Chapter 3

APD668, a G protein-coupled receptor 119 agonist improves fat tolerance and attenuates fatty liver in high trans-fat diet induced steatohepatitis model in C57BL/6 mice

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3.1 Abstract

G-protein coupled receptor 119 (GPR119) receptor is a rhodopsin-like, class A Gαs-coupled receptor, predominantly expressed in pancreatic islet cells and intestinal entero-endocrine cells. GPR119 has been emerged as a novel therapeutic target for the treatment of dyslipidemia in type 2 diabetes. In this study, we investigated the effect of APD668, a GPR119 agonist alone and in combination with linagliptin, a Dipeptidyl Peptidase IV (DPPIV) inhibitor on oral fat tolerance test. Our findings demonstrate that APD668, a GPR119 agonist inhibits the intestinal triglyceride absorption after acute fat load in mice. Single dose administration of APD668 increases incretin secretion and enhances total Peptide Tyrosine Tyrosine (PYY) levels in presence of fat load in mice. We found that, the anti-dyslipidemic action of APD668 was reversed in presence of exendin-3 in oral fat tolerance test. In addition, our results showed that exendin-3 (9-39) failed to block the effect of APD668 on gastric emptying indicating that gastric emptying effects of APD668 are indeed mediated through GPR119 receptor dependent mechanism. Combined administration of APD668 and linagliptin significantly increased plasma active Glucagon-Like Peptide-1 (GLP-1) levels in-vivo and showed improvement in fat tolerance. However, APD668 failed to show anti-dyslipidemic activity in tyloxapol-induced hyperlipidemia in mice. Furthermore, we investigated the chronic effects of APD668 on hepatic steatosis in high trans-fat (HTF) diet fed steatohepatitis model in mice. Oral administration of APD668 in HTF diet fed mice ameliorated hepatic endpoints such as plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), liver weight and steatosis. These findings suggest that GPR119 agonists may represent a promising therapeutic strategy for the treatment of dyslipidemia and non-alcoholic steatohepatitis.
3.2 Introduction

Increasing prevalence of postprandial dyslipidemia in type 2 diabetic patients has become a worldwide health concern. Dyslipidemia in diabetic patients is mainly associated with hyper-triglyceridemia, elevated triglyceride-rich lipoproteins (VLDL and chylomicrons), and increased levels of LDL and decreased levels of HDL [1]. Recently, several preclinical and clinical evidence suggest that incretin based anti-diabetes therapies such as GLP-1 receptor agonists and DPPIV inhibitors have shown improvement in fasting and postprandial lipemia. The amelioration of postprandial lipemia is achieved via multiple pathways such as GLP-1 effect on insulin secretion, gastric emptying, gastric lipase, gut motility, weight loss, and improvement in insulin sensitivity and glycemic control. Furthermore, GLP-1 receptor agonists and DPPIV inhibitors have been shown to reduce intestinal lipoprotein production [2, 3]. Since, G-protein coupled receptor 119 (GPR119) agonist increases plasma levels of the active forms of both GLP-1 and GIP in healthy and diabetic patients [4, 5], the present study was performed to assess the effects of APD668, a GPR119 agonist on postprandial lipemia and further explore the potential activity of GPR119 agonist in treatment of NAFLD/NASH mice model.

GPR119 agonists increase circulating GLP-1, GIP, and PYY in animal models and healthy humans. GPR119 agonists acts via dual mechanism of action 1) activation of GPR119 receptor in pancreatic β cells results in direct stimulation of glucose-dependent insulin secretion 2) activation of GPR119 in entero-endocrine cells results in stimulation of incretin release (GLP-1 & GIP), leading to improved acute glucose tolerance [6, 7]. GPR119 has recently attracted attention because of preclinical and clinical evidence of role of GPR119 receptor in lipid metabolism. GPR119 agonists have demonstrated improvement in lipid levels in db/db mice [8, 9] and
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hypertriglyceridemia and fatty liver in HFD/STZ diabetic mice model [10]. Recently, Yang et al. [11] demonstrated that GPR119 receptors expressed in hepatocytes and MBX2982, a GPR119 agonist inhibited the hepatic steatosis by inhibiting SREBP-1c-mediated pathway in obese mice. In addition, clinical trials of GSK1292263, a GPR119 agonist showed improvement in fasting triglyceride, LDL-cholesterol and increasing HDL-cholesterol levels in diabetic and non-diabetic dyslipidemic patients, suggesting that GPR119 agonist acts as an anti-dyslipidemic agent [12]. However, the exact mechanism of actions underlying these lipid effects are unknown.

In this study, we investigated the effects of APD668, a GPR119 agonist on the absorption and metabolism of lipids for which, two acute models of hyperlipidemia, viz. olive oil-induced hypertriglyceridemia and tyloxapol-induced hyperlipidemia were chosen. We also examined combination effects of APD668 and linagliptin on active GLP-1 and fat tolerance in mice. Furthermore, to explore the mechanism whereby APD668 treatment inhibit triglyceride absorption, the effect of APD668 was tested in presence of GLP-1 receptor antagonist in fat tolerance test and also on gastric emptying in mice. Finally, potential utility of APD668 was investigated in high trans-fat diet induced steatohepatitis model in C57BL/6 mice.

3.3 Materials and Methods

3.3.1 Chemicals

APD668, a GPR119 agonist and Linagliptin, a DPPIV inhibitor were obtained from ChemBo Pharma, Nanjing, China. Fenofibrate was procured from Sigma-Aldrich, St Louis, MO. Exendin-3 (9-39), a GLP-1 receptor antagonist was obtained from Tocris Bioscience, USA. D-Ala²-GIP, a GIP receptor agonist was obtained from (USV Laboratories, Mumbai). GSK1292263 and MBX2982 was synthesized at
Department of Medicinal Chemistry, Lupin Limited, Pune. All other chemicals were procured from Sigma-Aldrich, St Louis, MO.

3.3.2 Animals and experimental protocol

Male ICR (CD-1) and C57BL/6 mice, 5-6 weeks of age, were procured from Research Animal Facility (RAF), Lupin Limited (Research Park), Maharashtra, India. Animals were housed in an air-conditioned room at a temperature of 22 ± 2°C and a humidity of 50 ± 20%, with a 12 h light/dark cycle. They were allowed a normal diet (Altromin diet, Germany) and RO water *ad libitum* throughout the experimental period. Before experimentation, animals were acclimatized to experimental animal facility for one week. The guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India, were followed and all experimental procedures were approved by the Institutional Animal Ethics Committee.

