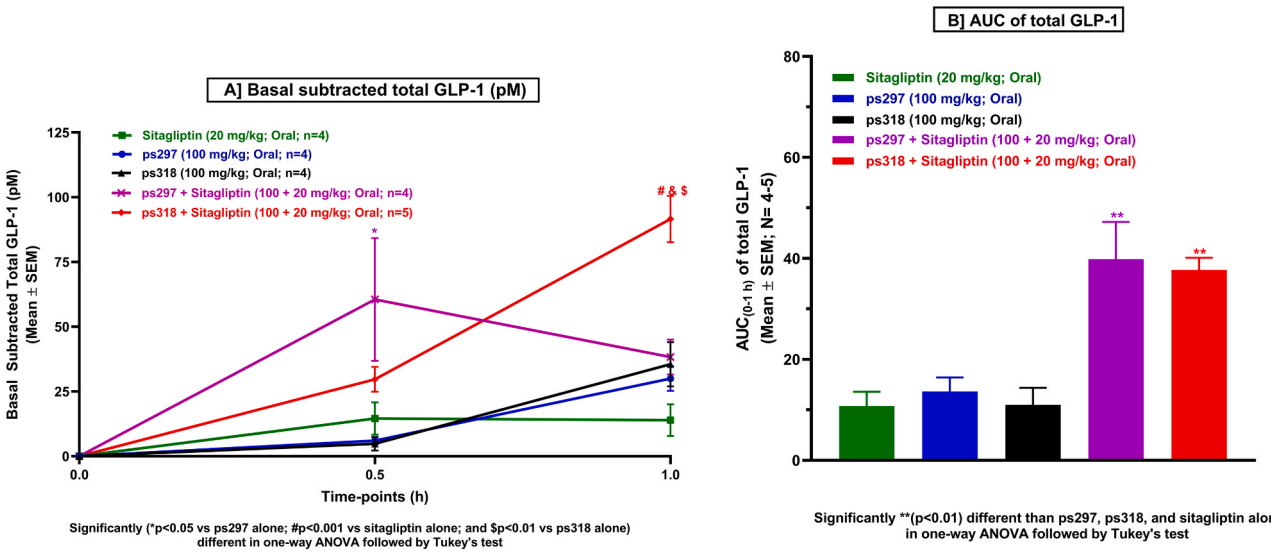

Fig. 4. GLP-1 secretion in db/db mice. A) Basal subtracted total plasmatic GLP-1; significantly (*p<0.05 *vs* ps297 alone; #p<0.001 *vs* sitagliptin alone; and $p<0.01 *vs* ps318 alone) different in one-way ANOVA followed by Tukey's test, B) AUC of basal subtracted GLP-1; Significantly **(p<0.01) different than ps297, ps318, and sitagliptin alone in one-way ANOVA followed by Tukey's test. Data presented as MEAN ± SEM.

Table 1
Unidirectional permeability of ps297 and ps318 across Caco-2 cell monolayers (n=3 replicates).

Compound	Permeability Classification	P_{app} (X 10^{-6} cm/s)	% Recovery	Test Concentration (μM)
		A>B	A>B	
ps297	Low	0.0	14.6	1
ps318	Low	0.0	8.4	1
Atenolol	Low	0.6	133.4	10
Propranolol	High	28.6	138.8	10
Digoxin	Moderate	1.1	94.8	10

Low = <1; High= >10; Moderate = 1–10 Papp (x 10–6 cm/s)

138.8 %, and 94.8 %, respectively. In contrast, the overall recovery for ps297 and ps318 was notably lower (14.6 % and 8.4 %, respectively). Post-assay measurement of TEER values exceeded 200 ohms.cm^2, indicating the maintenance of monolayer integrity throughout the incubation period. Additionally, the LY passage, indicative of paracellular permeability, remained below 1 % in the Caco-2 cell monolayer, further supporting the integrity and reliability of the experimental conditions (Supplementary data 2).

3.2.2. *In-vivo single oral dose PK in mice*

The PK measures (Table 2) of compounds ps297 and ps318 were derived from the plasma concentration-time profile (Fig. 5) following a single oral dose (10 mg/kg) administration in healthy fasted mice.

Compound ps297 exhibited poor oral absorption, as evidenced by a lower C_{max} of 23 ± 19 ng/mL at a median T_{max} of 1 hr. The corresponding AUC $_{(0–24\ h)}$ was 19.6 ± 21 (h*ng/mL). An excretion study in a parallel group revealed that a substantial proportion (25 ± 23 %) of the unchanged drug was eliminated through faeces. Similarly, compound ps318 also demonstrated poor oral availability as indicated by a low C_{max} value (75 ± 22 ng/mL) at a median T_{max} of 0.5 hr. The corresponding AUC $_{(0–24\ h)}$ was 35 ± 23 (h*ng/mL). Notably, a considerable amount of unchanged drug was excreted through faeces (3.9 ± 2.4 %). Due to insufficient drug concentrations detected in plasma, the half-life ($t_{1/2}$) was not calculated for both compounds.

