



Combination of APD668, a G protein-coupled receptor 119 agonist with linagliptin, a DPPIV inhibitor, prevents progression of steatohepatitis in a murine model of non-alcoholic steatohepatitis with diabetes

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Abstract

Non-alcoholic steatohepatitis (NASH) is characterized by the presence of hepatic steatosis, oxidative stress, inflammation, and hepatocyte injury with or without fibrosis. In this study, we explored the effect of APD668, a GPR119 agonist alone or in combination with linagliptin, a DPPIV inhibitor, on the progression of steatohepatitis in a murine model of NASH with diabetes. A novel NASH model with diabetes was generated by administration of streptozotocin injection to neonatal C57BL/6 mice (2–3 days old) combined with a high-fat diet feeding from the age of 4 weeks. The plasma biochemical parameters, oxidative stress, inflammation and histopathological changes were assessed. APD668 alone showed reduction in plasma glucose (–39%, $P < 0.05$) and triglyceride level (–26%) whereas a combined treatment of APD668 with linagliptin resulted in a more pronounced reduction in plasma glucose (–52%, $P < 0.001$) and triglyceride (–50%, $P < 0.05$) in NASH mice. In addition, co-administration of APD668 with linagliptin demonstrated a significant decrease in hepatic triglyceride, NAS score, hepatic TBARS and hepatic TNF- α in NASH mice with diabetes. These findings suggest that GPR119 receptor agonists in combination with DPPIV inhibitors may represent a promising therapeutic strategy for the treatment of NASH.

Keywords GPR119 agonist · GLP-1 · DPPIV inhibitor · NASH · Inflammation · Fibrosis

Abbreviations

NASH	Non-alcoholic steatohepatitis
NAFLD	Non-alcoholic fatty liver disease
GLP-1	Glucagon-like peptide-1
GIP	Gastric inhibitory peptide
DPPIV	Dipeptidyl peptidase IV
HFD/STZ	High-fat diet/streptozotocin
CDA	Choline-deficient, l-amino acid-defined

KO	Knockout
AMPK	AMP-activated protein kinase
SREBP-1c	Sterol regulatory element binding protein-1c
SCD-1	Stearoyl-CoA desaturase
FAS	Fatty acid synthase
SOCS3	Suppressor of cytokine signaling 3
ALT	Alanine aminotransferase
HTF	High trans-fat
TG	Triglyceride
TC	Total cholesterol
TBARS	Thiobarbituric acid reactants
NAS	NAFLD activity score

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Introduction

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, hepatocyte injury with or without fibrosis [1]. NASH is closely associated with metabolic

syndrome, which is a lifestyle-related disorder characterized by obesity, diabetes, dyslipidemia and hypertension [2]. At present, there is no approved drug for the treatment of NASH. Several pharmacotherapies have been attempted to treat NASH/NAFLD with limited overall benefit. Due to its high prevalence coupled with lack of approved treatments, there remains a strong unmet medical need in NASH patients [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 anti-steatotic activity in obese and HFD/STZ diabetic mice [4, 12], anti-inflammatory activity in CDAA diet-induced NASH model [13] and inhibited activation of hepatic stellate cells (unpublished results) [14], suggesting that they also have anti-fibrotic activity. Similarly, DPPIV inhibitors are a class of oral anti-diabetic agents which improved hepatic steatosis, insulin resistance, oxidative stress, inflammation and fibrosis in murine models of NASH with or without diabetes background [15–18]. Moreover, it has been reported that patients with NASH have increased DPPIV activity [19, 20].

We recently reported that APD668 alone or in combination with linagliptin shows beneficial effects on various parameters in high trans-fat (HTF) diet-induced steatohepatitis in C57BL/6 mice [5, 21]. We speculated that the enhanced effect observed with the combination treatment could be due to (1) direct activation of GPR119 receptors present in liver and intestine, (2) enhanced active GLP-1 levels and (3) decreased degradation of GLP-1 in vivo through DPPIV inhibition in HTF diet fed mice. However, combination treatment showed non-significant additive effects on different parameters (except body weight gain) which could be due to maximal or near maximal activity observed with single agents per se in HTF diet mice [21]. Moreover, the effect of APD668, linagliptin or their combination on plasma triglyceride, hepatic inflammation and fibrosis markers has not been evaluated in HTF diet fed mice. Fujii et al. [22] have developed a STAM™ mice model which closely mimics the human NASH disease progression and also exhibits NASH characteristics within a short duration of time. Therefore, to further extend our investigation, we hypothesized that APD668 in combination with linagliptin at lower doses will likely show beneficial effects in NASH mice. To our knowledge, no study to date has investigated the effect of GPR119 receptor agonist alone or in combination with DPPIV inhibitor on the progression of steatohepatitis in a murine model of NASH with diabetes. Our findings suggest that combination of APD668 with linagliptin shows beneficial effects such as

