Chapter 6

Effect of APD668, a GPR119 agonist on the development of steatohepatitis in mice fed on methionine and choline deficient diet
6.1 Abstract

Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the most common cause of chronic liver disease. Non-Alcoholic SteatoHepatitis (NASH) is the more severe form of NAFLD and is characterized by presence of steatosis, inflammation and hepatocyte injury with or without fibrosis. In the present study, we evaluated the effects of APD668, a GPR119 agonist on the development of steatohepatitis in mice fed on Methionine and Choline Deficient (MCD) diet. APD668 (6.25, 12.5 and 25 mg/kg) was administered by oral gavage twice daily for 5 weeks to mice. Our findings showed that APD668 at 6.25 and 12.5 mg/kg significantly reduced elevated levels of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and hepatic triglyceride whereas high dose (25 mg/kg) failed to show significant improvement in mice. To elucidate lack of activity at a high dose, we investigated time-dependent activity of different regimens of APD668 such as twice daily (b.i.d.), alternate day b.i.d. and once daily (q.d.) dosing in mice. We found that q.d. regimen of APD668 demonstrated greater and sustained reduction in ALT levels unlike b.i.d. and alternate day b.i.d. regimen. Furthermore, APD668 in combination with linagliptin, a Dipeptidyl Peptidase IV (DPPIV) inhibitor demonstrated greater reduction in ALT levels and raised High Density Lipoprotein (HDL) levels compared to linagliptin alone. However, greater reduction on ALT was not observed but significantly elevated HDL levels were seen when APD668 was combined with fenofibrate, a Peroxisome Proliferator-Activated Receptors-α (PPAR-α) agonist compared with APD668 monotherapy. Taken together, these findings suggest that optimal dosing regimen of GPR119 agonist alone and in combination with DPPIV inhibitor could be a novel therapeutic strategy for the treatment of NASH.
6.2 Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the most common cause of chronic liver disease. It ranges from simple hepatic steatosis (benign condition) to Non-Alcoholic SteatoHepatitis (NASH) which can result in fibrosis, cirrhosis, and ultimately, HepatoCellular Carcinoma (HCC). NASH is characterized by presence of hepatic steatosis, inflammation and hepatocyte injury with or without fibrosis [1]. A multiple parallel hit theory has been proposed for the pathogenesis of NASH where “first hit” is the development of hepatic fat accumulation and the “second hit” such as gut derived or adipocyte derived factors or many other hits may act in parallel caused oxidative stress followed by inflammation and hepatocyte injury [2]. Due to its high prevalence and lack of FDA approved treatments, there remains a strong unmet medical need in NASH patients. Recently, several anti-diabetic agents have been suggested for the treatment of NASH [3].

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]. GPR119 agonists act via a dual mechanism 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]. Recently, GPR119 has gained attention because of the emerging role of its receptor in lipid metabolism. GPR119 agonists have demonstrated improvement in lipid levels in preclinical and clinical settings, suggesting that they act as anti-dyslipidemic agent [8-10]. Some GPR119 agonists have demonstrated attenuation of fatty liver in obese and HFD/STZ diabetic mouse [11, 12], inhibited mRNA expressions of MCP-1 and pro IL-1β in choline deficient, amino acid fixed and HFD 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. Recently, we also reported that APD668, a GPR119 agonist improves fat tolerance and attenuates fatty liver in High Trans-Fat (HTF) diet induced steatohepatitis model [15].

Based on these reports, we investigated plausible beneficial effects of APD668 on the progression of NASH in MCD diet fed mice. To the best of our knowledge, this is the first report demonstrating that APD668, a GPR119 agonist, prevented MCD diet induced hepatic injury and steatosis. We also examined combination effects of APD668 with linagliptin or fenofibrate in mice. In the present study, APD668 in combination with linagliptin demonstrated greater reduction in hepatic injury markers and raised HDL levels in mice. However, greater reduction on ALT, AST levels were not observed when APD668 was combined with fenofibrate in mice. Taken together, these findings suggest that GPR119 receptor could be a novel target for the therapeutic management of NASH.

6.3 Materials and Methods

6.3.1 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) whereas linagliptin and fenofibrate were suspended in 1% Tween-80 + 99% Methyl Cellulose.

6.3.2 Animals and experimental protocol

Male C57BL/6 mice, 7-8 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. Before experimentation, animals were acclimatized to experimental animal facility for 2 weeks. They were fed on a normal diet (Altromin diet, Germany) or a Methionine and Choline Deficient (MCD) diet (Research Diets, New Brunswick, NJ, USA) and RO water *ad libitum* throughout the experimental period. 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.

