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LETTERS

Pioglitazone and Ezetimibe combination improves Liver histopathology and biochemistry in experimental MASH models

Santosh Kumar Rai*, Rakesh Kumar, Amit Panwar, Mohd Imran Khan, Srinivasa Reddy B, Bhavishya Vashist, Rakesh Ishwar Patil, Sazid Ali, Anil Kumar

New Drug Discovery Research, Mankind Research Centre, Mankind Pharma Limited, Sector 4-II, IMT Manesar, Gurugram, India.

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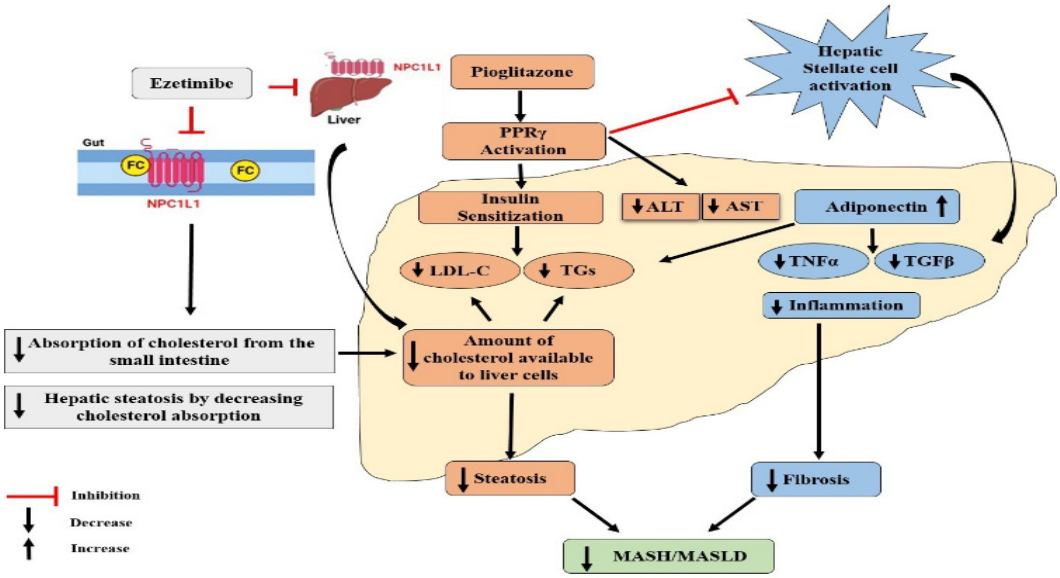
Article

ABSTRACT



Metabolic dysfunction-associated steatotic liver disease (MASLD) and metabolic dysfunction-associated steatohepatitis (MASH) are common clinico-pathological conditions affecting over 30% of adults worldwide. Previous studies have shown limited efficacy of activation of single PPARs (PPARα or PPARγ). However, ongoing clinical trials suggest that dual and pan-PPAR agonists have a broader and more potent therapeutic effect on MASH by simultaneously targeting different inter-related mechanisms in this multisystem disease. In the current study, we have investigated a novel combination of pioglitazone (a PPAR γ/α agonist) and ezetimibe (a cholesterol absorption inhibitor) in different MASH animal models. We tested pioglitazone at 2-3-fold reduced clinical dose (15mg/day) in combination to ezetimibe, since there are safety concerns associated with higher doses (30mg and 45mg, daily). Our results revealed that combination of low dose pioglitazone, with ezetimibe holds the ability to regulate steatosis, hepatocyte inflammation and ballooning, which resulted in superior effects in terms of NAS as well as fibrosis score compared to pioglitazone alone (30mg/kg). Moreover, in-vitro studies in human liver microsomes and mouse hepatocytes did not show any drug-drug interaction between pioglitazone and ezetimibe. Overall, this study provides a potential possibility for the clinical treatment of MASH with combination of pioglitazone and ezetimibe.