Overall, PK assessment suggests the limited systemic bioavailability of both agents after oral administration. Furthermore, a negligible proportion (<0.005 %) of drugs detected in the urine strengthens the poor bioavailability of these agents in the systemic circulation (Supplementary data 3).

Table 2
Plasma PK and excretion profile of ps297 & ps318 after single-oral dose (10 mg/kg) administration in healthy fasted mice (n=3).

PK measures	Compound ps297	Compound ps318
C_{max} (ng/mL)	23 ± 19	75 ± 22
T_{max} range (hr)	0.5 – 1	0.25 – 0.5
AUC_{0-t} (0–24 h*ng/mL)	19.6 ± 21	35 ± 23
$t_{1/2}$	NC	NC
% recovery in urine	0.0006 ± 0.0001	0.00064 ± 0.0006
% recovery in faeces	25 ± 23	3.9 ± 2.4

Values expressed as MEAN±SD; NC = not calculated due to insufficient plasma drug concentrations

3.3. *Toxicity assessments*

3.3.1. *Toxicity in zebrafish*

We utilized an established zebrafish platform as a screening model to test compound ps297-related toxicity and teratogenicity. Cisplatin, a chemotherapy agent with a known toxic profile was used as a reference. DMSO was used as a solvent and thus served as vehicle control. In this study, only one representative O-LPI mimetic agent (compound ps297) was screened along with other compounds; thus, the data obtained for DMSO and Cisplatin is the same as we reported earlier [21]. The results of one experiment, representative of three, are reported below.

Hatching and mortality rates are widely accepted measures in the zebrafish model while testing substance toxicity [23]. The normal hatching period for zebrafish embryos is around 3 days post-fertilization. At 72 h post fertilization, 100 % of the eggs treated with DMSO at all concentrations were open (Fig. 6A). Conversely, the lowest Cisplatin concentration (50 μM) resulted in only a 20 % hatching at 96 hpf, reaching only 70 % after 120 hpf. At 100 μM and 300 μM concentrations, Cisplatin treatment exhibited 20 % and 10 % embryos hatching at 72 hpf, which never increased over time. Moreover, none of the larvae broke the egg chorion in the presence of the highest Cisplatin (500 μM) concentration tested (Fig. 6B). In a compound ps297-treated plate, 70 % of the control embryos hatched at 48 hpf. At the same time-point, compound ps297 treatment with 50 μM and 100 μM concentrations showed 50 % and 80 % embryo hatching, while higher

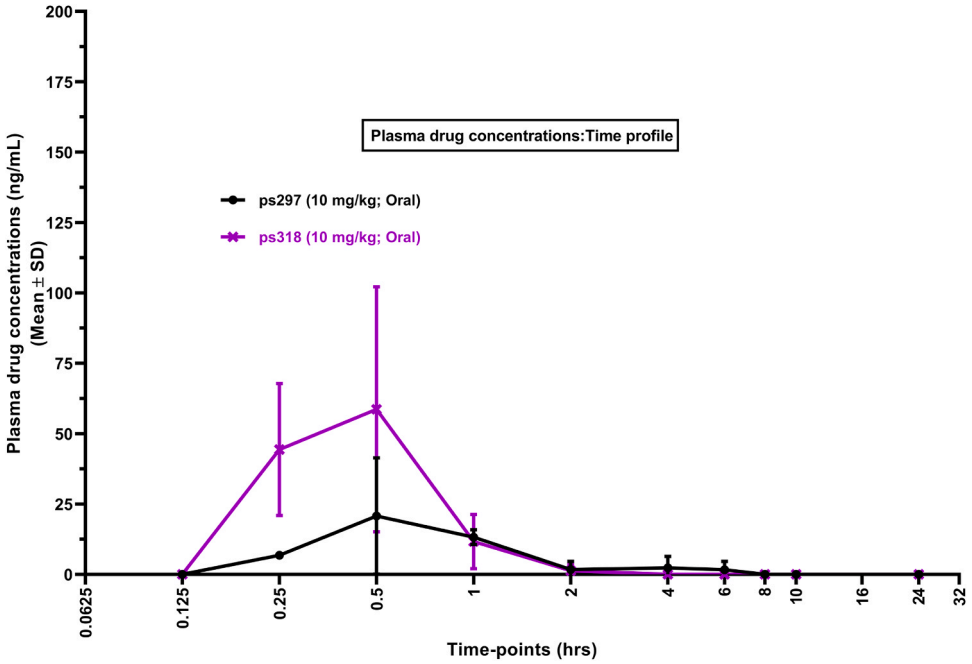

Fig. 5. Plasma drug concentration-time profile after single-oral dose (10 mg/kg) in healthy fasted mice (n=3). Data presented as MEAN ± SD.

concentrations (150, 200, 300, and 500 μM) achieved 90–100 % hatching. Overall, all embryos treated with compound ps297 were found to be completely hatched at 72 hpf (Fig. 6C).