anti-steatotic, anti-oxidant and anti-inflammatory activity in NASH associated with diabetes in mice.

Materials and methods

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. Linagliptin was suspended in 1% Tween-80 + 99% methyl cellulose (1:99). Vehicle or APD668 or linagliptin was administered by oral gavage twice daily for 4 weeks.

Animal experiments and induction of NASH

Animals were housed in an air-conditioned room at a temperature of 22 ± 2 °C and a humidity of $50 \pm 20\%$, with a 12 h light/dark cycle. The NASH model (hereafter termed NASH mice) was established according to the slightly modified protocol of Fujii et al. [22]. In brief, neonatal C57BL/6 male mice (2–3 days old) were injected with streptozotocin (STZ; 200 µg per mouse, s.c.) and then fed a high-fat diet (45% kcal fat, D12451, Research Diet Inc. NJ, USA) from 4 weeks after birth. NASH mice aged at 6 weeks were randomized based on body weight into four groups ($n = 6$ /group) as follows: group (1) vehicle; group (2) APD668 (6.25 mg/kg); group (3) Linagliptin (6.25 mg/kg) and group (4) APD668 (6.25 mg/kg) + linagliptin (6.25 mg/kg) group. Male C57BL/6 mice ($n = 6$) of the same age fed a normal diet (Altromin diet, Germany) without STZ injections, were assigned to the normal control group. Body weight was measured weekly. Post 4 weeks of treatment (post 14 h of last dose), 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.

Measurement of plasma biochemical and metabolic markers

Plasma ALT, triglyceride, glucose and cholesterol levels were measured using automated biochemical analyzer (Daytona, Randox Inc. UK). Plasma insulin was measured using commercially available ELISA kits (Mouse Ultrasensitive Insulin ELISA kit, ALPCO Diagnostics, USA).

Measurement of hepatic triglyceride and cholesterol

Lipids were extracted according to the method of Edvardsson et al. [23]. 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 685 g. Plasma triglyceride and cholesterol levels in the supernatants were measured using automatic biochemical analyzer (Daytona, Randox Inc. UK).

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 4–5 µm and stained with hematoxylin and eosin (H&E) for evaluation of the steatosis. Finally, the stained sections were observed and photographed under a light microscope (with 200× magnification). All stained sections were evaluated in a blinded manner by two pathologists for NAFLD activity score (NAS) including the components of steatosis, inflammation and hepatocyte ballooning. The scoring system was as follows: steatosis grade (0–3; 0: <5%, 1: 5–33%, 2: 33–66%, and 3: >66%), lobular inflammation (0–3; 0: no foci, 1: few foci/200×, 2: many foci/200×, and 3: >4 foci/200×scope), and ballooning (0–2; 0: no foci, 1: <2 foci/200×, and 2: 2–4 foci/200× [24]).

Measurement of hepatic thiobarbituric acid-reactants (TBARS) levels

The hepatic content of thiobarbituric acid-reactants, considered to be a marker of lipid peroxidation was measured using an OxiSelect™ TBARS Assay Kit (Cell Biolabs, Inc. USA). Briefly, frozen liver tissues (50 mg/mL) were homogenized in PBS containing butylated hydroxytoluene (BHT, 10 µL of 100× BHT to 1 mL of sample volume) to prevent further oxidation. Then, liver homogenates were centrifuged at 10,000g for 5 min. and the supernatants were collected. The supernatants were directly assayed for estimation of TBARS levels and results were expressed as µM/50 mg of liver tissue.

Measurement of hepatic TNF-α levels

Briefly, frozen liver tissues (50 mg/mL) were homogenized in lysis buffer. Then, liver homogenates were centrifuged at 12,000g for 10 min at 4 °C and the supernatants were collected. The concentrations of TNF-α levels in the supernatant were analyzed using commercially available ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The results were expressed as pg/mg of total protein. Total protein was estimated using Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, USA).

