Chapter 4

Co-administration of APD668, a G protein-coupled receptor agonist and Linagliptin, a DPPIV inhibitor, prevents progression of steatohepatitis in mice fed on a high trans-fat diet


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4.1 Abstract

Non-Alcoholic SteatoHepatitis (NASH) is the more severe form of Non-Alcoholic Fatty Liver Disease (NAFLD) and is characterized by the presence of hepatic steatosis, oxidative stress, inflammation and hepatocyte injury with or without fibrosis. Recently, G-protein coupled receptor 119 (GPR119) receptor has emerged as a novel therapeutic target for the treatment of dyslipidemia and non-alcoholic steatohepatitis. In the present study, we investigated the effect of APD668, a GPR119 agonist alone or in combination with linagliptin, a Dipeptidyl Peptidase IV (DPPIV) inhibitor on the progression of steatohepatitis in mice fed on a high trans-fat diet. In this study, monotherapy with either APD668 or linagliptin caused a reduction in the levels of alanine aminotransferase (ALT), alanine aminotransferase (AST), glucose, cholesterol and epididymal fat mass but the effect was more pronounced upon treatment with combination of both drugs. On the other hand, combined treatment of APD668 with linagliptin demonstrated a non-significant additive effect in reduction of hepatic triglyceride (−78%) and cholesterol (−56%) compared to monotherapy groups. Moreover, co-administration of APD668 and linagliptin resulted in enhanced levels of active Glucagon-Like Peptide-1 (GLP-1) with additional benefit of significant synergistic decrease in body weight gain (−19%) in mice. We speculated that the enhanced effect observed with the combination treatment could be due to either 1) direct activation of GPR119 receptors present in liver and intestine or 2) enhanced active GLP-1 levels or 3) decreased degradation of GLP-1 \textit{in-vivo} through DPPIV inhibition. Therefore, these findings clearly suggest that GPR119 receptor agonists in combination with DPPIV inhibitors may represent a promising therapeutic strategy for the treatment of non-alcoholic steatohepatitis.
4.2 Introduction

Non-Alcoholic SteatoHepatitis (NASH) is characterized by presence of hepatic steatosis, oxidative stress, inflammation and hepatocyte injury with or without fibrosis [1]. The prevalence of NASH is seen more frequently in people with diabetes, obesity, insulin resistance, hyperlipidemia and hypertension. Therefore, NASH can be considered as a hepatic manifestation of metabolic syndrome [2]. At present, there is no approved drug for the treatment of NASH, although weight loss is recommended. Several pharmacotherapies have been attempted to treat NASH/NAFLD with limited benefit overall [3].

Recently, G-protein coupled receptor 119 (GPR119) has emerged as a novel therapeutic target for the treatment of dyslipidemia and NASH [4, 5]. Previously, it has been reported that GPR119 agonists increased incretin (GLP-1 and GIP) levels [6-8] and improved circulating lipid levels in preclinical and clinical settings, suggesting that they act as anti-dyslipidemic agents [9-11]. Some GPR119 agonists have demonstrated attenuation of fatty liver in obese and HFD/STZ diabetic mice [4, 12], inhibited mRNA expressions of MCP-1 and pro IL-1β in choline deficient, amino acid fixed and high fat diet induced steatohepatitis model [13] and inhibited activation of hepatic stellate cells (unpublished results, [14]), suggesting that they have anti-steatotic, anti-inflammatory and anti-fibrotic activity. Similarly, DPPIV inhibitors are a class of oral anti-diabetic agents which also improved hepatic steatosis, insulin resistance, oxidative stress, inflammation and fibrosis in murine models of NASH with or without diabetes background [15-18]. Additionally, it has been reported that patients with NASH have increased DPPIV activity [19, 20].

We have demonstrated strong in-vivo evidence of protection of fatty liver by APD668 in HTF diet induced steatohepatitis model in C57BL/6 mice in our previous
study [5]. The beneficial effects of APD668 could be due to its combined effect on activation of intestinal and hepatic GPR119 receptors directly and elevated GLP-1 (indirect effect) levels which simultaneously contribute to the reduction in triglyceride uptake and hepatic lipogenesis in HTF diet fed mice [5]. Nevertheless, to our knowledge, no study to date has investigated the combined effect of GPR119 receptor agonist with DPPIV inhibitor on the progression of NASH. Our findings suggest that APD668 in combination with linagliptin could potentially improve hepatic injury markers, hepatic steatosis, metabolic markers and ultimately lead to decreased progression of steatohepatitis in mice fed on a high trans-fat (HTF) diet.