### 6.3.3 Effect of APD668 (6.25, 12.5 and 25 mg/kg) on the development of steatohepatitis in MCD diet fed mice

Post 2 weeks of acclimatization period, male C57BL/6 mice (9–10 weeks old) were randomly divided into five groups (9 mice/group) and treated for 5 weeks as follows: (1) Normal diet + vehicle (10 ml/kg); (2) MCD diet + vehicle (10 ml/kg); (3) MCD diet + APD668 (low dose, 6.25 mg/kg); (4) MCD diet + APD668 (medium dose, 12.5 mg/kg); (5) MCD diet + APD668 (high dose, 25 mg/kg). Vehicle or APD668 was administered by oral gavage twice daily for 5 weeks from the day mice were on MCD diet. Post 5 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.

### 6.3.4 Time-dependent activity of different regimens of APD668 (25 mg/kg) on the development of steatohepatitis in MCD diet fed mice

Male C57BL/6 mice (9–10 weeks of age) were randomly divided into four groups (8 mice/group) and treated for 6 weeks as follows: (1) MCD diet + vehicle (10 ml/kg,
MCD diet + APD668 (25 mg/kg, \textit{b.i.d.}); (3) MCD diet + APD668 (25 mg/kg, alternate day \textit{b.i.d.}); (4) MCD diet + APD668 (25 mg/kg, \textit{q.d.}). Vehicle or APD668 was administered by oral gavage for 6 weeks from the day mice were on MCD diet. Animals were fasted overnight (14 h) and blood samples were collected from retro orbital plexus under mild isoflurane anesthesia for biochemical and estimation of plasma levels of APD668 on day 14, day 28 and day 42 respectively. Post 6 weeks of treatment, the livers were isolated, immediately snap frozen in liquid nitrogen and stored at -80°C until further analysis.

6.3.5 \textbf{Effect of combination of APD668 with linagliptin or fenofibrate on the development of steatohepatitis in MCD diet fed mice}

Male C57BL/6 mice (9-10 weeks of age) were randomly divided into six groups (10 mice/group) and treated for 5 weeks as follows: (1) MCD diet + vehicle (10 ml/kg); (2) MCD diet + APD668 (12.5 mg/kg); (3) MCD diet + Linagliptin (12.5 mg/kg); (4) MCD diet + APD668 (12.5 mg/kg) + Linagliptin (12.5 mg/kg); (5) MCD diet + Fenofibrate (25 mg/kg) and (6) MCD diet + APD668 (12.5 mg/kg) + Fenofibrate (25 mg/kg). We chose the doses of linagliptin and fenofibrate based on in-house data in MCD diet fed mice model (data not shown). Vehicle or APD668 or linagliptin or fenofibrate were administered by oral gavage twice daily for 5 weeks from the day mice were on MCD diet. Post 5 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.

6.3.6 \textbf{Determination of plasma ALT, AST and lipid levels}

Please refer Chapter 4, as previously described in Section 4.3.3

6.3.7 \textbf{Determination of hepatic triglyceride and cholesterol content}
Please refer Chapter 3, as previously described in Section 3.3.7

6.3.8 APD668 levels in Plasma

Plasma concentrations of APD668 were determined by a high-performance liquid chromatography method developed in our laboratory. Briefly, blood was collected from retro-orbital plexus under isoflurane anesthesia in K$_2$EDTA (1 mg/0.1 ml per tube) containing microcentrifuge tubes. Plasma was obtained by centrifugation at 3192g for 8 min at 4°C. For time-dependent study, equal volume of plasma samples (15 µl) were pooled from each mouse from respective treatment groups. Plasma samples were collected post 14 h of last dose in b.i.d. treatment group and post 24 h of last dose in q.d. group. Plasma samples were processed by aliquoting each of 25 µl calibration standards, quality control and plasma study samples into a 96 well plate of 2 ml capacity. Then, 300 µl of internal standard working solution was added in all the samples except blank standard. In the blank standard, 300 µl of ammonium hydroxide in acetonitrile solution (0.1% v/v) was added and aspirated 3-4 times for proper mixing. The above mixture was centrifuged at 1096g for 10 min at 4°C. The supernatant was transferred to 1 ml 96-well plate and again centrifuged at 1096g for 10 min, then subjected to LC-MS/MS for bioanalysis. The LC-MS/MS system consisted of a Waters Acquity UPLC Separations Module with Xevo TQ-S Mass Spectrometer Detector equipped with Electrospray ionization source. APD668 was ionized in positive polarity. Separation was carried out at 40°C on a Waters Acquity BEH C18 column of 50 × 2.1 mm internal diameter and a particle size of 1.7 µm. Elution was carried out with a gradient of 0.1% v/v formic acid in acetonitrile and 0.1% v/v formic acid in water at a flow rate of 0.45 ml/min. The retention time for APD668 was 1.21 min. Calibration curve was obtained by dissolving a known quantity of APD668 in mice plasma to obtain concentrations ranging from 0.05 to 10
μm with lower, middle, higher and dilution quality control samples. A linear relationship ($r^2 = 0.9966$) was obtained when peak areas were plotted against plasma concentration. Accuracy ranged from 85% to 115% for calibration standard and quality control samples.