Next, we assessed treatment-related mortality rates throughout 120 hpf. Mortality was observed with the higher (1–5 %) DMSO concentrations tested at varying time points. However, a lower DMSO concentration of 0.5 % was found to be safe with all embryos surviving till 120 hpf (Fig. 6D); hence, the same DMSO concentration was used as a vehicle control to test compound ps297-related toxicity. At 48 hpf, Cisplatin resulted in 100 % mortality with 200, 300, and 500 μM concentrations. However, embryo survival was observed with lower Cisplatin concentrations (50, 100, 150 μM) till 96 hpf. By the end of 120 hpf, 30 % and 80 % mortalities were recorded with 50 and 100 μM concentrations of Cisplatin (Fig. 6E). Interestingly, till 120 hpf, zero mortality was recorded for the zebrafish treated with compound ps297 at all concentrations tested (50, 100, 150, 200 and 300 μM), strengthening the safety profile of this agent (Fig. 6F).

The zebrafish model is an established tool to predict drug-related cardiac toxicity [24]; therefore, we recorded heartbeats in zebrafish embryos exposed to compound ps297 at different concentrations (50, 100, 150, 200, and 300 μM). A control group received medium only (no treatment). 0.5 % DMSO served as a vehicle control. The reported zebrafish embryonic heartbeat range is 140–180 beats per minute (bpm) [24,25]. In this experiment, with the control arm (no treatment), we observed comparatively low heartbeats ranging from 120 to 145 bpm (average 130.6 bpm). However, the DMSO vehicle-treated arm recorded an average heartbeat of 126.6 bpm. Compared to the normal control and DMSO control group, no statistical difference was observed in compound ps297-treated zebrafish heartbeat at all concentrations tested. The average heartbeat noted in ps297 treatment was ranging 125–133 bpm. However, the highest concentration of ps297 (300 μM) recorded an average heartbeat of 122.3 bpm (Fig. 6G).

The effect of treatments on embryogenesis was recorded after 24 hrs of treatment. Treatment was initiated at 2 hpf. Differently from Cisplatin, compound ps297 up to 200 μM concentration did not cause any embryonic developmental defects (Fig. 6H). Overall, the toxicity assessment with compound ps297 in the zebrafish model revealed the safety of the compound and supported its further *in-vivo* testing in mice.

3.3.2. *Acute oral toxicity in mice*

Acute oral toxicity was examined in healthy mice by administering investigational compounds ps297 and ps318 (100 mg/kg) for five consecutive days at a once-a-day dosing frequency. During the treatment phase, body weight and feed intake were recorded daily. Drug treatment effect on vital organs was studied by measuring organ weights (Table 3) and gross morphological changes in histology (Fig. 7). Terminal blood collection was conducted to investigate the impact on haematology (Table 4) and plasma biochemistry (Table 5).

No dose-related mortality or morbidity was observed in the study. Moreover, both investigational agents were found to be well tolerated in healthy mice at the dose tested. At the end of the study, compound ps297 and ps318 treatment resulted in no significant change in body weights (25.4 ± 0.4 *vs* 24.5 ± 0.5 and 24.3 ± 0.3, respectively) when compared to the placebo control arm (Table 3). However, a retarded body weight gain was observed in both treatment arms when compared to their own basal body weight values (Day-0 *vs* Day-05). Over a treatment phase, cumulative food consumption was found to be significantly ($p<0.05$) low (18.1 ± 0.2 *vs* 15.2 ± 0.9) in the ps297 treated mice. Similarly, compound ps318 also resulted in low feed intake compared to placebo control (18.1 ± 0.2 *vs* 15.7 ± 0.8); albeit statistically non-significant. No significant change was observed in major vital organ weights when compared with a placebo control group (Table 3). Furthermore, no clinically relevant treatment-related morphological changes were noted in vital organs during the gross histological observation (Fig. 7). Irrespective of drug treatment, most lung tissues showed artefactual atelectasis which may have potentially resulted from insufficient post-mortal tissue perfusion.

Table 4 summarises the haematological changes observed in healthy mice after repeated administration of investigational compounds ps297 and ps318. Compared to the placebo control group, 5-day repeat administration of both compounds (100 mg/kg) resulted in no significant change in blood hemogram. Furthermore, no clinically relevant drug treatment-related changes were observed in fasted mice plasma samples during the biochemical evaluations (Table 5). Creatinine values were found to be significantly ($p<0.01$ and $p<0.001$, respectively) decreased in compound ps297 and ps318 treated mice compared to a placebo control group (56.5 ± 5.7 *vs* 37.3 ± 1.8 and 30.6 ± 2.7, respectively). Mice treated with investigational agents demonstrated