4.3 Materials and Methods

4.3.1 Chemicals

APD668, a GPR119 agonist and Linagliptin, a DPPIV inhibitor were obtained from ChemBo Pharma, Nanjing, China. All other chemicals were procured from Sigma-Aldrich, St Louis, MO. APD668 was suspended in 1% Tween-80 + 15% Gelucire 44/14 + 10% Propylene Glycol + 74% Type 1 ultrapure water (1:15:10:74). Linagliptin was suspended in 1% Tween-80 + 99% Methyl cellulose (1:99). Vehicle or APD668 or linagliptin were administered by oral gavage twice daily for 4 weeks.

4.3.2 Animals and experimental protocol

Please refer Chapter 3, Section 3.3.6 for detailed procedure. In this study, male C57BL/6 mice were fed on diet for 12 weeks, ad libitum [21]. Mice were randomly divided into five groups (8 mice/group) and treated for 4 weeks as follows: (1) LFD diet + vehicle (10 ml/kg); (2) HTF diet + vehicle (10 ml/kg); (3) HTF diet + APD668 (12.5 mg/kg); (4) HTF diet + Linagliptin (12.5 mg/kg) and (5) HTF diet + APD668 (12.5 mg/kg) + Linagliptin (12.5 mg/kg). To observe a better combination effect, the doses were chosen based on our previously published data in HTF diet model [5].
Body weight was measured weekly. Post 4 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 snap frozen in liquid nitrogen and stored at -80°C until further analysis.

### 4.3.3 Measurement of plasma biochemical and metabolic markers

Plasma ALT, AST, triglyceride, glucose and cholesterol levels were measured by using automatic biochemical analyzer (Daytona, Randox Inc. UK). Plasma insulin (Mouse Ultrasensitive Insulin ELISA kit, ALPCO Diagnostics, USA), leptin and active GLP-1 levels were measured by using commercially available ELISA kits (Millipore Inc. USA). To assess insulin resistance in mice, the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula:

\[
\text{HOMA-IR} = \frac{\text{fasting plasma glucose (mg/dL)} \times \text{fasting insulin (µU/mL)}}{405}
\]

### 4.3.4 Measurement of hepatic triglyceride and cholesterol

Please refer Chapter 3, as previously described in Section 3.3.7

### 4.3.5 Histological analysis

In brief, fresh liver tissue samples were fixed in 10% formalin and embedded in paraffin. The samples were cross-cut into slices of 4 to 5 µm and stained with hematoxylin and eosin (HE) for evaluation of steatosis. Finally, the stained sections were observed and photographed under a light microscope (with 200× magnification).

### 4.3.6 Statistical Analysis

All the results are expressed as Mean ± S.E.M. One-way ANOVA using Tukey’s post-hoc test was used for multiple comparisons. \(p < 0.05\) was considered to be statistically significant. Data was analyzed using GraphPad version 7.02 of GraphPad Prism for Windows, GraphPad software, San Diego, California, USA.
4.4 Results

Effect of APD668 and linagliptin on biochemical parameters

To characterize the development of murine model of steatohepatitis in the context of obesity and mild hyperglycemia associated with insulin resistance, we maintained C57BL/6 mice on LFD or HTF diet for 16 weeks respectively [21]. As expected, hepatic injury markers such as plasma ALT and AST levels were increased ~5 fold and ~2.5 fold by HTF diet as compared to LFD diet in mice (Fig. 4.1A and B; p < 0.001). Compared to vehicle treated HTF diet mice, both APD668 or linagliptin monotherapy reduced elevated plasma ALT and AST levels (~59% and ~69% in ALT, ~50% and ~48% in AST, both p < 0.001, Fig. 4.1A and B). However, combined treatment of APD668 with linagliptin demonstrated much better improvement in reduction of elevated ALT (~78%) and AST (~61%) as compared to monotherapy groups, as shown in Fig. 4.1A and B. Moreover, HTF diet fed mice developed mild hyperglycemia and hypercholesterolemia in comparison to LFD diet fed mice (p < 0.01, Fig. 4.1C and D). Co-administration of APD668 with linagliptin significantly reduced plasma glucose (~38%) and cholesterol (~31%) levels compared to vehicle treated HTF diet mice. In this study, mice on HTF diet did not develop hypertriglyceridemia which is in-agreement with our earlier study and published data (data not shown) [5, 21].
Figure 4.1 Effect of APD668, linagliptin or their combination on plasma biochemical parameters in mice fed on high trans-fat diet