### 6.3.9 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. The data were analyzed using GraphPad version 7.02 of GraphPad Prism for Windows, GraphPad software, San Diego, California, USA.
6.4 Results

Effect of APD668 on body weight and liver weight in mice fed on MCD diet

To investigate the effect of APD668 on the development of MCD diet induced steatohepatitis, varying doses of APD668 (6.25, 12.5 and 25 mg/kg) were administered orally twice daily for five weeks in mice. In the present study, body weight and liver weight of MCD diet fed mice were decreased as compared to mice kept on normal diet as shown in Table 6.1 (p < 0.001). Oral administration of APD668 did not show effect on body weight and liver weight except at the lowest dose of 6.25 mg/kg where significant reduction was observed in liver weight/body weight (%) as compared to MCD diet control mice.

Table 6.1 Effect of APD668 on body weight and liver weight in mice fed on MCD diet

<table>
<thead>
<tr>
<th>Treatment parameters</th>
<th>Normal diet + Vehicle (10 ml/kg)</th>
<th>MCD diet + Vehicle (10 ml/kg)</th>
<th>MCD diet + APD668 (6.25 mg/kg)</th>
<th>MCD diet + APD668 (12.5 mg/kg)</th>
<th>MCD diet + APD668 (25 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW (g)</td>
<td>26.1 ± 0.9</td>
<td>26.3 ± 0.7</td>
<td>26.3 ± 0.6</td>
<td>26.3 ± 0.6</td>
<td>26.3 ± 0.6</td>
</tr>
<tr>
<td>Final BW (g)</td>
<td>27.6 ± 0.6</td>
<td>18.7 ± 0.5 c</td>
<td>20.6 ± 0.5</td>
<td>19.3 ± 0.6</td>
<td>18.5 ± 0.7</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>0.93 ± 0.03</td>
<td>0.61 ± 0.03c</td>
<td>0.58 ± 0.03</td>
<td>0.57 ± 0.02</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>Liver weight / body weight (%)</td>
<td>3.38 ± 0.07</td>
<td>3.26 ± 0.12</td>
<td>2.80 ± 0.10 a</td>
<td>2.97 ± 0.12</td>
<td>2.91 ± 0.10</td>
</tr>
</tbody>
</table>

Data are represented as Mean ± S.E.M; n = 7-9 mice/group

a p < 0.05 vs. Normal diet + vehicle group. b p < 0.01 vs. Normal diet + vehicle group. c p < 0.001 vs Normal diet + vehicle group;

Effect of APD668 on MCD diet induced hepatic injury

To investigate the effect of APD668 on hepatic injury markers, we measured the plasma levels of ALT and AST in mice. As shown in Fig.6.1A and B, there was
increase in plasma ALT and AST levels in mice kept on MCD diet as compared those on the normal diet (p < 0.001). In our study, APD668 significantly reduced elevated levels of ALT and AST at a low dose 6.25 mg/kg (ALT: 72 ± 9% and AST: 41 ± 7%) and a medium dose 12.5 mg/kg (ALT: 75 ± 3% and AST: 41 ± 6%) as compared to MCD diet control mice. However, APD668 at a high dose 25 mg/kg (ALT: 37 ± 11% and AST: 12 ± 4%) showed slightly less activity as compared to low and medium dose in mice as shown in Fig 6.1A and B.