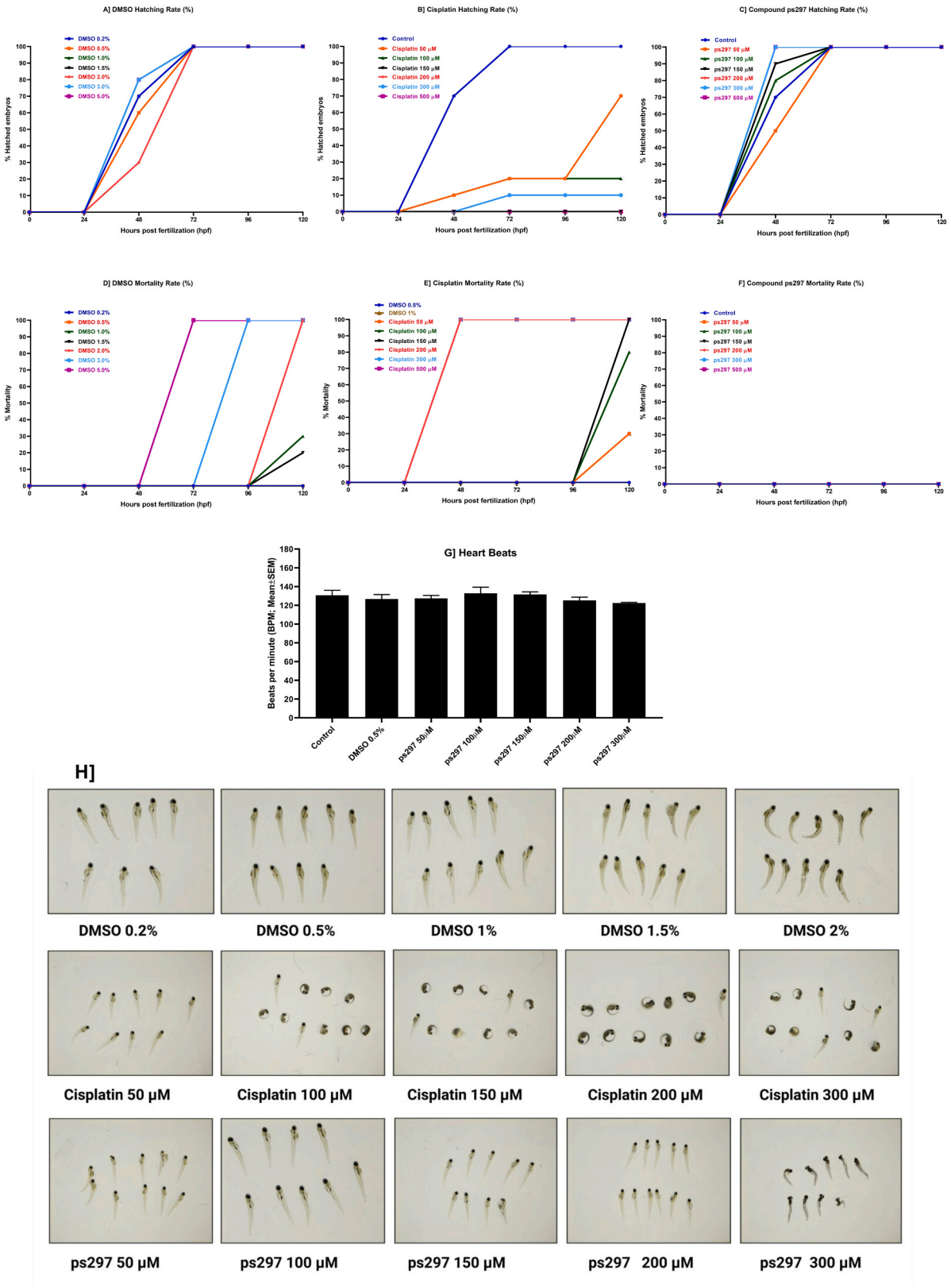

Fig. 6. Toxicity assessment of compound ps297 in zebrafish model. Graphs comparing the effect of a dose-response of DMSO control, Cisplatin, and ps297 on zebrafish hatching (Fig A-C) and embryo mortality (Fig. D-F) from 24 h post fertilisation (hpf) until 120 hpf. Hatching and mortality were expressed as an average of three independent experiments. Effect of dose-response of compound ps297 on zebrafish heartbeats (Fig. G) showing no significant difference up to 300 µM compared to DMSO. Data presented as MEAN ± SEM. Effect of compound ps297 on zebrafish embryo developmental morphology (Fig. H) showing no developmental effect up to 200 µM concentration. Treatment was initiated at 2 hpf and images were taken at 24 hpf. Images presented as representative from respective groups.

Table 3
Effect of O-LPI mimetics on body weight, feed intake, and vital organ weights after repeat (5 days) oral administration in healthy mice (n=8).