Mice were treated with vehicle or APD668 (12.5 mg/kg) or linagliptin (12.5 mg/kg) or their combination for 4 weeks twice daily. A) Plasma ALT, B) Plasma AST, C) Plasma glucose, D) Plasma cholesterol. The results are represented as the Mean ± S.E.M. n = 7-8 mice/group; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. Vehicle + LFD diet group; *p < 0.05, **p < 0.01, ***p < 0.001 vs. Vehicle + HTF diet group

Combined effect of APD668 and linagliptin on metabolic markers

Post 4 weeks of treatment (post 14 h of last dose), the plasma hormone levels were estimated in LFD and HTF diet fed mice. As shown in Fig. 4.2A and C, plasma insulin (0.40 ± 0.08 ng/mL vs. 0.12 ± 0.03 ng/mL, p < 0.01) and leptin levels (9.66 ± 0.78 ng/mL vs. 1.21 ± 0.29 ng/mL, p < 0.001) were elevated in mice fed on HTF diet compared to mice on LFD diet; suggesting that HTF diet fed mice developed insulin resistance and leptin resistance. In the present study, significant reduction in insulin levels were observed in APD668 monotherapy (0.18 ± 0.04 ng/mL vs. 0.40 ± 0.08 ng/mL, p < 0.05) and in combination group (0.19 ± 0.03 ng/mL vs. 0.40 ± 0.08 ng/mL, p < 0.05) compared to vehicle treated HTF diet mice as shown in Fig. 4.2A. Additionally, higher HOMA-IR values (p < 0.001) suggesting insulin resistance developed in vehicle treated HTF diet fed mice whereas treatment with APD668 alone (p < 0.01) alone or in combination with linagliptin (p < 0.01) improved insulin resistance in mice as shown in Fig. 4.2B. However, combination of APD668 and
linagliptin treatment decreased leptin levels (2.03 ± 0.71 ng/mL) more than APD668 alone (3.15 ± 0.34 ng/mL) or linagliptin alone (4.28 ± 0.74 ng/mL); although the reduction was not statistically significant compared to monotherapy groups (Fig. 4.2C). Furthermore, both APD668 and linagliptin failed to show significant elevation of active GLP-1 levels in mice. Interestingly, the combination treatment enhanced plasma active GLP-1 levels (26.96 ± 4.47 pM vs. 8.16 ± 2.32 pM, p < 0.01) compared with vehicle treated HTF diet fed mice as shown in Fig. 4.2D. Though, the combination group demonstrated additive effect in increase in active GLP-1 levels; however it failed to show statistical significance as compared to monotherapy groups (Fig. 4.2D).

Figure 4.2 Combined effect of APD668 with linagliptin on plasma metabolic markers in HTF diet fed mice
Mice were treated with vehicle or APD668 (12.5 mg/kg) or linagliptin (12.5 mg/kg) or their combination for 4 weeks twice daily. A) Plasma insulin, B) HOMA-IR C) Plasma leptin, D) Plasma active GLP-1. The results are represented as the Mean ± S.E.M. n = 7-8 mice/group;
#p < 0.05, ##p < 0.01, ###p < 0.001 vs. Vehicle + LFD diet group; *p < 0.05, **p < 0.01, ***p < 0.001 vs. Vehicle + HTF diet group

**Effect of combination of APD668 and linagliptin on body weight gain, liver to body weight (%), hepatic steatosis and fat pad weight in mice**

As expected, HTF diet fed mice exhibited significant increase in body weight gain, liver weight, liver to body weight (%), hepatic steatosis and fat mass as compared to those fed on LFD diet as depicted in Table 4.1. Both APD668 and linagliptin demonstrated reduction in body weight gain (APD668, –13%, p < 0.001 and linagliptin, –4%) following 4 weeks of treatment in mice. Notably, combined treatment of APD668 with linagliptin caused a significant synergistic reduction in body weight gain (–19%) compared to monotherapy groups as shown in Table 4.1.