3.3.3 Oral Fat Tolerance Test (OFTT)

Male ICR (CD-1) mice of age 6-8 weeks old were selected for OFTT study. Animals were fasted overnight (14 h) and on the next morning, animals were bled under mild isoflurane anesthesia from retro-orbital plexus and approximately 50-60 µL of blood was collected into microcentrifuge tubes containing anticoagulant (10% K$_2$EDTA; 1 mg/0.1 mL of blood) for measurement of pretreatment (basal) plasma triglyceride levels. Then, animals were randomized based on basal plasma triglyceride levels with approximately equal mean and equal variations into different treatment groups. Post randomization, animals were dosed orally or intraperitoneally with vehicle or test compounds at desired doses. Post 30 min of treatment, plasma triglyceride levels were measured (0 h) and then olive oil load (10 mL/kg, per oral) was given to all mice. Post olive oil load, plasma triglyceride levels were measured at
1, 2, 3 and 4 h. The plasma was separated by centrifugation at 8000 rpm for 8 min. at 4°C and plasma triglyceride level was measured by using automatic biochemical analyzer (Daytona, Randox Inc. UK). Delta AUC_(0-4h) was calculated using GraphPad Prism version 6.0. Percentage inhibition in delta AUC was calculated by using following formula:

\[
\text{Percent inhibition in delta AUC} = \frac{\text{Vehicle group mean} - \text{Test compound group individual value}}{\text{Vehicle group mean}} \times 100
\]

**Studies with Exendin-3 (9-39):** Similar study protocol was followed in presence of exendin-3 (9-39) with minor modification. Exendin-3 was given 45 min. before oral olive oil administration in mice.

**Measurement of active GLP-1 and Total GIP levels:** Male ICR (CD-1) mice of age 6-8 weeks old were selected for study. Animals were fasted overnight (14 h) and on the next morning, animals were randomized based on body weight with approximately equal mean and equal variations into different treatment groups. Post randomization, animals were dosed orally with vehicle or test compounds at desired doses. Post 30 min. of treatment, animals were bled under mild isoflurane anesthesia from retro-orbital plexus and approximately 100-150 µL of blood (for GLP-1 assay) and approximately 20-30 µL of blood (for total GIP assay) was collected into microcentrifuge tubes containing anticoagulant (10% K₂EDTA; 1 mg/0.1 mL of blood) and DPPIV inhibitor (Millipore, USA; catalog no. DPP4–010, 10 µL/mL of blood) for measurement of plasma hormone levels (0 h). Then, olive oil load (10 mL/kg, per oral) was given to all mice. Post olive oil load, plasma active GLP-1 and total GIP levels were measured at 0.5, 1 and 2 h. Plasma samples were obtained via centrifugation and assayed for active GLP-1 and Total GIP using commercially available kit (Millipore, USA).
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*Measurement of Total PYY levels:* Similar study protocol was followed as mentioned above with minor modification. Approximately 50-60 µL of blood was collected into microcentrifuge tubes containing anticoagulant (10% K₂EDTA; 1 mg/0.1 mL of blood) and Total PYY levels were measured at 0, 15, 30 and 60 min by using commercially available kit (ALPCO, USA).

**3.3.4 Effect of APD668 on gastric emptying in mice**

Briefly, 8-9 weeks old male ICR (CD-1) mice were selected for study [13]. Mice deprived of food for 22 h and water for 2 h prior to testing (n = 6-7/treatment group) were dosed orally or intraperitoneally with vehicle or test compounds at desired doses. Post 30 min. of treatment, each mouse from all the groups were dosed orally with 0.15 ml of phenol red solution. Standard control mice were killed immediately after phenol red dosing. After 30 min. of phenol red dosing, vehicle or test compound group mice were killed by cervical dislocation. The stomach was immediately removed, collected in tube containing 2 ml of 0.1 N NaOH and cut into several pieces.

The stomach was homogenized followed by addition of 20 µl of trichloro-acetic acid (20 % w/v), the mixture was centrifuged for 15 min at 3000 rpm, and the supernatant (50 µl) added to 0.5 N NaOH (200 µl). A pink colour develops whose absorbance was measured with a spectrophotometer at a wavelength of 560 nm. The % gastric emptying was calculated by using following formula.

\[
\% \text{ Gastric Emptying} = 100 - \frac{A}{B} \times 100
\]

Where A is the amount of phenol red remaining in the stomach 30 min. after administration of the phenol red solution, and B is the amount of phenol red in the stomach immediately after administration of the phenol red solution.
Similar study protocol was followed in presence of exendin-3 (9-39) with minor modification such as exendin-3 was administered 45 min. before phenol red administration in mice.

### 3.3.5 Tyloxapol induced acute hyperlipidemia in mice

Briefly, 6-8 weeks old male ICR (CD-1) mice were selected for the study. Animals were fasted overnight (12 h) and on the next morning, animals were bled under mild isoflurane anesthesia from retro-orbital plexus and approximately 50-60 µL of blood was collected into microcentrifuge tubes containing anticoagulant (10% K$_2$EDTA; 1 mg/0.1 mL of blood) for measurement of pretreatment (basal) plasma triglyceride levels. Then, animals were randomized based on basal plasma triglyceride levels with approximately equal mean and equal variations into different treatment groups. Post randomization, animals were dosed orally or intraperitoneally with vehicle or test compounds at desired doses. Post 15 min. of treatment, Triton WR-1339 (400 mg/kg; prepared in PBS) was administered to all animals intraperitoneally. Post Triton WR-1339 administration, plasma triglyceride levels were measured at 2, 4 and 6 h by using automatic biochemical analyzer (Daytona, Randox Inc. UK). Delta AUC (0-6h) was calculated using GraphPad Prism version 6.0.

### 3.3.6 Repeated administration of APD668 in HTF diet induced steatohepatitis model in mice

Briefly, 7-8 weeks old male C57BL/6 mice were fed Low Fat Diet (LFD, no fructose or cholesterol, cat. no. D09100301) and High Trans Fat (HTF) diet comprising high fat (40 % kcal), high fructose (22 % by weight), and high cholesterol (2 % by weight), where the source of fat was either trans-fat (Primex partially hydrogenated vegetable oil shortening, cat. no. D09100301) for 8 weeks, ad libitum. Mice with normal observations were randomly divided into five groups (9
mice/group) which were treated for 6 weeks as follows: (1) LFD diet + vehicle; (2) HTF diet + vehicle; (3) HTF diet + APD668 (6.25 mg/kg); (4) HTF diet + APD668 (12.5 mg/kg) and (5) HTF diet + APD668 (25 mg/kg). APD668 was suspended in 1% Tween-80 + 15% Gelucire 44/14 + 10% Propylene Glycol + 74% Type 1 ultrapure water (1:15:10:74). Vehicle or APD668 were administered by oral gavage twice daily for 6 weeks. Body weight was measured weekly. Post 6 weeks of treatment, animals were fasted overnight (14 h) and blood samples were collected from retro-orbital plexus under mild isoflurane anesthesia and the livers were isolated, immediately freeze-clamped in liquid nitrogen, and stored at -80°C until analysis. Plasma ALT, AST, triglyceride, glucose and cholesterol were measured by using automatic biochemical analyzer (Daytona, Randox Inc. UK). Plasma insulin and leptin levels were measured by using commercial kits (Millipore Inc. USA).