**Figure 6.1 Effect of APD668 on plasma ALT and AST levels in mice fed on MCD diet.** Data are represented as Mean ± S.E.M; n = 7-9 mice/group. ### p < 0.001 vs. normal diet + vehicle treated mice. *** p < 0.001 vs. MCD diet + vehicle treated mice

**Effect of APD668 on hepatic and plasma lipid levels in mice fed on MCD diet**

As shown in Fig 6.2A and B, the hepatic triglyceride and cholesterol contents were increased in mice fed on MCD diet as compared to mice on normal diet (p < 0.001). APD668 (6.25 and 12.5 mg/kg) decreased hepatic triglyceride (p < 0.001) whereas significant reduction was observed in hepatic cholesterol only at 12.5 mg/kg dose in mice. Paradoxically, a high dose of APD668 (25 mg/kg) lacked these inhibitory effects as shown in Fig. 6.2A and B. As expected, plasma lipid levels were significantly lowered in MCD diet fed mice as compared to normal diet mice as shown in Fig. 6.2C, D and E. In the present study, APD668 (6.25 and 12.5 mg/kg)
treatment inhibited the reduction in plasma HDL-cholesterol whereas no effect was observed at a high dose (25 mg/kg) in mice (Fig. 6.2C). In addition, administration of APD668 (6.25 and 12.5 mg/kg) treatment reversed plasma cholesterol levels to normal (p < 0.001, Fig. 6.2D) whereas reversal effect was not observed on plasma triglyceride levels as shown in Fig. 6.2E.

Figure 6.2 Effect of APD668 on hepatic and plasma lipid levels in mice
Data are represented as Mean ± S.E.M; n = 7-9 mice/group.
# p < 0.01, ## p < 0.001 vs. normal diet + vehicle treated mice
** p < 0.01, *** p < 0.001 vs. MCD diet + vehicle treated mice
Time-dependent activity of different regimens of APD668 (25 mg/kg) on the development of steatohepatitis in MCD diet fed mice

In an effort to comprehend the lack of activity of APD668 at a high dose (25 mg/kg), we performed the experiment using three different regimens as follows: APD668 was administered twice daily (*b.i.d.*), alternate day *b.i.d.* and once daily (*q.d.*) for 6 weeks to MCD diet fed mice. Control group mice received vehicle twice daily (*b.i.d.*) for 6 weeks. Plasma biochemical parameters and plasma concentrations of APD668 were measured post 2, 4 and 6 weeks of treatment in mice. In our study, post 2 weeks, APD668 *b.i.d.* treatment demonstrated greater reduction in ALT (78 ± 4%) as compared to alternate day *b.i.d.* (63 ± 3%) and *q.d.* treatment (45 ± 8%) in mice as shown in Table 6.2 and Fig. 6.3. Interestingly, post 4 weeks, we found that *q.d.* treatment of APD668 showed greater reduction in ALT (79 ± 3%) as compared to *b.i.d.* (39 ± 5%) and alternate day *b.i.d.* (46 ± 7%) regimen. Then, post 6 weeks, the *q.d.* treatment of APD668 demonstrated sustained greater reduction in ALT (77 ± 3%) compared to *b.i.d.* (55 ± 13%) and alternate day *b.i.d.* (38 ± 13%) regimen as shown in Table 6.2 and Fig. 6.3. Furthermore, post 2 weeks, APD668 *b.i.d.* regimen reduced elevated plasma AST levels (*p* < 0.001, Table 6.2) but not following post 4 and 6 weeks of treatment period. On the other hand, alternate day *b.i.d.* and *q.d.* treatment of APD668 failed to show a decrease in AST levels in mice. Also, post 2 weeks, APD668 *b.i.d.* and alternate day *b.i.d.* treatment demonstrated significant increase in plasma HDL levels compared with vehicle treated mice, but post 4 and 6 weeks of treatment period failed to show significant increase in HDL levels. In contrast, *q.d.* treatment (post 4 and 6 weeks) demonstrated significant rise in plasma HDL levels compared with vehicle treated mice as shown in Table 6.2. We also found that, post 6 weeks of APD668 *b.i.d.* and *q.d.* treatment showed significant increase in plasma
HDL levels as compared to alternate day *b.i.d.* regimen. However, in this study, none of the APD668 regimen showed significant effect on liver weight, hepatic triglyceride and cholesterol in mice as shown in Table 6.2.