Treatment Group (n=8)	Body weight (g)		Cumulative feed intake	Organ weights (% of body weight)				
	Onset (Day-0)	**End (Day-5)**	**(g/mouse)**	**Liver**	**Heart**	**Kidneys**	**Lung**	**Spleen**
Placebo Control (0.25 % CMC; Oral; OD)	24.5 ± 0.3	25.4 ± 0.4	18.1 ± 0.2	4.03 ± 0.14	0.58 ± 0.02	1.12 ± 0.04	0.63 ± 0.02	0.32 ± 0.02
ps297 (100 mg/kg; Oral; OD)	24.5 ± 0.2	24.5 ± 0.5	15.2 ± 0.9*	4.05 ± 0.12	0.52 ± 0.02	1.07 ± 0.04	0.58 ± 0.03	0.31 ± 0.04
ps318 (100 mg/kg; Oral; OD)	24.2 ± 0.2	24.3 ± 0.3	15.7 ± 0.8	3.84 ± 0.15	0.54 ± 0.01	1.04 ± 0.04	0.58 ± 0.03	0.28 ± 0.03

Data expressed as MEAN±SEM;Significantly (*p<0.05) different from placebo control in ordinary one-way ANOVA followed by Tukey's test; OD=once-a-day

higher plasma GLP-1 concentrations compared to the placebo control arm (12.4 ± 1.8 *vs* 18.3 ± 3.1 and 20.2 ± 2.3, respectively), this elevation in basal GLP-1 levels may have contributed to retarded body weight gain and numerical reduction in feed-intake, plasma glucose, liver enzymes (ALT and AST) levels observed in these groups (Tables 3 and 5).

4. Discussion

Despite extensive explorations, no synthetic GPR119 agonists have emerged as an approved therapy. Retrospective analysis points towards the suboptimal physiochemical and/or physiological properties, off-target effects, and tachyphylaxis as major concerns to resolve with future synthetic drug candidates. High lipophilic agents may enhance the potency but are likely to be accompanied by off-target effects [13]. Gut-restricted GPR119 receptor activation by next-generation non-systemic agonists could be a safer approach that may overcome historical constraints and challenges. Although addressing only intestinal GPR119 for incretin secretion may restrict the efficacy due to the lack of direct impact on pancreatic β-cells, in chronic use, this mechanism may safely modulate glucose and energy homeostasis. On the same line, recently, it has been shown that activation of gut GPR119 by microbiota-derived metabolites is associated with satiety control and energy homeostasis in mice [26].

Previously, we reported synthesis, structural-activity relationship (SAR), and preliminary pharmacology of synthetic O-LPI mimetic small-molecule entities that interact with the orthosteric site of gut-located GPR119 receptors for potentiating endogenous intestinal GLP-1 secretion [15]. The primary objective of the current study was to investigate the acute safety, efficacy, and toxicity of lead candidates, ps297 and ps318, in preclinical models to determine the feasibility of their future evaluations in chronic settings.

In the present work, the pharmacodynamic effect of both agents was evaluated in the ipGTT and MMTT protocols by measuring glucose, insulin, and incretin handling. Earlier, we reported that lower concentrations (20 and 50 mg/kg) of both agents have no significant impact on GLP-1 secretion in healthy mice. However, in diabetic db/db mice, compound ps297, but not ps318, significantly increased physiological GLP-1 secretion [15]. Therefore, we employed a considerably higher dose regimen (100 mg/kg) of both agents in the current experiment to evaluate their robust single-dose efficacy in healthy and diabetic db/db mice models. Indeed, PD data demonstrated that both agents considerably improved glucose disposal (30 % and 18 %, respectively, Fig. 2A-C) during ipGTT. Although both agents raised physiological GLP-1 secretion, a significant impact on insulin levels was not observed (Fig. 2F-G). As mentioned earlier, gut-restricted agonism limits the direct action of β-cells, thus restricting insulin-secreting capability by relying solely on incretin secretion. Therefore, glucose control through this mechanism is anticipated to be an indirect process via incretin secretion. We speculate that the observed GLP-1 spike after a single oral dose administration of these agents may be associated with limited insulin secretion, which was not captured in the study, possibly due to the faster insulin utilization in healthy mice. Similarly, the compound effect on post-prandial GLP-1 secretion was studied in MMTT, wherein both agents demonstrated glucose lowering and GLP-1 modulatory activity but lacked a prominent effect on insulin secretion (Fig. 3). In both protocols, the glucose regulatory action of the agents appears to be more dependent on indirect GLP-1 effects rather than direct insulin secretion.

Co-administration of DPP-IV inhibitor therapy is recommended to achieve maximal metabolic benefits from GPCR-driven endogenous GLP-1 secretion by extending its half-life [27–29]. As expected, coupling the DPP-IV inhibition mechanism of sitagliptin with compounds ps297 and ps318 exhibited synergism on GLP-1 secretion in db/db mice (Fig. 4). However, this study has limitations, lacking outcomes on glucose and insulin due to the sample scarcity and the availability of a limited set of db/db mice population. Despite such limitations, the observed synergistic effect on GLP-1 suggests that chronic concurrent use of DPP-IV inhibitors with oral GLP-1 secretagogue agents, ps297 and ps318, may produce synergistic metabolic outcomes by reinforcing GLP-1-driven insulin secretion and energy control. It is worth noting that DPP-IV inhibitors as monotherapy are clinically established to be weight-neutral [30]. Also, compounds ps297 and ps318 lack a direct stimulatory effect on β-cells for insulin secretion due to their limited systemic bioavailability. Therefore, when used together, these two distinct mechanisms may complement each other, providing a potent treatment strategy for managing T2D and obesity. Moreover, reports suggest the extended role of the DPP-IV enzyme in the degradation process of energy-regulating gut peptide hormones such as GIP and PYY [31]. High intestinal GPR119 expression is known to potentiate endogenous GIP secretion by synthetic GPR119 agonists [11,12]. Therefore, in the long run, DPP-IV inhibitor co-therapy with a gut-restricted GPR119 agonist may also modulate the regulation of other gut peptides. Thus, future extensive chronic studies are warranted in disease animal models such as diet-induced obese, db/db, and ob/ob mice to study the therapeutic utility of these agents as monotherapy and in conjunction with DPP-IV inhibition.