Measurement of hepatic hydroxyproline levels

For hepatic hydroxyproline measurement, 10 mg of frozen liver tissue was homogenized in 100 µL of deionized water. Then, liver homogenate was added in glass screw-thread vial containing 100 µL of concentrated HCl (10 N). Further, samples were incubated at 120 °C for 24 h on heat block and mixed periodically during incubation. If the hydrolyzed black residue is still present in the samples, transfer to a microcentrifuge tube and spin at 2740g for 3 min. The concentrations of hydroxyproline levels in the supernatant were analyzed using commercially available ELISA kits according to the manufacturer's instructions (Chondrex, Redmond, WA, USA). The results were expressed as µg/mg of liver tissue.

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 were analyzed using GraphPad version 7.02 of GraphPad Prism for Windows, GraphPad software, San Diego, California, USA.

Results

Effect of APD668, linagliptin or their combination on body weight and biochemical parameters

As shown in Table 1, the body weight tended to decrease in diabetic mice as compared to control group mice. At the end of study, none of the treatment groups showed a significant effect on body weight in mice. As expected, NASH mice showed a significant increase in plasma ALT, glucose, triglyceride and cholesterol levels as compared to control group mice (Table 1). APD668 or linagliptin monotherapy reduced elevated plasma glucose levels (39%, $P < 0.05$ and –30%) and plasma triglyceride (–26 and –33%) in NASH mice. However, the effect was more pronounced upon treatment with combination of both drugs on plasma glucose (–52%, $P < 0.001$) and plasma triglyceride (–50%, $P < 0.05$), but failed to reach statistical significance as compared to monotherapy groups (Table 1). In addition, APD668 or linagliptin monotherapy non-significantly reduced elevated plasma ALT and cholesterol levels in NASH mice. Surprisingly, combination treatment failed to demonstrate more pronounced reduction in plasma ALT (–16%) and cholesterol (–13%) as compared to monotherapy with either APD668 (ALT: –31% and cholesterol: –18%) or linagliptin (ALT: –21% and cholesterol: –16%) in mice, as shown in Table 1.

Table 1 Effect of APD668, linagliptin or combination therapy on body weight and biochemical parameters in mouse model of NASH with diabetic background

Parameters	Control	Vehicle	APD668 (6.25 mg/kg)	Linagliptin (6.25 mg/kg)	APD668 + linagliptin
Body weight (g)	22.0±0.3	18.8±1.0	17.1±1.0	20.6±1.8	19.5±1.5
ALT (U/l)	23±5	50±6 [#]	34±7	39±4	42±3
Glucose (mg/dL)	127±9	488±30 ^{###}	297±65*	340±37	235±26 ^{***}
Triglyceride (mg/dL)	49±5	115±14 [#]	86±14	78±19	58±7*
Cholesterol (mg/dL)	85±2	150±16 ^{##}	123±9	126±11	130±9
Insulin (ng/mL)	0.26±0.08	0.20±0.03	0.18±0.02	0.16±0.02	0.16±0.03

Data are represented as mean ± SEM, *n* = 5–6 mice/group

[#]*P* < 0.05, ^{##}*P* < 0.01, ^{###}*P* < 0.001 vs control group

P* < 0.05, *P* < 0.01, ****P* < 0.001 vs vehicle group

Moreover, NASH mice displayed hypoinsulinemia phenotype and APD668, linagliptin or combination treatment was unable to show increase in plasma insulin in mice (Table 1).

Effect of APD668, linagliptin or their combination on hepatic steatosis and NAS score