**Table 6.2 Effect of time-dependent activity of different regimens of APD668 (25 mg/kg) on biochemical parameters and hepatic steatosis in MCD diet fed mice**

<table>
<thead>
<tr>
<th>Treatment parameters</th>
<th>Weeks</th>
<th>MCD diet + Vehicle (10 ml/kg; b.i.d.)</th>
<th>MCD diet + APD668 (25 mg/kg; b.i.d.)</th>
<th>MCD diet + APD668 (25 mg/kg; alternate day b.i.d.)</th>
<th>MCD diet + APD668 (25 mg/kg; q.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/I)</td>
<td>2</td>
<td>133 ± 15</td>
<td>29 ± 6&lt;sup&gt;x&lt;/sup&gt;</td>
<td>50 ± 4</td>
<td>73 ± 11</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>279 ± 48</td>
<td>171 ± 15&lt;sup&gt;x&lt;/sup&gt;</td>
<td>150 ± 20&lt;sup&gt;y&lt;/sup&gt;</td>
<td>58 ± 10&lt;sup&gt;x,q&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>229 ± 51</td>
<td>103 ± 30&lt;sup&gt;x&lt;/sup&gt;</td>
<td>141 ± 29</td>
<td>52 ± 8&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>AST (U/I)</td>
<td>2</td>
<td>185 ± 26</td>
<td>64 ± 8&lt;sup&gt;z&lt;/sup&gt;</td>
<td>134 ± 8</td>
<td>126 ± 13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>170 ± 25</td>
<td>166 ± 11</td>
<td>156 ± 18</td>
<td>153 ± 14</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>192 ± 28</td>
<td>125 ± 24</td>
<td>161 ± 64</td>
<td>133 ± 22</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>2</td>
<td>8 ± 2</td>
<td>15 ± 8&lt;sup&gt;y&lt;/sup&gt;</td>
<td>17 ± 1&lt;sup&gt;z&lt;/sup&gt;</td>
<td>11 ± 2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3 ± 1</td>
<td>9 ± 1</td>
<td>7 ± 1</td>
<td>11 ± 2&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5 ± 1</td>
<td>11 ± 1&lt;sup&gt;t&lt;/sup&gt;</td>
<td>4 ± 1</td>
<td>12 ± 2&lt;sup&gt;x,u&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>6</td>
<td>0.50 ± 0.03</td>
<td>0.55 ± 0.01</td>
<td>0.48 ± 0.01</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td>Liver TG (mg/g of tissue)</td>
<td>6</td>
<td>133.5 ± 16.1</td>
<td>115.1 ± 11.4</td>
<td>118.1 ± 15.0</td>
<td>103.4 ± 11.2</td>
</tr>
<tr>
<td>Liver TC (mg/g of tissue)</td>
<td>6</td>
<td>5.7 ± 0.4</td>
<td>5.3 ± 0.6</td>
<td>5.2 ± 0.5</td>
<td>4.7 ± 0.3</td>
</tr>
</tbody>
</table>

Data are represented as Mean ± S.E.M; n = 6-8 mice/group

<sup>x</sup><i>p</i> < 0.05 vs. MCD diet + vehicle group, <sup>y</sup><i>p</i> < 0.01 vs. MCD diet + vehicle group, <sup>z</sup><i>p</i> < 0.001 vs. MCD diet + vehicle group; <sup>4</sup><i>p</i> < 0.05 vs. APD668 *b.i.d.* group, <sup>5</sup><i>p</i> < 0.01 vs. APD668 *b.i.d.* group, <sup>6</sup><i>p</i> < 0.001 vs. APD668 *b.i.d.* group; <sup>7</sup><i>p</i> < 0.05 vs. APD668 alternate day *b.i.d.* group, <sup>8</sup><i>p</i> < 0.01 vs. APD668 alternate day *b.i.d.* group.

Further, to investigate whether lack of activity with *b.i.d.* regimens was due to decreased levels of APD668 over the treatment period due to induction of metabolizing enzymes if any, we estimated plasma levels of APD668 in mice. As
shown in Table 6.3, we did not observe a decrease in levels of APD668 post 4 and 6 weeks in \textit{b.i.d.} regimens compared to week 2. In fact, we noticed increased levels with time possibly due to accumulation of APD668 in plasma. Interestingly, accumulation of APD668 over the treatment period was not observed in \textit{q.d.} regimen as shown in Table 6.3.