**Table 4.1 Effect of APD668, linagliptin or their combination on body weight, liver to body weight (%), hepatic steatosis and fat pad weight in mice fed on high trans-fat diet**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle + LFD diet</th>
<th>Vehicle + HTF diet</th>
<th>APD668 (12.5 mg/kg)</th>
<th>Linagliptin (12.5 mg/kg)</th>
<th>APD668 + Linagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.3 ± 0.2</td>
<td>36.3 ± 0.4</td>
<td>31.3 ± 0.6</td>
<td>34.7 ± 0.6</td>
<td>29.3 ± 0.2</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.07 ± 0.04</td>
<td>2.45 ± 0.11</td>
<td>1.18 ± 0.06</td>
<td>1.34 ± 0.07</td>
<td>1.09 ± 0.05</td>
</tr>
<tr>
<td>Liver to body weight (%)</td>
<td>3.79 ± 0.13</td>
<td>6.74 ± 0.29</td>
<td>3.75 ± 0.14</td>
<td>3.85 ± 0.19</td>
<td>3.72 ± 0.16</td>
</tr>
<tr>
<td>Liver TG (mg/g of tissue)</td>
<td>92.0 ± 4.7</td>
<td>327.4 ± 2.5</td>
<td>174.6 ± 15.8</td>
<td>179.3 ± 23.7</td>
<td>134.2 ± 21.9</td>
</tr>
<tr>
<td>Liver TC (mg/g of tissue)</td>
<td>5.7 ± 0.4</td>
<td>26.9 ± 0.5</td>
<td>15.8 ± 1.0</td>
<td>16.1 ± 1.8</td>
<td>12.0 ± 2.2</td>
</tr>
<tr>
<td>Epididymal fat mass (g)</td>
<td>0.61 ± 0.06</td>
<td>2.25 ± 0.09</td>
<td>1.16 ± 0.11</td>
<td>1.35 ± 0.16</td>
<td>0.98 ± 0.06</td>
</tr>
<tr>
<td>Retroperitoneal fat mass (g)</td>
<td>0.13 ± 0.02</td>
<td>0.34 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

Data are represented as Mean ± S.E.M; n = 7-8 mice/group

*ap < 0.001 vs. Vehicle + LFD diet group; bp < 0.001 vs. Vehicle + HTF diet group; cp < 0.05 vs. APD668 group; dp < 0.001 vs. Linagliptin group
In addition, APD668, linagliptin or their combination treatment significantly reduced liver weight, liver/body weight ratio, hepatic steatosis and fat pad weight compared to vehicle treated HTF diet fed mice. Nevertheless, we found that treatment with the combination showed non-significant additive effect in reduction of hepatic triglyceride (combination group, −78% vs. monotherapy, −46%) and hepatic cholesterol (combination group, −56% vs. monotherapy, −40%) in mice. Similarly, combination treatment also caused slightly better reduction in epididymal fat mass (combination group, −56% vs. APD668, −48% and linagliptin, −40%) and additive reduction in retro-peritoneal fat mass (−65%, p < 0.001) compared with linagliptin alone (−26%) in mice (Table 4.1). However, we observed slightly lesser additive effect caused by combination treatment in reduction of hepatic TG, TC and epididymal fat mass than the predicted additive effect in mice. As shown in Fig. 4.3A-E, hepatic accumulation of fat was higher in HTF diet group compared with the LFD diet group and was decreased by treatment with APD668, linagliptin and combination of APD668 and linagliptin in HTF diet fed mice.

Figure 4.3 Effect of APD668, linagliptin or their combination on hepatic steatosis assessed by histopathology in mice fed on high trans-fat diet Hematoxylin and Eosin staining of liver sections from representative mice from each groups; A) LFD diet + vehicle, B) HTF diet + vehicle, C) HTF diet + APD668, D) HTF diet + linagliptin E) HTF diet + APD668 + linagliptin, respectively (magnification, 200×)
4.5 Discussion

NASH is closely associated with metabolic syndrome, which is a lifestyle related disease characterized by obesity, diabetes, dyslipidemia and hypertension. Due to high prevalence and lack of approved treatments, there remains a strong unmet medical need in NASH patients. Given the multifactorial nature of the disease, combination therapies may be needed to achieve best results in NASH management. Therefore, in this study, for the first time, we explored the effect of APD668 in combination with linagliptin on the progression of NASH in mice fed on HTF diet.