3.3.7 Measurement of hepatic triglyceride and cholesterol

Lipids were extracted according to the method of Edvardsson et al [14]. Frozen livers were homogenized in isopropanol (1 ml/50 mg tissue) and incubated at 4°C for 1 h. The samples were centrifuged at 4°C for 5 min. at 2500 rpm. Plasma triglyceride and cholesterol levels in the supernatants were measured by using automatic biochemical analyzer (Daytona, Randox Inc. UK).

3.3.8 Statistical Analysis

All the results are expressed as Mean ± S.E.M. Differences between two groups were analyzed by Student's t-test. For multiple comparisons, One-way or Two-way ANOVA were used. Statistical analysis applied are described in each table and figure legend. p < 0.05 was considered to be statistically significant. Data was analyzed using GraphPad version 6.07 of GraphPad Prism for Windows, GraphPad software, San Diego, California, USA.
3.4 Results

Effect of APD668, a GPR119 agonist on oral fat tolerance test in mice

To investigate the effect of APD668 on intestinal lipid absorption, we conducted an oral fat tolerance test in normal mice. As shown in Fig. 3.1A, olive oil administration elevated plasma triglyceride levels in vehicle treated mice. Treatment with APD668 (1, 3, 10 and 30 mg/kg, p.o.) significantly reduced the elevated plasma triglyceride levels at 2 h as compared to vehicle treated mice. Next, we calculated area under curve and APD668 at tested doses non-significantly inhibited the lipid excursion by approximately 25%, 38%, 45% and 42% in mice (Fig 3.1A and B).

Figure 3.1 APD668 improves fat tolerance in mice
Overnight fasted male mice were orally administered vehicle or APD668 (1, 3, 10 and 30 mg/kg) 30 min before the fat load. Plasma triglyceride levels (A) were measured during OFTT and AUCs were calculated from 0 to 4 h (B) respectively. The results are represented as the Mean ± S.E.M. n = 6-7 mice/group; Plasma triglyceride values between the APD668 treated group and control group were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma triglyceride among all the groups were compared by One-
way ANOVA using Tukey’s *post-hoc* test. $^{\text{^p}} p < 0.01$ (1 mg/kg), $^{\text{###}} p < 0.001$ (3 mg/kg), $^{\text{$$p}} < 0.01$ (10 mg/kg), $^{\text{***}} p < 0.001$ (30 mg/kg) vs. vehicle treated mice

**Effect of APD668 on plasma active GLP-1, Total GIP and Total PYY levels in OFTT**

To test whether the improved fat tolerance was due to enhanced incretin release, we estimated incretin levels in mice. Post olive oil load, plasma active GLP-1 level peaked at 0.5 h in vehicle treated mice. APD668 (30 mg/kg; p.o.) showed non-significant increases in plasma active GLP-1 levels at 1 h and 2 h whereas linagliptin (1 mg/kg; p.o.) showed significant increases in plasma active GLP-1 levels at 0.5 h, 1 h and 2 h (Fig. 3.2A). As shown in Fig. 3.2B, APD668 demonstrated non-significant increase in AUC of active GLP-1 as compared to vehicle treated mice. On the other hand, linagliptin demonstrated increase in AUC of active GLP-1 as compared to vehicle treated mice ((p < 0.001). Similar to active GLP-1 levels, APD668 treatment also increased plasma total GIP levels (p < 0.01) and significantly increased AUC compared with vehicle treated mice (Fig. 3.2C and D). Linagliptin showed non-significant increase in plasma total GIP levels and AUC as compared to vehicle treated mice. We also observed APD668 showed non-significant increase in GLP-1 and GIP levels before administration of olive oil load (0 h) in mice. In addition, GPR119 receptor activation with APD668 significantly enhanced total PYY levels after acute fat load in mice while linagliptin failed to enhance PYY in mice (Fig. 3.2E and F).
Figure 3.2 APD668 treatment increases plasma active GLP-1, Total GIP and Total PYY levels in mice

Overnight fasted male mice were orally administered vehicle or APD668 (30 mg/kg) or linagliptin (1 mg/kg) 30 min before the fat load. Plasma active GLP-1, Total GIP and PYY levels were measured during OFTT and AUCs were calculated. The results are represented as the Mean ± S.E.M. For GLP-1 study; n = 7-8 mice/group; For GIP and PYY study; n = 5-7 mice/group; the plasma hormone levels between the APD668 treated group and control group were compared by Two-way ANOVA using Bonferroni’s post test. * p < 0.01,** p < 0.01, *** p < 0.001 vs. vehicle treated mice

Effect of GLP-1 receptor and GIP receptor agonist on OFTT in mice

Next, we measured effect of GLP-1 receptor agonist (exendin-4) and GIP receptor agonist (D-Ala²-GIP) on intestinal lipid absorption in mice. We chose doses of exendin-4 and D-Ala²-GIP based on published literature data [15]. As shown in Fig. 3.3A, exendin-4 (100 µg/kg; i.p.) reduced circulating triglyceride level after acute fat
load (p < 0.01, p < 0.001). In contrast, D-Ala²-GIP (100 µg/kg; i.p.) slightly reduced (non-significant) plasma triglyceride levels in mice (Fig. 3.3A). Next, we calculated area under curve and exendin-4 significantly inhibited the lipid excursion by approximately 85% in mice (Fig 3.3A and B). Therefore, our results suggest that GLP-1 receptor plays a major role in regulation of intestinal lipid absorption after acute fat load.

**Figure 3.3 Effect of D-Ala²-GIP and exendin-4 on oral fat tolerance in mice**

Overnight fasted male mice were intraperitoneally administered vehicle or D-Ala²-GIP and exendin-4 (100 µg/kg) 30 min before the fat load. Plasma triglyceride levels (A) were measured during OFTT and AUCs were calculated from 0 to 3 h (B) respectively. The results are represented as the Mean ± S.E.M. n = 7 mice/group; Plasma triglyceride values between the D-Ala²-GIP or exendin-4 treated group and control group were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma triglyceride among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle treated mice

**Effect of APD668 on oral fat tolerance in presence of GLP-1 receptor antagonist (Exendin-3) in mice**

Since GLP-1 peptide plays a major role in regulation of intestinal lipid absorption after acute fat load, we investigated whether the efficacy of APD668 in fat tolerance test required GLP-1 receptor signaling. We used high dose of exendin-3 (300 µg/kg; i.p.) which fully antagonizes GLP-1 signaling in mice [16]. APD668 at 30 mg/kg
reduced the plasma triglyceride levels at 2 h and 3 h after a fat load (p < 0.001; Fig. 3.4A). The GLP-1 receptor antagonist, exendin-3 blocked the APD668 mediated reduction of plasma triglyceride levels at 2 h (p < 0.01; Fig. 3.4A). Next, we calculated AUC for all treatment groups. As shown in Fig. 3.4B, APD668 in combination with normal saline inhibited lipid excursion by 49%, but this effect was blocked when APD668 was administered in combination with exendin-3. Similar studies were performed to evaluate the effect of linagliptin in presence of exendin-3 in mice. Linagliptin also inhibited lipid excursion by 55%, but this effect was blocked when linagliptin was administered in combination with exendin-3 (Fig. 3.4C and D).