Keywords: MASLD; MASH; PPARα; PPARγ; Pioglitazone; Ezetimibe

INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) includes a spectrum of progressive steatotic liver conditions, ranging from isolated hepatic steatosis to metabolic dysfunction-associated steatohepatitis (MASH) with varying amounts of liver fibrosis, which may progress to cirrhosis.[1,2]

Pharmacological approaches for treating MASLD are directed towards diverse signalling process involved in MASLD progression. These approaches aimed at interfering with MASLD progression by targeting liver steatosis, inflammation or fibrosis.[3] Due to complex pathophysiology of MASLD, multiple targets including PPARs, FXR, ACC, GLP1-R, SGLT-2, FGF-21, ASK1, CCR2/CCR5, THRβ, Caspase and GALECTIN have been explored so far for the treatment of MASH,[4] among these Resmetirom (THRβ agonist) has been approved by FDA as a first medication to treat MASH.[5] In addition, the 2022 guidance from the American Association of Clinical Endocrinology

*Corresponding Author: Dr. Santosh Kumar Rai,
New Drug Discovery Research, Mankind Research Centre, Plot No 191-E, Sector 4-II, IMT Manesar, Gurugram, India-122051
Email: Santosh.rai@mankindpharma.com

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recommends the use of GLP-1 receptor agonist or pioglitazone in MASLD patients with type 2 diabetes.[6]

Several drugs developed as monotherapy for the treatment of MASLD have not yet met success in the clinical trials. Hence, a combination approach has been considered as a best option to enhance efficacy and slow down the disease progression or reversing fibrosis.[2] Although previous studies have shown limited efficacy of activation of single PPARs (PPARα, PPARγ), ongoing clinical trials suggest that dual (Pioglitazone, Saroglitazar, Elafibranor) and pan-PPAR agonists (lanifibranor) may have a broader and more potent therapeutic effect on MASH by simultaneously targeting different inter-related mechanisms in this multisystem disease. Saroglitazar, a dual PPARα/γ agonist approved in India for MASLD treatment, reduced ALT levels and demonstrated an absolute reduction in MRI-PDFF compared to placebo[7,8] Since, dual or pan-PPAR agonists have a broader and more potent therapeutic effect on MASH, combining PPARγ agonist (to enhance insulin sensitivity) with lipid lowering drug (similar to PPARα) could have better strategy to target MASH.

Among the major lipid lowering drugs, statins and ezetimibe have been shown to be effective in MASLD/MASH in different clinical trials. Since, inflammatory and oxidative mechanisms are involved in the pathogenesis of MASLD or MASH, statins have been investigated as therapy for patients with either MASLD or MASH.[9] Although, efficacy and safety for many statins have been proved in many clinical trials, it has also been observed that long term statin treatment may worsen hepatic histology in patients with MASLD. Moreover, decompensated cirrhosis and acute liver failure are contraindications for statin therapy.[10,11] In contrast to statins, long-term ezetimibe therapy improved the metabolic, biochemical, and histological abnormalities of MASLD and was well-tolerated with no clinically meaningful differences between the adverse events profiles of ezetimibe and placebo in MASLD patients.[12]

In the present study, we investigated the effects of novel combination of Pioglitazone and ezetimibe in two different rodent models of MASH and showed that reduction in pioglitazone dose by half or one third, in combination with ezetimibe, could hold the ability to regulate the steatosis, hepatocyte inflammation and ballooning, which ultimately resulted in reduction of NAS and fibrosis scores.

Materials and Methods

Materials

Streptozotocin (Cat # 100780, Medkoo), CCl4 (Cat # 289116, Sigma-Aldrich), Pioglitazone Hydrochloride (Cat # I868, AK scientific), Rodent Diet with 60 kcal% Fat (Cat # D12492, Research Diets Inc.), Ezetimibe (Cat # SML1629, Sigma-Aldrich).

Animal Experiments

Effect of Pioglitazone and Ezetimibe combination in STAM mouse model for MASH

STAM mouse model is one of the widely used chemically induced models for preclinical studies of MASH. Pathological analysis revealed that these mice have mild steatosis, more severe inflammation and ballooning.[13,14] The STAM mouse is a model that demonstrates MASH progression resembling the disease in humans. STAM mice manifest MASH at 8 weeks, which progresses to fibrosis at 12 weeks and finally develop hepatocellular carcinoma.