As shown in Table 4.1, co-administration of APD668 and linagliptin resulted in a significant synergistic decrease in HTF diet induced body weight gain (−19%) following 4 weeks of treatment in mice. Consistent with the body weight reduction, the combination caused a significant reduction in epididymal fat mass (−56%) and retro-peritoneal fat mass (−65%) compared to monotherapy groups, as shown in Table 4.1. Importantly, combination of APD668 with linagliptin resulted in enhanced active GLP-1 levels and better reduction in leptin levels compared to monotherapy groups in mice (Fig. 4.2C and D). We presume that the reduction in body weight gain elicited by combination of APD668 with linagliptin was mainly due to either significant reduction in fat pad weight or significant increase in GLP-1 levels which in turn would cause reduction in feed intake in mice. This is consistent with a previous report by Al-Barazanji et al. [23] that chronic treatment of GSK2041706, a GPR119 agonist in combination with metformin caused a synergistic reduction in body weight gain mainly due to significant reduction in feed intake and leptin levels associated with greater loss of fat mass in diet induced obese mice.

Furthermore, APD668 or linagliptin alone showed reduction in ALT, AST, glucose and cholesterol levels whereas treatment with combination caused a slightly better
effect on these parameters in mice (Fig. 4.1). On the other hand, biochemical and histological analysis confirmed that combined treatment of APD668 with linagliptin demonstrated a non-significant additive effect in reduction of hepatic triglyceride (−78%) and cholesterol (−56%) compared to monotherapy groups, as shown Fig.4.3 and Table 4.1. We speculated that non-significant additive effect could be due to maximal or near maximal activity observed with single agents per se in this study. Thus, further investigative studies at lower doses are required to verify potential synergistic effects between APD668 and linagliptin. Surprisingly, even at the selected doses, we found that combination treatment caused significant effect on GLP-1 levels, fat mass and body weight gain in mice. It has been reported previously that 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 liver specific GPR119 KO mice [4]. Therefore, we hypothesized that APD668 might be inhibiting hepatic steatosis via direct activation of GPR119 receptors in the liver of HTF diet fed mice. In addition, it was suggested that chronic treatment with DPPIV inhibitors (sitagliptin and linagliptin) reduced hepatic steatosis via inhibition of lipogenesis related genes (SREBP-1c, FAS, and SCD-1) in diet induced obese mice [15, 16]. However, we cannot rule out the role of incretin hormones specifically GLP-1, on protection of fatty liver in mice. Previous studies have reported that chronically elevated GLP-1 levels in DPPIV deficient rats and GLP-1 agonist (exendin-4) exhibit improvement in hepatic steatosis by downregulation of de novo lipogenesis, activation of AMPK pathways and improving insulin sensitivity in rodent species [24, 25]. In our study, we found that chronic treatment with APD668 or linagliptin alone non-significantly elevated GLP-1 levels whereas co-administration of APD668 and
linagliptin caused a significant increase in active GLP-1 levels in HTF diet fed mice (Fig. 4.2C). Thus, it is tempting to speculate that anti-steatotic effect of combination therapy could be partly due to release of GLP-1 through activation of GPR119 receptors present on intestine and partly via prevention of degradation of GLP-1 \textit{in-vivo} through DPPIV inhibition.

In this study, we have not evaluated the effect of APD668, linagliptin or their combination on hepatic inflammation and fibrosis markers in HTF diet fed mice. It has been reported that significant inflammation and fibrosis was only observed in diabetic Lep\textsuperscript{ob}/Lep\textsuperscript{ob} mice whereas it was less evident in normal C57BL/6 mice on HTF diet feeding for up to 16 weeks [21]. Furthermore, Clapper \textit{et al.} [26] reported that high trans-fat diet induces all stages of NAFLD for periods >20 weeks in normal C57BL/6 mice.

In summary, this is the first study to report the combined effect of APD668, a GPR119 agonist and linagliptin, a DPPIV inhibitor, on the progression of simple steatosis to steatohepatitis in a murine model of NASH. Our findings demonstrated enhanced effects on reduction in hepatic injury markers, hyperglycemia, hypercholesteremia, hepatic steatosis, fat mass and leptin levels caused by combination of APD668 with linagliptin in HTF diet fed mice. Of note, co-administration of APD668 with linagliptin also caused synergistic reduction in body weight gain and increased GLP-1 levels which might be beneficial for patients with NASH/NAFLD. Thus, these findings suggest potential beneficial therapeutic effects of GPR119 agonists in combination with DPPIV inhibitors for treatment of NASH.
4.6 References