A

B
Figure 3.4 Effect of APD668 and linagliptin on oral fat tolerance in presence of GLP-1 receptor antagonist (Exendin-3) in mice

Overnight fasted male mice were treated with either normal saline or exendin-3 (300 µg/kg, delivered i.p.) 45 min before fat load (time -45 min). Then, animals were treated with either vehicle or APD668 (30 mg/kg, p.o.) or linagliptin (1 mg/kg; p.o.) 30 min prior to fat load (time -30 min). Plasma triglyceride levels (A and C) were measured during OFTT and AUCs were calculated from 0 to 3 h (B and D) respectively. The results are represented as the Mean ± S.E.M. and average of 3 independent studies; n = 19-20 mice/group for APD668 and average of 2 independent studies; n = 9-12 mice/group for linagliptin; The plasma triglyceride values among all the groups were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma triglyceride among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. ** p < 0.01, *** p < 0.001 vs. saline + vehicle treated mice. # p < 0.05, ## p < 0.01 vs. APD668 or linagliptin treated mice.
Effect of APD668 on gastric emptying in mice

As GPR119 agonists increase plasma GLP-1 levels in-vivo and further GLP-1 regulates glucose homeostasis via inhibition of gastric emptying, we measured the effect of APD668, linagliptin and exendin-4 on gastric emptying in mice. As shown in Fig. 3.5A, APD668 (45 ± 6%; p < 0.05) and exendin-4 (17 ± 7%; p < 0.001) treatment inhibited gastric emptying in mice compared with vehicle treated mice (65 ± 2%). In contrast, linagliptin did not show any effect on gastric emptying in mice. To check whether the effects of APD668 on gastric emptying were mediated through GLP-1 receptor dependent mechanism, we repeated these studies in presence of GLP-1 receptor antagonist. As shown in Fig. 3.5B, APD668 alone (41 ± 10%) and in presence of exendin-3 (43 ± 10%) continued to inhibit gastric emptying compared with vehicle treated mice (68 ± 3%); though the effect was not statistically significant in this experiment. However, exendin-4 inhibited gastric emptying (18 ± 7%; p < 0.01) but its effect was partially reversed (non-significant) in presence of exendin-3, as shown in Fig. 3.5B. Our results indicate that APD668 inhibits gastric emptying via GPR119 receptor mediated dependent mechanism.

A
Figure 3.5 GPR119 activation inhibits gastric emptying and control gastric emptying independent of GLP-1 receptor
Overnight fasted male mice were orally administered vehicle or APD668 (30 mg/kg) or linagliptin (30 mg/kg) 30 min before phenol red administration in mice. Post 30 min of phenol red administration, stomach excised and homogenized in 0.1N NaOH, followed by addition of 20 % trichloroacetic acid and centrifuged. Then, supernatant mixed with 0.5N NaOH and assessed the absorbance at 560 nm by spectrophotometry. Similar study protocol followed in presence of exendin-3 with minor modifications as mentioned above. The results are represented as the Mean ± S.E.M. n = 5-7 mice/group; the % gastric emptying values among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle control; $ p < 0.05 vs. exendin-4 + exendin-3 (9-39)

Effect of combination of APD668 and linagliptin on plasma active GLP-1 levels and oral fat tolerance test
To confirm the role of GLP-1 in the APD668 mediated anti-dyslipidemic effects in OFTT in mice, we evaluated the potential combination effects of APD668, a GPR119 agonist and linagliptin (0.3 mg/kg), a DPPIV inhibitor on plasma active GLP-1 levels. We choose the lower doses of APD668 and linagliptin based on our in-house data in OFTT model (data not shown). We found that at a low dose of APD668 (1 mg/kg) did not show increase in plasma GLP-1 levels while linagliptin (0.3 mg/kg) showed increase in plasma GLP-1 levels after acute fat load in mice (p < 0.001 at 0.5 h only; Fig. 3.6A). However; combination therapy induced significantly higher and
sustained increases in plasma GLP-1 levels as compared to linagliptin treated mice (Fig. 3.6A). Further, combinative approach of APD668 and linagliptin therapy exhibited increase in AUC of plasma GLP-1 levels as compared to APD668 or linagliptin alone treatment (p < 0.001 vs APD668 alone; p < 0.05 vs linagliptin alone Fig. 3.6B). Because combined delivery of APD668 and linagliptin enhanced GLP-1 release, further we examined whether this regimen might also result in improved fat tolerance in mice. As shown in Fig. 3.7A, APD668 and linagliptin alone showed non-significant reduction in plasma triglyceride levels compared with vehicle treated mice. However, combined administration of 1 mg/kg APD668 and 0.3 mg/kg linagliptin significantly reduced elevated plasma triglyceride levels at 2 h, 3 h and 4 h in comparison to vehicle treated mice (Fig. 3.7A). Next, we calculated area under curve for all treatment groups. Single dose administration of APD668 (1 mg/kg) showed less than 20% improvement while linagliptin (0.3 mg/kg) demonstrated approximately 35% improvement in fat tolerance test. Our findings demonstrate that combination of APD668 and linagliptin inhibited the lipid excursion by greater than 60% which is significantly higher as compared to APD668 or linagliptin alone treatment as shown in Fig. 3.7B (p < 0.01).
A

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

B

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

Figure 3.6 Combination effects of APD668 with linagliptin on acute plasma active GLP-1 in response to oral fat load in mice
Overnight fasted male mice were orally administered vehicle or APD668 (1 mg/kg) or linagliptin (0.3 mg/kg) 30 min before the fat load. The combination treatment group was orally administered with APD668 (1 mg/kg) and linagl

Figure 3.7 APD668 and linagliptin cooperatively improve fat tolerance in mice
Overnight fasted male mice were orally administered vehicle or APD668 (1 mg/kg) or linagliptin (0.3 mg/kg) 30 min before the fat load. The combination treatment group was orally administered with APD668 (1 mg/kg) and linagliptin (0.3 mg/kg). Plasma active GLP-1 levels were measured during OFTT and AUCs were calculated from 0 to 2 h (B) respectively (Fig. 3.6A & B). The results are represented as the Mean ± S.E.M. n = 4-5 mice/group; the plasma GLP-1 levels among all the groups were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma active GLP-1 among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. *** p < 0.001 vs. vehicle treated mice. # p < 0.05 vs. linagliptin treated mice. $$$ p < 0.001 vs. APD668 treated mice.