![Graph](image)

**Figure 6.3 Effect of APD668 on plasma ALT levels in mice**
Data are represented as Mean ± S.E.M.; \(n = 6\)-8 mice/group.
** \(p < 0.01\) vs. APD668 (25 mg/kg; \textit{b.i.d.}); $ \ p < 0.01\) vs. APD668 (25 mg/kg; alternate day \textit{b.i.d.}); # \(p < 0.05\) vs. APD668 (25 mg/kg; \textit{q.d.})

**Table 6.3 Plasma concentrations of APD668 in fasted state in MCD diet fed mice**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Dose ((mg/kg; p.o.))</th>
<th>Plasma Conc. ((\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 2</td>
</tr>
<tr>
<td>APD668</td>
<td>25, \textit{b.i.d.}</td>
<td>21.62</td>
</tr>
<tr>
<td>APD668</td>
<td>25, alternate day \textit{b.i.d.}</td>
<td>17.77</td>
</tr>
<tr>
<td>APD668</td>
<td>25, \textit{q.d.}</td>
<td>5.17</td>
</tr>
</tbody>
</table>

APD668 plasma levels were estimated from pooled mixture of equal volume of plasma from each mouse from respective treatment groups; samples were collected post 14 h of last dose in \textit{b.i.d.} treatment groups and post 24 h of last dose in \textit{q.d.} group; ND – Not determined
Effect of combination of APD668 with linagliptin or fenofibrate on the development of steatohepatitis in MCD diet fed mice

Further, we investigated the combined effect of APD668, a GPR119 agonist with linagliptin, a DPPIV inhibitor or with fenofibrate, a PPAR-α agonist on the progression of steatohepatitis induced by MCD diet in mice. In the present study, APD668 (12.5 mg/kg) alone reduced elevated plasma ALT and AST levels compared with vehicle treated mice (p < 0.01, Table 6.4). Linagliptin (12.5 mg/kg) and fenofibrate (25 mg/kg) alone treatment also demonstrated reduction in ALT and AST levels (p < 0.05, p < 0.001) in mice. Interestingly, combination of APD668 with linagliptin demonstrated greater reduction in plasma ALT levels compared with linagliptin alone (ALT: APD668 + linagliptin: 27 ± 2 vs. linagliptin alone: 94 ± 11; p < 0.01; Table 6.4). Conversely, the combination of APD668 with fenofibrate did not demonstrate a similar trend in ALT reduction as compared to APD668 or fenofibrate monotherapy groups. Paradoxically, APD668 in combination with fenofibrate failed to decrease plasma AST levels (Table 6.4). In our study, combination of APD668 both with linagliptin or fenofibrate significantly increases plasma HDL levels compared with their respective treatment groups in mice as shown in Table 6.4. Next, we found that APD668, linagliptin and fenofibrate alone demonstrated significant reduction in hepatic triglyceride and cholesterol content as compared to vehicle treated mice. On the contrary, combined effect of APD668 with linagliptin or fenofibrate failed to demonstrate synergistic or additive effect on hepatic steatosis as compared to their monotherapy groups as shown in Table 6.4. APD668 and linagliptin alone or in combination did not show any effect on liver weight whereas as expected, fenofibrate alone or in combination with APD668 showed significant increase in liver weight compared with vehicle treated mice (Table 6.4).
Table 6.4 Effect of APD668, linagliptin and fenofibrate alone or in combination on biochemical parameters and hepatic steatosis in MCD diet fed mice