STAM mice model were generated to study the effect of Pioglitazone and Ezetimibe combinations on MASH. Male neonatal C57BL/6 mice of age 2 days were used for the study. On day 1 of study, each neonatal mouse was administered streptozotocin 200 µg/mouse subcutaneously. Mice were fed on rodent diet with 60 kcal %Fat (Cat # D12492, Research Diets Inc.) 4th week onwards except Naïve control, which were fed on standard chow diet. Mice were randomized based on body weight and blood glucose level into seven groups on 6th week: Group-1 Naïve control, Group-2 disease control/vehicle control (0.5% v/v Tween-80 and 0.5% Na-CMC in a ratio 0.5:99.5), Group-3 Pioglitazone 15 mg/kg and Ezetimibe 10 mg/kg, Group-4 Pioglitazone 15 mg/kg and Ezetimibe 5 mg/kg, Group-5 Pioglitazone10 mg/kg and Ezetimibe 10 mg/kg, Group-6 Pioglitazone 10 mg/kg and Ezetimibe 5 mg/kg and Group-7 Pioglitazone alone 30 mg/kg. The drugs were administered once daily by oral gavage for a period of 6 weeks. During the study body weight of the animals was recorded twice a week.

Effect of Pioglitazone and Ezetimibe combination in Western diet and CCl4 induced mouse model for MASH

We have also used CCl4 induced mouse model fed on western diet to study the effect of Pioglitazone and Ezetimibe combination on MASH. Carbon tetrachloride (CCl4) is a hepatotoxic chemical which causes liver injury, liver fibrosis and cirrhosis in experimental animals. Repeated administration of CCl4 to HFD-fed obese mice successfully induced chronic oxidative stress, triggered inflammation and led to liver fibrosis. Notably, under feeding with Western diet (WD) supplemented with 5% fructose (WDF), CCl4 reduced the induction time and aggravated liver fibrosis in mice.[15,16,17] This preclinical model of moderate and advanced MASH closely mimics human disease and exhibits almost all the characteristics of advanced human MASH after 10 weeks and cirrhotic MASH after 24 weeks.

Male neonatal C57BL/6 mice of age 2 days were used for the study. On Day-1 to 12 weeks of the study, all the mice except naive control animals were fed daily with western diet (WD) along with high sugar solution (23.1 g/L d-fructose and 18.9 g/L d-glucose) and 0.2 ml/kg of CCl4 injected intraperitoneally once weekly. Naïve control animals were provided with standard chow diet along with normal RO water. Mice were randomized on 4th week based on body weight into six different treatment groups: Group-1 Naïve control, Group-2 disease control/vehicle control (0.5% v/v Tween-80 and 0.5% Na-CMC in a ratio 0.5:99.5), Group-3 Pioglitazone 15 mg/kg and Ezetimibe 10 mg/kg, Group-4 Pioglitazone 15 mg/kg and Ezetimibe 5 mg/kg, Group-5 Saroglitazar 4 mg/kg and Group-6 Pioglitazone 30 mg/kg. The drugs were administered once daily by oral gavage for a period of 8 weeks. During the study, the body weight of animals was recorded twice a week.

Biochemical analysis

At end of study i.e. 12th week, blood was collected by retro-orbital plexus under isoflurane anaesthesia. Plasma was separated and collected for the estimation of plasma ALT, AST, ALP, Glucose, Cholesterol and Triglycerides. Hepatic cholesterol and hepatic triglycerides were also estimated from liver samples. All the parameters estimation was done either by Selectra Pro M Lite - Fully Auto Biochemistry Analyzer or assay kits.

Histological assessment

Histology was performed for liver samples using MT (Masson's Trichome) staining for Ashcroft score and Hematoxylin and Eosin (H&E) staining for inflammatory and fibrosis markers. Briefly, liver from all the experimental groups were fixed in 10% buffered neutral formalin for one week at room temperature. Thereafter, tissues were dehydrated in a graded series of alcohol, cleaned in xylene and then embedded in paraffin. Serial sections of 4-mm-thickness were prepared from each tissue embedded paraffin blocks using a rotary microtome and employed for histological examination using light microscope after being stained by a MT or H&E procedure. Blind histopathological evaluation of samples was performed by the pathologist.

Specimens were scored for the severity of hepatocellular steatosis, ballooning, inflammation and fibrosis according to the scoring method described by Kleiner et al. and Brunt et al.[18,19] Briefly, hepatocellular steatosis (grade 0: no fat; grade 1: steatosis occupying <33% of the hepatic parenchyma; grade 2: 34-66%; grade 3: more than 66%). Inflammatory cell infiltration (grade 0: none; grade 1: 1-2 foci per 200× field; grade 2: 3-4 foci per 200× field; grade 3: more than 4 foci per 200× field). Hepatocellular ballooning (grade 0: none; grade 1: few balloon cells; grade 2: many balloon cells). Staging of hepatic fibrosis (stage 0: none; stage 1: mild perisinusoidal or periportal; stage 2: moderate perisinusoidal or periportal; stage 3: bridging fibrosis; stage 4: cirrhosis). Total MASH score was calculated by summation of all scores for the severity of hepatocellular steatosis, ballooning, inflammation and fibrosis by H&E staining for individual animals and then group mean ± SEM was calculated and effect of treatment was evaluated. Fibrosis score for all treatment groups was also calculated on the basis of Ishak Scoring system for MT staining.