A

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

B

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

Overnight fasted male mice were orally administered vehicle or APD668 (1 mg/kg) or linagliptin (0.3 mg/kg) 30 min before the fat load. The combination treatment group was orally administered with APD668 (1 mg/kg) and linagliptin (0.3 mg/kg). Plasma active GLP-1 levels were measured during OFTT and AUCs were calculated from 0 to 2 h (B) respectively (Fig. 3.6A & B). The results are represented as the Mean ± S.E.M. n = 4-5 mice/group; the plasma GLP-1 levels among all the groups were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma active GLP-1 among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. *** p < 0.001 vs. vehicle treated mice. # p < 0.05 vs. linagliptin treated mice. $$$ p < 0.001 vs. APD668 treated mice.

A

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

B

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

Overnight fasted male mice were orally administered vehicle or APD668 (1 mg/kg) or linagliptin (0.3 mg/kg) 30 min before the fat load. The combination treatment group was orally administered with APD668 (1 mg/kg) and linagliptin (0.3 mg/kg). Plasma active GLP-1 levels were measured during OFTT and AUCs were calculated from 0 to 2 h (B) respectively (Fig. 3.6A & B). The results are represented as the Mean ± S.E.M. n = 4-5 mice/group; the plasma GLP-1 levels among all the groups were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma active GLP-1 among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. *** p < 0.001 vs. vehicle treated mice. # p < 0.05 vs. linagliptin treated mice. $$$ p < 0.001 vs. APD668 treated mice.

A

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

B

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

Overnight fasted male mice were orally administered vehicle or APD668 (1 mg/kg) or linagliptin (0.3 mg/kg) 30 min before the fat load. The combination treatment group was orally administered with APD668 (1 mg/kg) and linagliptin (0.3 mg/kg). Plasma active GLP-1 levels were measured during OFTT and AUCs were calculated from 0 to 2 h (B) respectively (Fig. 3.6A & B). The results are represented as the Mean ± S.E.M. n = 4-5 mice/group; the plasma GLP-1 levels among all the groups were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma active GLP-1 among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. *** p < 0.001 vs. vehicle treated mice. # p < 0.05 vs. linagliptin treated mice. $$$ p < 0.001 vs. APD668 treated mice.

A

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

B

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

Overnight fasted male mice were orally administered vehicle or APD668 (1 mg/kg) or linagliptin (0.3 mg/kg) 30 min before the fat load. The combination treatment group was orally administered with APD668 (1 mg/kg) and linagliptin (0.3 mg/kg). Plasma active GLP-1 levels were measured during OFTT and AUCs were calculated from 0 to 2 h (B) respectively (Fig. 3.6A & B). The results are represented as the Mean ± S.E.M. n = 4-5 mice/group; the plasma GLP-1 levels among all the groups were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma active GLP-1 among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. *** p < 0.001 vs. vehicle treated mice. # p < 0.05 vs. linagliptin treated mice. $$$ p < 0.001 vs. APD668 treated mice.

A

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

B

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

Overnight fasted male mice were orally administered vehicle or APD668 (1 mg/kg) or linagliptin (0.3 mg/kg) 30 min before the fat load. The combination treatment group was orally administered with APD668 (1 mg/kg) and linagliptin (0.3 mg/kg). Plasma active GLP-1 levels were measured during OFTT and AUCs were calculated from 0 to 2 h (B) respectively (Fig. 3.6A & B). The results are represented as the Mean ± S.E.M. n = 4-5 mice/group; the plasma GLP-1 levels among all the groups were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma active GLP-1 among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. *** p < 0.001 vs. vehicle treated mice. # p < 0.05 vs. linagliptin treated mice. $$$ p < 0.001 vs. APD668 treated mice.

A

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

B

Vehicle (10 ml/kg; p.o.)
APD668 (1 mg/kg; p.o.)
Linagliptin (0.3 mg/kg; p.o.)
APD668 (1 mg/kg; p.o.) + Linagliptin (0.3 mg/kg; p.o.)

Overnight fasted male mice were orally administered vehicle or APD668 (1 mg/kg) or linagliptin (0.3 mg/kg) 30 min before the fat load. The combination treatment group was orally administered with APD668 (1 mg/kg) and linagliptin (0.3 mg/kg). Plasma active GLP-1 levels were measured during OFTT and AUCs were calculated from 0 to 2 h (B) respectively (Fig. 3.6A & B). The results are represented as the Mean ± S.E.M. n = 4-5 mice/group; the plasma GLP-1 levels among all the groups were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma active GLP-1 among all the groups were compared by One-way ANOVA using Tukey’s post-hoc test. *** p < 0.001 vs. vehicle treated mice. # p < 0.05 vs. linagliptin treated mice. $$$ p < 0.001 vs. APD668 treated mice.
Effect of APD668, GSK1292263 and MBX2982 on oral fat tolerance test

Next, to investigate whether the effect of APD668 on intestinal triglyceride absorption was due to either compound specific or target specific effect; we measured the effect of other GPR119 agonists i.e. GSK1292263 and MBX2982 in oral fat tolerance test in mice. Treatment with APD668 (30 mg/kg; p.o.) reduced elevated plasma triglyceride levels at 2 h, 3 h and 4 h (p < 0.05, p < 0.01, Fig. 3.8A) and showed 55% improvement in fat tolerance (p < 0.05, Fig. 3.8B) whereas, GSK1292263 reduced elevated plasma triglyceride levels at 2 h (p < 0.01) and showed 49% improvement in fat tolerance test. In addition, MBX2982 demonstrated significant reduction in elevated plasma triglyceride (p < 0.01, p < 0.001, Fig. 3.8A) and showed 65% improvement in fat tolerance (p < 0.05, Fig. 3.8B).