<table>
<thead>
<tr>
<th>Treatment parameters</th>
<th>Vehicle + Vehicle</th>
<th>Vehicle + APD668</th>
<th>Vehicle + Linagliptin</th>
<th>APD668 + Linagliptin</th>
<th>Vehicle + Fenofibrate</th>
<th>APD668 + Fenofibrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/I)</td>
<td>130 ± 18</td>
<td>62 ± 18&lt;sup&gt;y&lt;/sup&gt;</td>
<td>94 ± 11</td>
<td>27 ± 2&lt;sup&gt;x,f&lt;/sup&gt;</td>
<td>80 ± 7</td>
<td>65 ± 10&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>AST (U/I)</td>
<td>163 ± 20</td>
<td>105 ± 12&lt;sup&gt;y&lt;/sup&gt;</td>
<td>112 ± 7&lt;sup&gt;x&lt;/sup&gt;</td>
<td>79 ± 4&lt;sup&gt;y&lt;/sup&gt;</td>
<td>90 ± 8&lt;sup&gt;z&lt;/sup&gt;</td>
<td>121 ± 9</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>7 ± 1</td>
<td>11 ± 1</td>
<td>6 ± 1</td>
<td>12 ± 1&lt;sup&gt;x,f&lt;/sup&gt;</td>
<td>15 ± 1&lt;sup&gt;z&lt;/sup&gt;</td>
<td>16 ± 1&lt;sup&gt;x,i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>0.51 ± 0.02</td>
<td>0.49 ± 0.02</td>
<td>0.53 ± 0.02</td>
<td>0.55 ± 0.02</td>
<td>0.67 ± 0.02</td>
<td>0.66 ± 0.02</td>
</tr>
<tr>
<td>Liver TG (mg/g of tissue)</td>
<td>135.2 ± 11.8</td>
<td>76.9 ± 8.1&lt;sup&gt;y&lt;/sup&gt;</td>
<td>74.1 ± 1.5&lt;sup&gt;z&lt;/sup&gt;</td>
<td>77.4 ± 1.5&lt;sup&gt;z&lt;/sup&gt;</td>
<td>61.2 ± 1.5&lt;sup&gt;z&lt;/sup&gt;</td>
<td>60.7 ± 4.6&lt;sup&gt;z&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver TC (mg/g of tissue)</td>
<td>5.0 ± 0.6</td>
<td>3.1 ± 0.1&lt;sup&gt;y&lt;/sup&gt;</td>
<td>3.8 ± 0.4</td>
<td>3.3 ± 0.3&lt;sup&gt;y&lt;/sup&gt;</td>
<td>2.7 ± 0.2&lt;sup&gt;z&lt;/sup&gt;</td>
<td>2.5 ± 0.1&lt;sup&gt;z&lt;/sup&gt;</td>
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Data are represented as Mean ± S.E.M; n = 8-10 mice/group

<sup>x</sup>p < 0.05 vs. Vehicle + Vehicle group, <sup>y</sup>p < 0.01 vs. Vehicle + Vehicle group, <sup>z</sup>p < 0.001 vs. Vehicle + Vehicle group; <sup>z</sup>x<p < 0.05 vs. Vehicle + Linagliptin group, <sup>y</sup>x<p < 0.01 vs. Vehicle + Linagliptin group, <sup>z</sup>y<p < 0.001 vs. Vehicle + Linagliptin group; <sup>i</sup>p < 0.05 vs. Vehicle + APD668 group, <sup>j</sup>i<p < 0.01 vs. Vehicle + APD668 group, <sup>k</sup>i<p < 0.001 vs. Vehicle + APD668 group.
6.5 Discussion

The Methionine and Choline Deficient (MCD) diet model is a well-established, reproducible and commonly used dietary model for studying NASH. The impaired hepatic VLDL (Very Low-Density Lipoprotein) secretion may play a role in MCD diet induced hepatic steatosis in mice. In addition, due to low calorie intake, mice fed on MCD diet have significant body weight loss, reduced circulating levels of glucose, triglyceride, cholesterol, leptin, insulin and fat pad weight. Consequently, mice fed on MCD diet do not exhibit obesity, insulin resistance, dyslipidemia and diabetes, which are recognized features of human NASH [17, 18]. However, MCD diet induces severe phenotype of hepatic injury markers (ALT and AST) and hepatic steatosis within a short period in rodents. The present study demonstrated for the first time that APD668, a GPR119 agonist alone and in combination with linagliptin, a DPPIV inhibitor, demonstrated hepatoprotective and anti-steatotic activity in a murine model of NASH.

In the present study, APD668 (6.25 and 12.5 mg/kg) significantly reduced MCD diet induced elevated levels of ALT, AST and hepatic triglyceride in mice (Fig. 6.1 and 6.2). Also, APD668 (6.25 and 12.5 mg/kg) significantly inhibited the reduction in HDL-cholesterol levels and reversed the plasma cholesterol to normal levels in mice (Fig. 6.2C and D). Conversely, APD668 failed to show an effect on body weight, liver weight and plasma triglyceride levels in mice (Fig. 2E and Table 6.1). Recently, Yang JW et al. [12] reported that GPR119 receptors were also expressed in liver tissues with predominant expression in gastrointestinal tract and pancreas. MBX2982, another 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 obese mice and hepatic anti-lipogenesis effects of MBX2982 were abolished
in liver specific GPR119 KO mice. Therefore, we hypothesized that APD668 might be inhibiting hepatic steatosis via direct activation of GPR119 receptors in the liver of MCD diet fed mice. Additionally, APD668 might be inhibiting steatosis indirectly via release of GLP-1 through activation of GPR119 receptors present on the intestine. Previously, it has been reported that GLP-1 plays a major role in improvement of fatty liver in rodent studies [19, 20]. Thus, further studies are warranted for better understanding of the mechanism of action of APD668 in attenuation of hepatic steatosis in MCD diet fed mice.