As shown in Fig. 1a–c, there was a significant increase in liver-to-body weight ratio, hepatic triglyceride and cholesterol content in NASH mice compared to control group mice. The liver-to-body weight ratio tended to decrease in the combination treatment group, but not in monotherapy groups (Fig. 1a). Treatment with APD668 alone caused a reduction in hepatic TG (−60%, *P* < 0.01) and TC contents (−53%, *P* < 0.05) whereas linagliptin showed a non-significant reduction in hepatic TG (−41%) and TC (−34%) in mice. Nevertheless, the combination treatment caused a significant reduction only in hepatic TG (−56%), but not in TC content (−41%) as compared to vehicle treated NASH mice as shown in Fig. 1b, c. Furthermore, hematoxylin and eosin stained liver sections showed hepatic fat accumulation (*P* < 0.05), inflammatory infiltration (*P* < 0.01), hepatocyte ballooning (*P* < 0.01) and the aggregate NAS score (*P* < 0.001) were higher in vehicle treated NASH mice as compared to control group mice as shown in Fig. 2a, b and Supplementary Table S1. In this study, APD668 alone reduced hepatocyte ballooning grade (*P* < 0.05) and aggregate NAS score (*P* < 0.05) whereas linagliptin monotherapy caused non-significant reduction in aggregate NAS score as compared to vehicle treated NASH mice. However, combination of APD668 with linagliptin significantly decreased hepatic steatosis grade and aggregate NAS score compared to vehicle treated NASH mice (Fig. 2a, b and Supplementary Table S1).

Combined effect of APD668 and linagliptin on oxidative stress marker

As shown in Fig. 3a, a significant increase in hepatic TBARS levels was observed in NASH mice as compared to control group (8.46 ± 0.88 μM/50 mg of tissue vs 4.22 ± 0.38 μM/50 mg of tissue, *P* < 0.01). APD668 or linagliptin monotherapy showed a trend towards decrease in hepatic TBARS level in mice. On the other hand, co-administration of APD668 with linagliptin caused a significant reduction in hepatic TBARS levels compared to vehicle treated NASH mice (5.35 ± 0.38 μM/50 mg of tissue vs 8.46 ± 0.88 μM/50 mg of tissue, *P* < 0.05, Fig. 3a).

Combined effect of APD668 and linagliptin on hepatic inflammatory cytokine

As shown in Fig. 3b, there was an increase in hepatic TNF-α level in NASH mice as compared to control group mice (1.92 ± 0.28 pg/mg of total protein vs 1.15 ± 0.09 pg/mg of total protein, *P* < 0.05). In this study, APD668, linagliptin and their combination significantly reduced elevated hepatic TNF-α levels as compared to NASH mice (Fig. 3b).

Effect of APD668, linagliptin or their combination on hepatic fibrosis marker

Furthermore, we investigated the effect of APD668, linagliptin or their combination on hepatic fibrosis marker, i.e., hepatic hydroxyproline level. As shown in Fig. 3c, a non-significant increase in hepatic hydroxyproline levels was observed in NASH mice as compared to control group mice (3.18 ± 0.39 μg/mg of tissue vs 1.91 ± 0.27 μg/mg of tissue). APD668, linagliptin and combination treatment demonstrate a marginal decrease in hepatic hydroxyproline