Figure 3.8 Effect of APD668, GSK1292263 and MBX2982 on oral fat tolerance test
Overnight fasted male mice were orally administered vehicle or APD668 or GSK1292263 or MBX2982 (30 mg/kg) 30 min before the fat load. Plasma triglyceride levels (A) were measured during OFTT and AUCs were calculated from 0 to 4 h (B) respectively. The results are represented as the Mean ± S.E.M. n = 6-7 mice/group; Plasma triglyceride values between the APD668 or GSK1292263 or MBX2982 treated group and control group were compared by Two-way ANOVA using Bonferroni’s post test. Then, AUCs of plasma triglyceride between APD668 or GSK1292263 or MBX2982 treated group and control group were compared by One-way ANOVA using Tukey’s post-hoc test. For APD668; * p < 0.05, ** p <
Effect of APD668 on plasma triglyceride in tyloxapol induced hyperlipidemia model

As shown in Fig. 3.9A, tyloxapol administration in mice elevated the plasma triglyceride level as compared to sham vehicle group at 2 h, 4 h and 6 h (p < 0.001). APD668 failed to show reduction in plasma triglyceride levels at all time-points (Fig. 3.9A). Linagliptin demonstrated a reduction of circulating triglyceride levels (Fig. 3.9A) and showed 25% improvement in AUC (Fig. 3.9B). In the present study, we used fenofibrate (50 mg/kg) as a reference standard. As expected, fenofibrate decreased plasma triglyceride levels compared with tyloxapol vehicle treated group at 4 h and 6 h (p < 0.001, Fig. 3.9A) and demonstrated ~70% improvement in AUC (p < 0.001, Fig. 3.9B). Next, we investigated the effects of exendin-4 and D-Ala²-GIP on plasma triglyceride levels in tyloxapol induced hyperlipidemia model. Exendin-4 decreased plasma triglyceride levels compared with tyloxapol vehicle treated group at 6 h (p < 0.01, Fig. 3.9C) and demonstrated 45% improvement in AUC (p < 0.01, Fig. 3.9D). In contrast, D-Ala²-GIP marginally lowered (non-significant) plasma triglyceride levels with minimal improvement in AUC (Fig. 3.9C and D).
Effect of APD668 on biochemical, metabolic indices and hepatic steatosis in diet induced steatohepatitis model in C57BL/6 mice

To characterize the development of murine model of steatohepatitis in the context of obesity and diabetes, we maintained C57BL/6 mice on LFD or HTF diet for 14 weeks respectively [17]. C57BL/6 mice on HTF diet gained significant body weight compared with LFD diet (p < 0.001, Table 3.1). HTF diet fed mice showed significant increase in liver weight, liver (% of body weight), hepatic steatosis and fat mass as compared with LFD diet as shown in Fig. 3.10A, B and C. In addition, hepatic injury markers such as plasma ALT and AST levels were increased ~10 fold by HTF diet compared with LFD diet (p < 0.001, Table 3.1). HTF diet fed mice developed mild hyperglycemia and hypercholesterolemia in comparison to LFD diet (p < 0.001, Table...
3.1). However, HTF fed mice did not develop hypertriglyceridemia. Furthermore, plasma leptin levels increased in HTF diet (13.26 ± 1.22 vs 0.87 ± 0.20, p < 0.001) while non-significant increase in plasma insulin levels was observed in HTF diet (0.42 ± 0.05 vs 0.25 ± 0.03) compared with LFD diet; suggesting that HTF mice developed insulin resistance (Table 3.2). We confirm that HTF diet induces several key markers of NAFLD/NASH such as hepatomegaly, hepatic injury, steatosis, hypercholesterolemia, mild hyperglycemia, insulin resistance and obesity in C57BL/6 mice.

Table 3.1 Effect of APD668 on biochemical parameters and body weight in HTF diet induced steatohepatitis model

<table>
<thead>
<tr>
<th>Diet + Drug</th>
<th>A. No.</th>
<th>ALT (U/I)</th>
<th>AST (U/I)</th>
<th>Glucose (mg/dL)</th>
<th>Triglyceride (mg/dL)</th>
<th>Cholesterol (mg/dL)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFD + Vehicle</td>
<td>8</td>
<td>10 ± 3</td>
<td>64 ± 7</td>
<td>117 ± 10</td>
<td>131 ± 6</td>
<td>116 ± 4</td>
<td>25.8 ± 0.5</td>
</tr>
<tr>
<td>HTF + Vehicle</td>
<td>8</td>
<td>106 ± 13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>150 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>170 ± 13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>197 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.3 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HTF + APD668 (6.25 mg/kg)</td>
<td>8</td>
<td>72 ± 13</td>
<td>140 ± 13</td>
<td>169 ± 7</td>
<td>105 ± 8</td>
<td>182 ± 7</td>
<td>32.6 ± 0.5</td>
</tr>
<tr>
<td>HTF + APD668 (12.5 mg/kg)</td>
<td>8</td>
<td>48 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>111 ± 13</td>
<td>144 ± 11</td>
<td>70 ± 4</td>
<td>168 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.2 ± 0.8</td>
</tr>
<tr>
<td>HTF + APD668 (25 mg/kg)</td>
<td>8</td>
<td>71 ± 13</td>
<td>119 ± 14</td>
<td>132 ± 10</td>
<td>76 ± 6</td>
<td>187 ± 4</td>
<td>31.3 ± 0.7</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; AST, aspartate aminotransferase; LFD, low fat diet; HTF, high trans-fat, high fructose, high cholesterol diet; Data are represented as Mean ± S.E.M. <sup>a</sup>p < 0.05 vs. LFD diet + vehicle group; <sup>b</sup>p < 0.05 vs. HTF diet + vehicle group
APD668 (6.25, 12.5 and 25 mg/kg, p.o.) was administered twice daily for 6 weeks to HTF fed mice and body weight was recorded weekly. In the present study, APD668 did not show significant effect on body weight gain (Table 3.1). Treatment with APD668 at a tested doses demonstrated reduction in liver weight and liver (% of body weight) as shown in Fig. 3.10A (p < .001 vs HTF control). In addition, our findings demonstrate that APD668 reduced hepatic triglyceride (6.25 mg/kg: 308 ± 13 vs 326 ± 20; 12.5 mg/kg: 260 ± 19 vs 326 ± 20 and 25 mg/kg: 251 ± 19 vs 326 ± 20, p < 0.05) and hepatic cholesterol (6.25 mg/kg: 23 ± 3 vs 26 ± 2; 12.5 mg/kg: 18 ± 1 vs 26 ± 2, p < 0.01 and 25 mg/kg: 20 ± 1 vs 26 ± 2, p < 0.01) in comparison to HTF control mice (Fig. 3.10B). At the end of the experiment, epididymal and retroperitoneal fat depots were removed and weighed. APD668 reduced epididymal fat mass (6.25 mg/kg: 1.72 ± 0.05 vs 1.77 ± 0.05; 12.5 mg/kg: 1.41 ± 0.10 vs 1.77 ± 0.05, p < 0.01 and 25 mg/kg: 1.47 ± 0.09 vs 1.77 ± 0.05, p < 0.05) and retroperitoneal fat mass (6.25 mg/kg: 0.29 ± 0.01 vs 0.32 ± 0.02; 12.5 mg/kg: 0.24 ± 0.02 vs 0.32 ± 0.02, p < 0.05 and 25 mg/kg: 0.25 ± 0.02 vs 0.32 ± 0.02) compared with HTF control mice (Fig. 3.10C).