We noted variability during the compounds-induced GLP-1 secretion regardless of the models utilized (ipGGT, MMTT, and db/db mice). It may be due to the nature of the mechanism of action of these agents that requires drug local contact with luminal EEC cells to stimulate GLP-1 secretion. The extent of drug local contact is likely to be varied between individual animals. We also suspect that the feeding status of animals contributed to the variability in GLP-1 secretion. For example, we observed comparatively higher GLP-1 secreting variability during the MMTT than ipGTT, possibly due to the oral load of a mixed meal in this protocol. In this context, the GLP-1 secreting activity of compound ps318 appears to be more affected by the meal load compared to compound ps297. Compound ps318 resulted in GLP-1 rise at early time points (5 and 10 mins) only (Fig. 3C and D). Surprisingly, glucose control in ps318-treated mice was comparable to that in ps297 arm (Fig. 3A and B). We speculate that the food presence in the gut interfered with ps318-induced EEC cell stimulation activity but did not completely abolish it, leading to a non-significant rise in GLP-1 (Fig. 3D) while countering hyperglycaemia. Also, the possible involvement of other gut

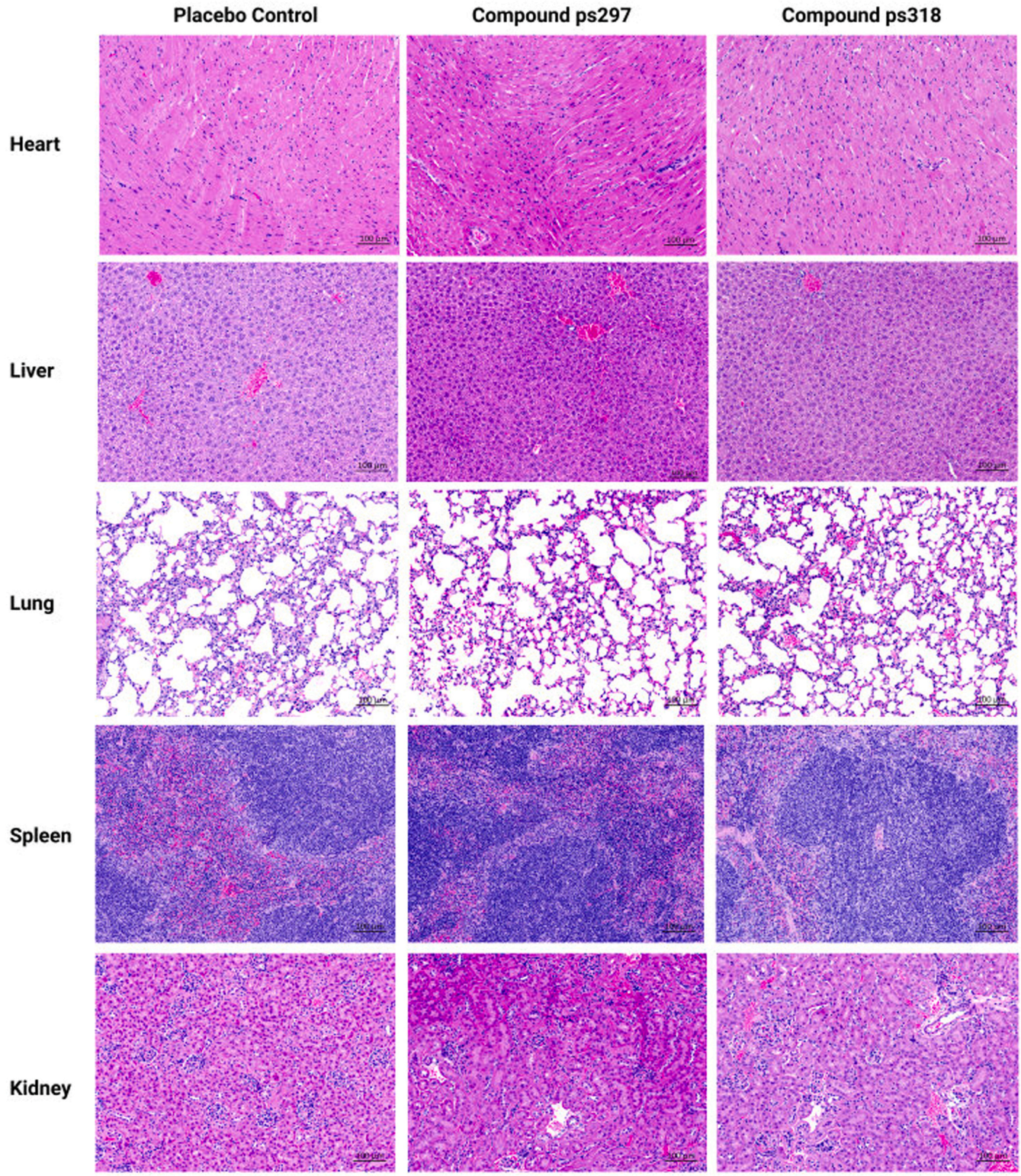

Fig. 7. Acute toxicity of compounds ps297 and ps318 in healthy mice (5-day). Group representative images (x20) of H/E stained, vital organ histology (heart, liver, lung, spleen, and kidney). Scale bar 100 μm.

metabolic hormones, such as GIP and PYY, in ps318-induced glucose control cannot be ruled out. Overall, future long-term preclinical assessments with these agents are needed to predict the clinical translatability of the GLP-1 variability issue and harness the therapeutic benefit of these agents.

PK investigations of compounds ps297 and ps318 in healthy mice revealed restricted systemic bioavailability after a single oral dose administration (Fig. 5 and Table 2). It may be due to the poor gut cell permeability observed in the Caco-2 cells (Table 1). Additionally, the drug excretion pattern in mice showed a substantial recovery (around 25 %) of unchanged drug in faeces and negligible (<0.005 %) drug amounts in the urine (Table 2) that further supports poor absorption and