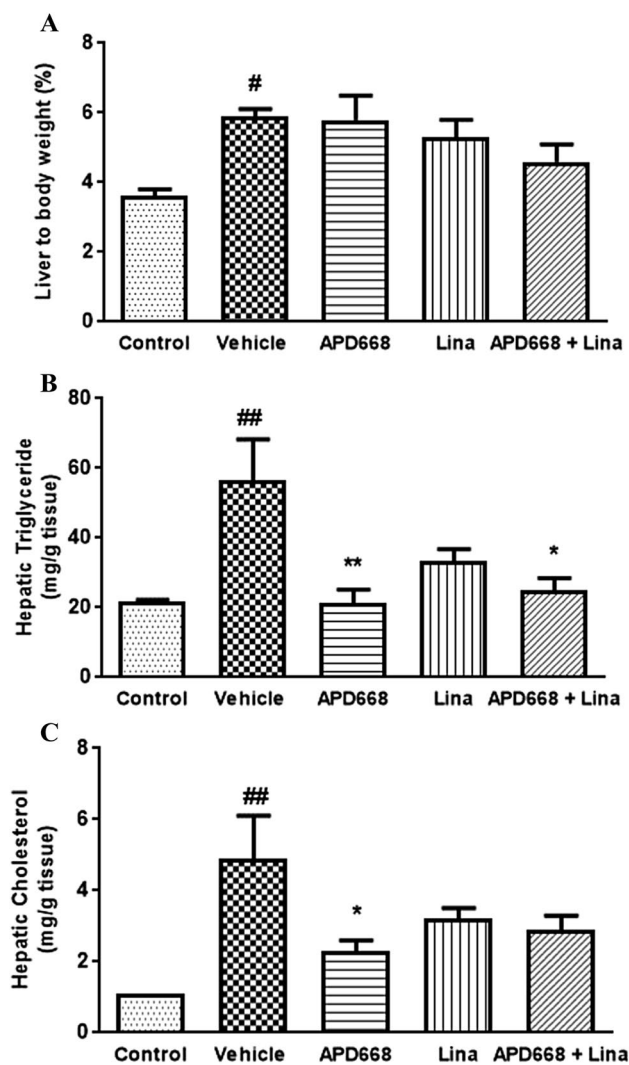


Fig. 1 Effect of APD668, linagliptin or their combination on liver-to-body weight ratio and hepatic steatosis in mouse model of NASH with diabetes. Mice were treated with vehicle or APD668 (6.25 mg/kg) or linagliptin (6.25 mg/kg) or their combination for 4 weeks twice daily. **a** Liver-to-body weight ratio, **b** hepatic triglyceride, **c** hepatic cholesterol. The results are represented as the mean \pm SEM, $n=5-6$ mice/group; [#] $P < 0.05$, ^{##} $P < 0.01$, ^{###} $P < 0.001$ vs control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$ vs vehicle group

levels compared with vehicle treated NASH mice, as shown in Fig. 3c.

Discussion

Recently, we have reported the beneficial effects of AP668 alone or in combination with linagliptin in high trans-fat diet-induced steatohepatitis in the context of obesity with mild hyperglycemia and insulin resistance in C57BL/6 mice [5, 21]. However, the major limitation of this model is that high trans-fat diet induces all stages of NAFLD for

periods > 20 weeks including lack of hypertriglyceridemia in normal C57BL/6 mice [25]. It is well-known that no single animal model so far has managed to recapitulate the full spectrum of human NASH disease progression, but individual animal models can mimic particular characteristics of human disease [26]. Therefore, it was tempting to assess effect of APD668 alone or in combination with linagliptin in a murine model of NASH with strong hyperglycemia, hypertriglyceridemia, moderately elevated liver enzymes, inflammation and fibrosis.

In the present study, APD668 alone or in combination with linagliptin significantly decreased plasma glucose in mice. Importantly, combination treatment, but not monotherapy caused significant reduction in plasma triglyceride in NASH mice, as shown in Table 1. Furthermore, APD668 alone or in combination with linagliptin, significantly reduced hepatic triglyceride and this was further supported by histological analysis with significant reduction in NAS score in mice (Figs. 1b, 2a, b). On the contrary, combination treatment failed to show enhanced or additive reduction in plasma ALT and hepatic triglyceride in NASH mice. These findings are in contrast with HTF diet study, where we found enhanced effects on hepatic injury markers and hepatic triglyceride caused by APD668 in combination with linagliptin in HTF diet fed mice [21]. Recently, Yang et al. [4] for the first time demonstrated that GPR119 receptors were expressed in mouse and human hepatocytes and the expression of lipogenic genes (FAS and ACC) was upregulated in GPR119 KO hepatocytes. Authors also reported that MBX2982, a GPR119 agonist, inhibited hepatic steatosis via inhibition of lipogenesis genes (SREBP-1c, FAS, ACC and SCD-1) in obese mice. Another GPR119 agonist, DA-1241, also attenuated hepatic steatosis via AMPK-mediated SREBP-1c inactivation and further inhibited the downstream lipogenic signals [27]. Additionally, we also demonstrated that APD668 alone or in combination with linagliptin enhanced active GLP-1 levels in HTF diet fed mice [21] and it has been reported that GLP-1 plays a major role in improvement of fatty liver in rodent studies [28, 29]. Therefore, we hypothesized that anti-steatotic effect of APD668 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 NASH mice.