A
Figure 3.10 Effect of APD668 on hepatic lipid accumulation in mice fed on HTF diet

Male C57BL/6 mice were initially maintained on LFD or HTF for 8 weeks. Post randomization, mice were treated with vehicle or APD668 (6.25, 12.5 and 25 mg/kg; twice daily) for 6 weeks. A) Liver weight and liver (% of body weight) B) effect of APD668 on hepatic triglyceride and cholesterol and C) epididymal and retro-peritoneal fat mass. The results are represented as the Mean ± S.E.M. n = 7-8 mice/group; ### p < 0.001 vs LFD vehicle control group; * p < 0.05, ** p < 0.01, *** p < 0.001 vs. HTF vehicle control groups

In addition to hepatic steatosis, we assessed the plasma markers associated with NAFLD/NASH, including ALT and AST, and circulating levels of triglyceride, glucose and cholesterol in LFD and HTF diet fed mice. As shown in Table 3.1, APD668 at a dose of 6.25 mg/kg and 25 mg/kg showed decreased trend in reduction in plasma ALT levels (72 ± 13 vs 106 ± 13; 32% reduction and 71 ± 13 vs 106 ± 13; 32% reduction) whereas at a dose of 12.5 mg/kg showed significant reduction in ALT levels (48 ± 11 vs 106 ± 13; 55% reduction; p < 0.01) as compared to HTF control
mice. Similarly, APD668 at a tested doses reduced plasma AST levels (7%, 27% and 20% reduction) compared with HTF control mice (Table 3.1). In this model, HTF diet fed mice did not develop hypertriglyceridemia compared with LFD fed mice. Still, APD668 at a dose of 12.5 and 25 mg/kg showed decreased trend in reduction in circulating triglyceride levels in mice (70 ± 4 vs 85 ± 3 and 76 ± 6 vs 85 ± 3). However, APD668 reduced the hypercholesterolemia induced by HTF diet (6.25 mg/kg: 182 ± 7 vs 197 ± 8; 12.5 mg/kg: 168 ± 6 vs 197 ± 8, p < 0.01 and 25 mg/kg: 187 ± 4 vs 197 ± 8, p < 0.05) as shown in Table 3.1. Furthermore, our findings also demonstrated reduction in plasma leptin levels by APD668 treatment (at 12.5 mg/kg: 5.06 ± 0.91 vs 13.26 ± 1.22, p < 0.001 and at 25 mg/kg: 9.54 ± 1.05 vs 13.26 ± 1.22, p < 0.05; Table 3.2) in HTF fed mice. In addition, APD668 displayed non-significant improvement in insulin sensitivity only at a dose of 12.5 mg/kg as compared to HTF control mice (Table 3.2).

**Table 3.2 Effect of APD668 on metabolic indices in HTF diet induced steatohepatitis model**

<table>
<thead>
<tr>
<th>Diet + Drug</th>
<th>Leptin (ng/mL)</th>
<th>Insulin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFD + Vehicle</td>
<td>0.87 ± 0.20</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>HTF + Vehicle</td>
<td>13.26 ± 1.22\textsuperscript{a}</td>
<td>0.42 ± 0.05</td>
</tr>
<tr>
<td>HTF + APD668 (6.25 mg/kg)</td>
<td>-ND-</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>HTF + APD668 (12.5 mg/kg)</td>
<td>5.06 ± 0.91\textsuperscript{b}</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>HTF + APD668 (25 mg/kg)</td>
<td>9.54 ± 1.05\textsuperscript{b}</td>
<td>0.45 ± 0.04</td>
</tr>
</tbody>
</table>

Data are represented as Mean ± S.E.M. for n = 6-8 mice/group
\textsuperscript{a}p < 0.05 vs. LFD diet + vehicle group; \textsuperscript{b}p < 0.05 vs. HTF diet + vehicle group, ND - Not Determined
3.5 Discussion

In the present study, we demonstrated anti-dyslipidemic and anti-steatotic activity of APD668, a GPR119 agonist in acute and chronic animal models. First, we investigated the effect of APD668, a GPR119 agonist in acute models of hyperlipidemia. To the best of our knowledge, this is the first study to report the effect of APD668 on acute oral fat tolerance test. Our findings demonstrate that APD668 improved fat tolerance in mice (Fig. 3.1A and B). We hypothesized that improved fat tolerance by APD668 was due to either direct GPR119 receptor activation or via incretin release in mice. Therefore, we measured the plasma active GLP-1 and total GIP levels in-vivo. APD668 promoted incretin secretion and also enhanced total PYY secretion in acute fat tolerance test (Fig. 3.2A-F). Previous reports suggest that both incretin regulates plasma lipid levels [15, 18, 19], therefore we investigated the effect of GLP-1 receptor agonist (exendin-4) and GIP receptor agonist (D-Ala²-GIP, resistant to DPPIV enzyme) on oral fat tolerance in mice. Our results suggest that exendin-4 significantly improved fat tolerance while D-Ala²-GIP showed marginal improvement in fat tolerance (Fig. 3.3A and B). However, we have not seen further worsening of fat tolerance with administration of D-Ala²-GIP in mice as reported by Hsieh et al. [15]. This discrepancy could be due to difference in animal species (C57BL/6 mice vs CD-1 mice) or study design (fasting duration, no tyloxapol administration in our study) etc. However, it is clearly indicated that GLP-1 receptor plays a major role in comparison to GIP receptor in improvement of fat tolerance in mice. To further elucidate the findings, we tested the effect of APD668 induced decrease in elevated triglyceride levels in presence of GLP-1 receptor antagonist on oral fat tolerance test. We found that the hypolipidemic effects of APD668 were reversed in presence of exendin-3 (Fig. 3.4A and B).
Next, we determined the potential combination effects of APD668 and linagliptin on plasma active GLP-1 and fat tolerance in mice. Our studies also demonstrate that APD668 and linagliptin additively increased plasma active GLP-1 levels and improved fat tolerance as compared to APD668 or linagliptin alone treatment in mice (Fig. 3.6 and 3.7). Our findings also demonstrated that all three GPR119 agonists (APD668, GSK1292263 and MBX2982) improved fat tolerance suggesting a GPR119 receptor mediated effect (Fig. 3.8A and B). Interestingly, APD668 treatment did not show any effect on plasma triglyceride while linagliptin showed decreased trend on reduction of elevated plasma triglyceride levels in tyloapol induced hyperlipidemia model. (Fig. 3.9A and B). As expected, fenofibrate showed significant reduction in plasma triglyceride levels in mice as shown in Fig. 3.9A and B. In the same model, exendin-4 demonstrated significant reduction in plasma triglyceride while D-Ala²-GIP marginally lowered plasma triglyceride in mice as shown in Fig. 3.9C and D. Taken together, these novel findings suggest that APD668 reduced circulating triglyceride levels in postprandial state but did not reduced synthesized triglyceride (VLDL-TG) secreted from liver in tyloapol induced hyperlipidemia model. However, linagliptin and exendin-4 demonstrated reduction in plasma triglyceride in both acute models of hyperlipidemia. In summary, all these findings together suggest that GLP-1 plays an important role in the improvement of fat tolerance by APD668. Additionally, APD668 and exendin-4 significantly inhibited gastric emptying whereas linagliptin did not show any effect on gastric emptying in mice (Fig. 3.5A). We evaluated the effect of APD668 in presence of exendin-3 on gastric emptying in mice. Surprisingly, APD668 still inhibited gastric emptying while effect of exendin-4 on gastric emptying was reversed in presence of exendin-3 (Fig. 3.5B). This clearly suggest that APD668 reduced gastric emptying and its effect may be mediated through GPR119 receptor.
activation in mice. These observations are in-line with previously published results in which AR231453, a prototype GPR119 agonist inhibited gastric emptying in GLP-1 receptor\textsuperscript{-/-}, DIRKO, GLP-2 receptor\textsuperscript{-/-} mice and even in presence of Y2 receptor antagonist in mice. Also, AR231453 reduced gastric emptying in wild type but not in GPR119\textsuperscript{-/-} mice [20]. Overall, all these findings suggest that APD668 inhibit gastric emptying independent of GLP-1 receptor, GLP-2 receptor and Y2 receptor. However, further investigative studies are required for coupling of GPR119 activation to control intestinal lipid absorption and gastric emptying in mice.