Surprisingly, we observed that repeated administration of APD668 at a high dose (25 mg/kg) showed slightly less activity or lack of significant activity as compared to low dose (6.25 mg/kg) and medium dose (12.5 mg/kg) on different markers such as plasma ALT, AST, hepatic triglyceride and cholesterol content in mice (Fig. 6.1 and 6.2). Previously, we reported similar trend of lack of activity or lesser activity at a high dose of APD668 on parameters such as plasma ALT, AST, cholesterol, leptin and insulin levels in high trans-fat (HTF) diet fed obese mice [15]. We speculated that the lack of activity at a high dose could be due to desensitization of GPR119 receptors in mice. Some evidence for GPR119 desensitization has been previously reported in literature [21-23]. Our findings are in-line with an earlier report that oleoylethanolamide (OEA), an endogenous ligand of GPR119 receptor showed a bell shaped concentration-response curve on GLP-1 secretion in mGLUTag cells suggesting GPR119 receptor desensitization at a higher concentration [21]. Further, to support the hypothesis of GPR119 receptor mediated desensitization as one of the major mechanisms behind the lack of efficacy at a high dose, we studied time-dependent effect of different regimens of APD668 in mice. Our findings clearly indicate that b.i.d. treatment of APD668 led to a decreased activity on ALT reduction.
(post 4 and 6 weeks vs. post 2 weeks) over the treatment period. On the other hand, *q.d.* regimen of APD668 showed greater and sustained ALT reduction as compared to *b.i.d.* and alternate day *b.i.d.* regimen throughout the 6 weeks treatment period (Table 6.2 and Fig. 6.3). The above experimental findings strongly suggest that *b.i.d.* regimen probably results in desensitization of GPR119 receptors (lack of activity) but not the *q.d.* regimen. It is interesting to note that plasma APD668 levels also did not decrease on chronic treatment in any regimen (Table 6.3). Thus, these findings led us to suggest that lack of activity (desensitization) is due to pharmacodynamic phenomenon. Additional studies are required to determine whether desensitization is due to defect in receptor signaling or down regulation of receptors or a combination of these factors. The time-dependent desensitization phenomenon was also observed with APD668 on reduction in AST levels and elevation of HDL levels in mice (Table 6.2). To the best of our knowledge, we are the first to report time dependent desensitization phenomenon with a GPR119 agonist in mice. However, extensive studies are warranted to verify whether desensitization is compound specific or a target related phenomenon with respect to end parameter(s) and dosage regimen.

Furthermore, we evaluated the effects of APD668 in combination with linagliptin or fenofibrate on the development of steatohepatitis induced by MCD diet in C57BL/6 mice. Previously, it has been demonstrated that linagliptin, a DPPIV inhibitor [24] and Wy-14643, a PPAR-α agonist [25] improved steatohepatitis in MCD diet fed mice. As shown in Table 6.4, our findings suggest that combination of APD668 with linagliptin demonstrated greater reduction in ALT levels and increase in plasma HDL levels in mice. In contrast, co-dosing with fenofibrate failed to show comparable reduction in hepatic injury markers in mice (Table 6.4). Surprisingly, both
combination groups did not demonstrate synergistic or additive effect in attenuation of hepatic steatosis as compared to alone treatment groups in mice.

In summary, the present studies for the first-time report that APD668, a GPR119 agonist at an optimum dose demonstrated attenuation of fatty liver in MCD diet fed mice. Additionally, once daily regimen of APD668 (25 mg/kg) showed greater and sustained reduction in ALT levels compared with b.i.d. and alternate day b.i.d. regimen in mice. APD668 treatment alone or in combination with linagliptin improved hepatic injury markers and also increased HDL-cholesterol, which might be beneficial effect for patients with non-alcoholic steatohepatitis. It would be an interesting avenue of future investigation to assess the effect of APD668 alone or in combination with linagliptin over prolonged period on inflammatory and fibrosis markers in MCD diet fed mice. Taken together, these data suggest that optimal dosing regimen with GPR119 agonists could be a potential strategy for the treatment of nonalcoholic steatohepatitis.

6.6 References


Preserved Beta Cell Mass in a Progressive Diabetic Mice Model. 75th ADA 2015;280-LB.


Chapter 6