Furthermore, it has been suggested that oxidative stress-induced lipid peroxidation followed by inflammation is another possible mechanism in the progression of non-alcoholic steatohepatitis [30]. As shown in Fig. 3a, combination of APD668 with linagliptin significantly reduced hepatic thiobarbituric acid-reactive substances (TBARS) in NASH mice. Moreover, APD668, linagliptin and their combination significantly reduced hepatic TNF- α in mice (Fig. 3b). The

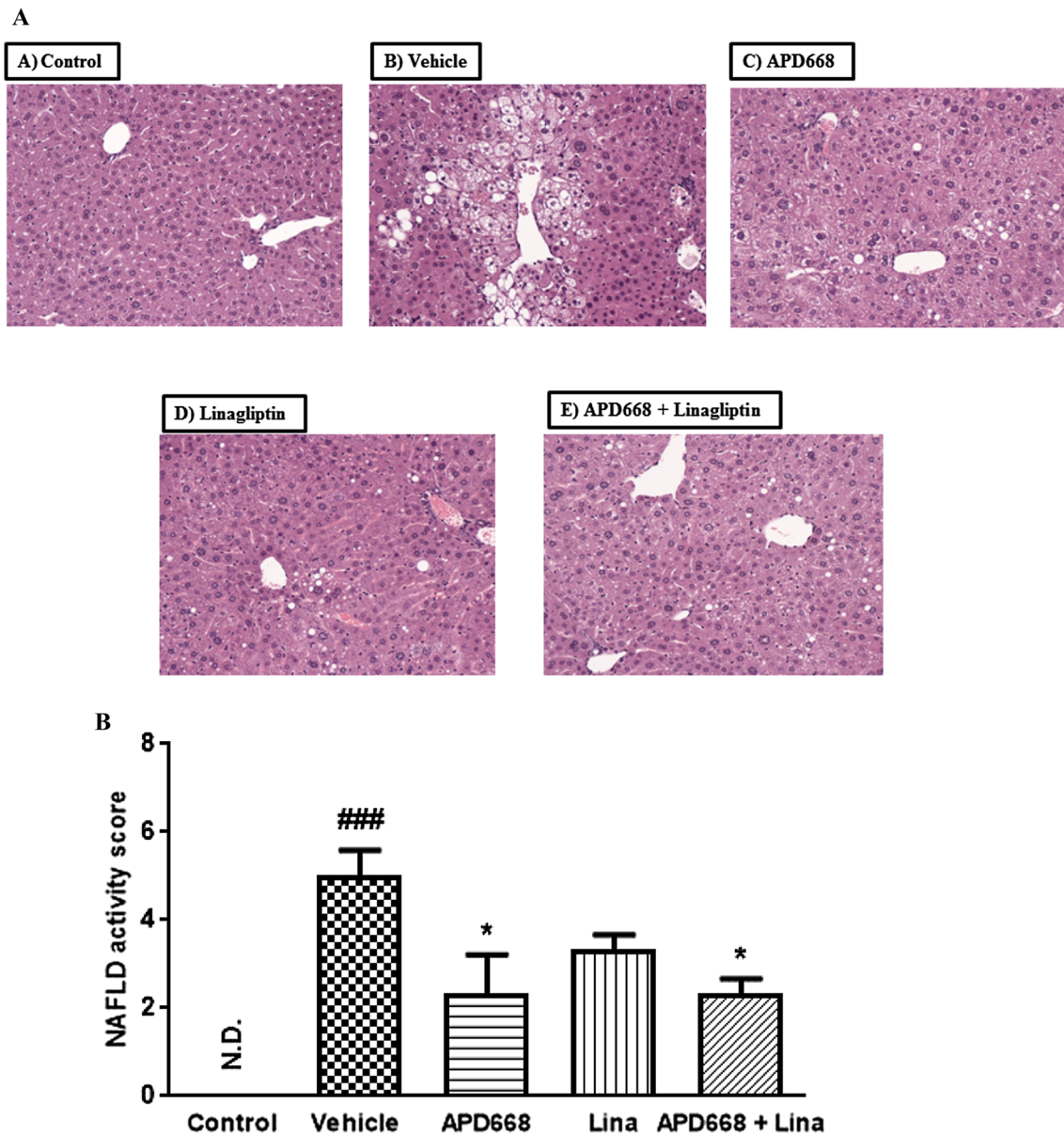


Fig. 2 Effect of APD668, linagliptin or their combination on hepatic steatosis and NAS score assessed using histopathologic analysis in NASH mice. Mice were treated with vehicle or APD668 (6.25 mg/kg) or linagliptin (6.25 mg/kg) or their combination for 4 weeks twice daily. **a** Hematoxylin and eosin staining of liver sections from rep-

resentative mice from each group (magnification, $\times 200$), **b** NAFLD activity Score (NAS). *N.D.* not determined. The results are represented as the mean \pm SEM, $n = 3$ mice/group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs control group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs vehicle group