Next, we explored the effect of APD668 on hepatic endpoints in HTF diet induced steatohepatitis model in C57BL/6 mice. In the present study, APD668, a GPR119 agonist reduced hepatic injury markers such as ALT and AST levels in HTF diet fed mice (Table 3.1). As shown in Fig. 3.10A, APD668 significantly reduced liver weight and liver (% of body weight) compared with HTF control mice. In addition, APD668 also inhibited hepatic triglyceride, cholesterol content and fat mass in HTF diet fed mice (Fig. 3.10B and C). To our knowledge, this is the first study to report the effect of APD668, a GPR119 agonist on hepatic injury, steatosis and fat mass in HTF diet induced steatohepatitis model. GPR119 receptors are predominantly expressed in gastrointestinal tract and pancreas, but are also expressed in muscle, adipose tissue and liver where a direct effect of GPR119 agonist on hepatic tissues recently has been proposed. MBX2982, a GPR119 agonist demonstrated reduction in hepatic steatosis via inhibition of SREBP-1c and other lipogenesis related genes (FAS, ACC and SCD-1) in high fat diet fed mice and hepatic anti-lipogenesis effects of MBX2982 were abolished in GPR119 KO mice [11]. Therefore, we hypothesized that APD668 might be inhibiting hepatic steatosis via direct activation of GPR119 receptor in liver of HTF diet fed mice. However, we also can’t rule out the role of
incretin specifically GLP-1 on protection of fatty liver in mice. Previous studies reported that chronically elevated GLP-1 (in DPPIV deficient rats) and GLP-1 agonist (exendin-4) demonstrated improvement in hepatic steatosis by downregulation of de novo lipogenesis, activation of AMPK pathways and improving insulin sensitivity in rodent species [21, 22]. As in the present study, we found that APD668 promotes GLP-1 secretion after an acute fat load (Fig. 3.2A and B); suspecting chronically elevated GLP-1 secretion may be happened in-vivo by repeated administration of APD668 in HTF diet fed mice. Thus, it is tempting to speculate that anti-steatotic effects of APD668 could be partly due to increased GLP-1 levels. Furthermore, APD668 inhibited intestinal lipid absorption in acute oral fat tolerance test (Fig. 3.1A and B) which might be another possible mechanism of action to attenuate fat accumulation in hepatic tissues. APD668 treatment showed marginal weight loss which clearly suggest that contribution of weight loss to the observed improvement in hepatic endpoints would be minimum. Further, we found that young C57BL/6 mice fed on HTF diet developed mild hyperglycemia, hypercholesterolemia but no hypertriglyceridemia [17]. In the present study, APD668 reduced circulating glucose and cholesterol levels which are a beneficial effect in NAFLD/NASH patients. In contrast, mice did not developed hypertriglyceridemia, still APD668 (12.5 and 25 mg/kg) slightly lowered plasma triglyceride levels in HTF diet fed mice as shown in Table 3.1. APD668 demonstrated significant reduction in plasma leptin levels and marginal improvement in insulin sensitivity at a tested doses as shown in Table 3.2.

It is important to note that repeated administration of APD668 at a high dose (25 mg/kg) showed slightly lesser activity as compared to 6.25 and 12.5 mg/kg on different parameters such as plasma ALT, AST, cholesterol, leptin and insulin levels in HTF diet fed mice as shown in Table 3.1 and 3.2. However, we have not seen such
a loss of efficacy on improvement of hepatic triglyceride and cholesterol in HTF diet fed mice. These findings together suggest that trend in loss of efficacy is clearly parameter specific under the experimental conditions. Whether long-term and higher dose treatment also develops tolerance to these specific parameters is worthy of further investigation. In fact, such a loss of efficacy (antidiabetic) was reported with GSK1292263 in a clinical trial upon repeated administration [23, 24]. Further studies are warranted for better understanding of this phenomenon.

In summary, the present studies for the first time report that GPR119 agonist inhibits intestinal lipid absorption in oral fat tolerance test in mice. APD668 improved fat tolerance could be due to increased GLP-1 levels and inhibition of gastric emptying through via GPR119 receptor activation in mice. In addition, our results demonstrate strong in-vivo evidence of protection of fatty liver by APD668 in HTF diet induced steatohepatitis model in C57BL/6 mice. APD668 also showed reduction in circulating cholesterol, glucose, triglyceride and hepatic injury markers which might be beneficial for patients with NAFLD/NASH. Taken together, APD668 showed beneficial effects could be due to combination of direct activation of GPR119 receptors in intestine and hepatic tissues and elevated GLP-1 (indirect effect) may simultaneously contribute to the reduction in triglyceride uptake and hepatic lipogenesis in HTF diet fed mice. Thus, these data suggest that GPR119 agonists may be useful for the treatment of dyslipidemia and non-alcoholic steatohepatitis.

3.6 References


Chapter 3