Table 4
Effect of O-LPI mimetics on blood haematology after repeat (5 days) oral administration in healthy mice (n=8).

Blood haematology			
Parameters (count unit)	Placebo control (0.25 % CMC; OD)	Compound ps297 (100 mg/kg; Oral; OD)	Compound ps318 (100 mg/kg; Oral; OD)
White blood cells (10^9/L)	8.33 ± 0.32	6.76 ± 0.46	7.10 ± 0.56
Lymphocytes (10^9/L)	6.80 ± 0.50	6.33 ± 0.28	6.08 ± 0.23
Monocytes (10^9/L)	0.29 ± 0.04	0.33 ± 0.02	0.28 ± 0.03
Granulocytes (10^9/L)	3.36 ± 0.19	3.24 ± 0.53	3.14 ± 0.54
Red blood cells (10^{12}/L)	10.15 ± 0.29	10.02 ± 0.49	10.89 ± 0.23
Haemoglobin (g/L)	156.63 ± 4.7	145.88 ± 6.99	156.88 ± 2.80
Haematocrit (%)	50.46 ± 1.42	49.14 ± 2.32	53.35 ± 1.09
Mean corpuscular volume (fL)	49.80 ± 0.46	49.15 ± 0.31	49.05 ± 0.48
Mean corpuscular haemoglobin (pg)	15.38 ± 0.15	14.90 ± 0.23	15.11 ± 0.22
Mean corpuscular haemoglobin concentration (g/L)	309.75 ± 1.56	307.70 ± 1.82	306.00 ± 1.57
Red cell distribution width (%)	15.13 ± 0.13	15.25 ± 0.16	14.88 ± 0.14
Platelets (10^9/L)	679.63 ± 34.90	869.29 ± 131.19	919.5 ± 136.95
Mean platelet volume (fL)	5.35 ± 0.15	5.13 ± 0.12	5.05 ± 0.14
Platelet distribution width	16.33 ± 0.11	16.49 ± 0.24	16.0 ± 0.15
Procalcitonin (%)	0.43 ± 0.04	0.36 ± 0.06	0.45 ± 0.04

Data expressed as MEAN±SEM

Table 5
Effect of O-LPI mimetics on plasma biochemistry after repeat (5 days) oral administration in healthy mice (n=8).

Biochemical Measure (Unit)	Placebo control (0.25 % CMC; Oral; OD)	Compound ps297 (100 mg/kg; oral; OD)	Compound ps318 (100 mg/kg; oral; OD)
Glucose (mg/dL)	167.2 ± 4.8	151.9 ± 6.9	154.8 ± 4.2
Triglycerides (mg/dL)	56.5 ± 6.3	44.1 ± 2.9	48.0 ± 4.0
Cholesterol (mg/dL)	71.3 ± 5.5	75.4± 4.5	85.5 ± 4.8
Alanine aminotransferase (ALT) (U/l)	31.9 ± 3.5	24.4 ± 1.4	26.1 ± 1.4
Aspartate transferase (AST) (U/l)	105.5 ± 11.8	89.8 ± 7.4	87.1 ± 4.4
Creatinine (μmol/l)	56.5 ± 5.7	37.3 ± 1.8**	30.6 ± 2.7***
Albumin (g/l)	26.9 ± 0.9	28.0 ± 1.0	27.5 ± 0.3
Blood urea nitrogen (BUN) (mmol/l)	8.8 ± 0.4	8.1 ± 0.3	9.1 ± 0.3
Total protein (g/l)	46.1 ± 1.0	47.3 ± 0.4	48.0 ± 1.4
Total GLP-1 (pM)	12.4 ± 1.8	18.3 ± 3.1	20.2 ± 2.3