mechanism related to inhibition of hepatic oxidative stress and inflammation by APD668 alone or in combination with linagliptin could be due to improvement in hepatic steatosis in NASH mice. Combination treatment also showed a trend towards reduction in hepatic hydroxyproline levels, suggesting that APD668, a GPR119 agonist may have potential to inhibit hepatic fibrosis in NASH mice, as shown in Fig. 3c. Previously, it has been suggested that linagliptin attenuated hepatic steatosis and inflammation through inhibition of lipogenesis genes (SREBP-1c, FAS and SCD-1), inhibition

of macrophage infiltration as well as TNF- α , SOCS3 gene expressions in rodent studies [15, 17, 18].

The present study had some limitations. In contrast to HTF diet study, combination treatment failed to show significant synergistic or additive effect in reduction of biochemical parameters, hepatic steatosis, NAS score, oxidative stress, inflammation and fibrosis markers as compared to monotherapy groups in NASH mice. These differential findings could be due to differences in murine models of NASH or due to small sample size failed to detect statistical

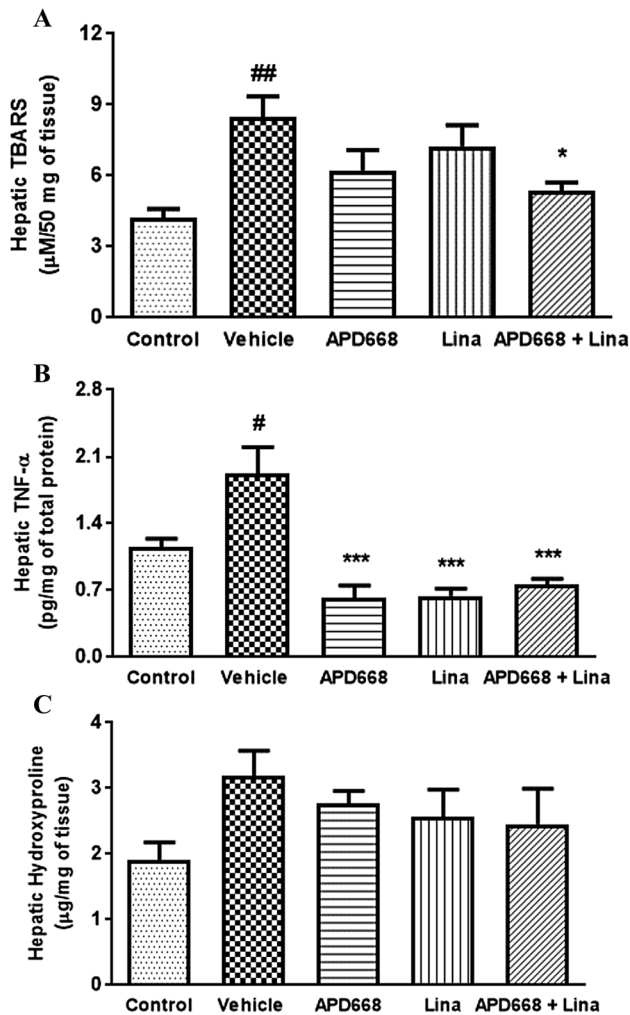


Fig. 3 Effect of APD668, linagliptin or their combination on hepatic TBARS, inflammatory cytokine (TNF- α) and fibrosis marker (hydroxyproline) in NASH mice. Mice were treated with vehicle or APD668 (6.25 mg/kg) or linagliptin (6.25 mg/kg) or their combination for 4 weeks twice daily. The results are represented as the mean \pm SEM, $n=5-6$ mice/group; $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$ vs control group; $*P<0.05$, $**P<0.01$, $***P<0.001$ vs vehicle group

significant differences. Thus, further investigative studies are required to confirm the significant differences between combination and monotherapy groups by increasing the sample size in this murine model of NASH with diabetes.

In summary, our findings demonstrated that co-administration of APD668 with linagliptin significantly improved hyperglycemia, hypertriglyceridemia and hepatic steatosis in NASH mice. Also, combination treatment significantly reduced hepatic TBARS and TNF- α levels in mice. Therefore, these findings together with our recent reports in HTF diet mice model suggest that GPR119 agonists in combination with DPPIV inhibitors may be useful for the management of non-alcoholic steatohepatitis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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