Data expressed as MEAN±SEM; Significantly (**p<0.01; ***p<0.001) different than placebo control in one-way ANOVA followed by Tukey's test; OD=once-a-day

limited systemic availability of these entities. The overall PK findings indicate the food-prototype nature of investigational entities with minimal systemic bioavailability after oral administration and EEC stimulation for GLP-1 release.

The poor permeability of compounds ps297 and ps318 in Caco-2 gut cells, along with their limited oral bioavailability in mice, implies that the observed moderate glucose-lowering effect in acute models is likely due to the local stimulation of gut receptors for GLP-1 secretion rather than the direct insulin secretion from pancreatic β-cells. Therefore, we hypothesise that the chronic use of a gut-restricted GPR119 agonism strategy may efficiently and safely regulate glucose and energy homeostasis in type-2 diabetes and obesity. Indeed, the current PK profiling is limited by a lack of information on drug metabolism, metabolite formation, and tissue distribution. However, these preliminary observations are expected to guide future extensive PK investigations with these agents.

Acute toxicity profiling of investigational agents was conducted in zebrafish and healthy mice models. Cardiac toxicity and teratogenicity for compound ps297 were tested in zebrafish, confirming no drug-related toxicity and cardiac abnormality (Fig. 6). Based on these findings, a 5-day repeat oral dose (100 mg/kg/day) administration study was conducted on healthy mice, revealing no drug-related mortality or morbidity. Furthermore, no alterations in haematological (Table 4), biochemical (Table 5), or histological (Fig. 7) profiles were observed. A numerical increase in basal total GLP-1 levels was noted in investigational agent-treated mice compared to the placebo control arm, which may have contributed to a numerical reduction in feed intake, plasma glucose, and liver enzyme levels (Tables 3 and 5). Since it was the first time these new chemical entities were orally administered in mice for five consecutive days, we adopted a prudential approach by testing a 100 mg/kg dose for the preliminary safety assessment of these agents. Indeed, further extensive toxicity screening is needed with higher dose regimens to establish chronic safety. However, the current toxicity findings established the acute oral safety of the investigational agents, providing a basis for future toxicological assessments with higher dose regimens and longer treatment duration. In particular, toxicokinetic assessments will be crucial in studying the health impact of the systemic drug concentration upon its chronic use.

5. Conclusions

This study evaluated the acute safety, efficacy, and toxicity of lead synthetic O-LPI derivatives, ps297 and ps318 in preclinical settings. The observed acute preclinical PK, PD, and toxicological profiles of O-LPI mimetic agents support the future pursuit of these drug candidates. Further extensive pharmacological explorations are needed to evaluate the chronic impact of these agents on T2D and obesity. The current PD assessment is limited to the acute effect of the investigational agents on glucose, GLP-1, and insulin. However, it will be interesting to study the chronic impact of these agents on other gut peptide hormones that govern glucose and energy regulation, such as GIP and PYY. Also, multiple-dose PK and chronic toxicity assessment is warranted with higher dose regimens in different species/models with a comparatively large number set of animals to confirm the therapeutical utility of the agents belonging to this class. Indeed, the current findings on the acute safety, efficacy, and toxicity of gut-oriented GPR119 agonist agents pave the way for future explorations on these agents to treat metabolic disorders.

Ethics approval

All animal experiments were in accordance with institutional animal care guidelines and conducted according to the committee-approved protocols of the Animal Ethics Approval number ARE2022-12 filed under Curtin University.

Funding

Curtin University received funding from Little Green Pharma Ltd. to support MF to conduct this study. The funder played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report. The study was independently investigator-led, and all authors had full access to all data (including statistical reports and tables) in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

CRediT authorship contribution statement

Ilaria Casari: Writing – review & editing, Methodology,

Investigation, Formal analysis, Data curation. **Leon Warne:** Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Dinesh Thapa:** Methodology, Investigation, Formal analysis. **Massimiliano Massi:** Writing – review & editing, Supervision, Resources, Conceptualization. **Elena Dallerba:** Resources, Methodology, Investigation. **Rodrigo Carlessi:** Writing – review & editing, Supervision, Data curation. **Mohan Patil:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Marco Falasca:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

MF and MM are inventors (MF is owner) of a patent related to oleoyl-LPI mimetics. Curtin University received funding from Little Green Pharma Pty Ltd. to support MF to conduct the submitted work. LW was a paid employee of Little Green Pharma Pty Ltd. No other relationships or activities that could appear to have influenced the submitted work.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117077.

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