TGF-β and TNF-α Immunohistochemistry (IHC)

Liver samples were collected for immunohistochemistry of TGF-β and TNF-α. For immunostaining, paraffin wax embedded tissue blocks were sectioned at 4-6 µm thickness with the Rotary Microtome and placed on slides coated with Poly-L-Lysine and incubate overnight at 37°C. Further these sections were deparaffinised, rehydrated and incubated with citrate buffer, pH 6 at decloaking chamber. Slides were incubated in 3% hydrogen peroxide block for 20 minutes to block endogenous peroxidase. TGF-β antibody (ab190503), and TNF-α antibody (ab1793) were applied as the primary antibodies and peroxidase-labeled goat anti-rabbit IgG as secondary antibody. The staining was visualized by reaction with diaminobenzidine colour reagent and then counterstained with hematoxylin. Finally, the sections were rinsed with tris-buffered saline (TBS), then dehydrated in alcohol and cleared in xylene prior to mounting using DPX. All the sections were examined under the light microscope to record the intensity of antigen antibody reaction.

Drug-drug interactions

Quantitative in-vitro drug-drug interaction for Pioglitazone and Ezetimibe was carried in human liver microsomes and hepatocytes as per reported literature.[20]

Drug-drug interactions using human liver microsomes

The compounds at a final concentration were mixed with 0.5 mg/mL human liver Microsomes (Xenotech #H0630) containing 3.3µM MgCl2 and incubated at 37°C in presence and absence of cofactor NADPH (Sigma #N1630) at various time points. Reaction was stopped by the addition of 233 µL of acetonitrile containing internal standard, lansoprazole and samples for 0, 5, 15, 30, 60 and 90 min were analysed by LC-MS/MS to calculate the half-life and intrinsic clearance.

Drug-drug interactions using mouse hepatocytes

1 x 10^6 mouse hepatocyte cells/mL (>95% viability) were added to individual wells in 24 well plates and incubated in a CO2 incubator at 37°C and 5% CO2 for 15 min. Zero min incubation was terminated by adding 500 µL of ice-cold acetonitrile containing 0.45µM lansoprazole. All the reactions were initiated by adding 250 µL of test compounds diluted in KHB (pre-warmed at 37°C in CO2 incubator) and further incubated for 0, 30, 60 and 90 min in CO2 incubator with gentle shaking. At the end of each time points incubation reactions were terminated by adding 500 µL of ice-cold acetonitrile and samples were analysed by LC-MS/MS to calculate the half-life and intrinsic clearance.

RESULTS

Pioglitazone and ezetimibe combination reverses MASH and improves fibrosis in STAM mouse model

STAM mice maintained on rodent diet with 60 kcal %Fat for 2 weeks were treated with Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) or Pioglitazone and Ezetimibe (15 mg/kg and 5 mg/kg) or Pioglitazone and Ezetimibe (10 mg/kg and 10 mg/kg) or Pioglitazone and Ezetimibe (10 mg/kg and 5 mg/kg) or Pioglitazone (30 mg/kg) or vehicle for the 6 following weeks. At the end of 12 weeks, plasma ALT, AST, ALP, Glucose, Cholesterol and Triglycerides levels were estimated. Hepatic triglycerides were also estimated from liver samples. Data from treatment groups was compared to vehicle/disease control.

Serum AST, ALT, and ALP

Alanine transaminase (ALT) and aspartate aminotransferase (AST) are indicators of hepatocellular injury. Several studies have demonstrated that high ALT levels are correlated with a higher risk of MASH.[21,22] Also, MASLD significantly associated with higher ALT and gamma-glutamyl transferase (GGT) but not ALP levels in IGT and T2DM patients.[23] At the end of the study, the disease control animals that were fed only on high fat diet exhibited significant increase in plasma ALT and AST levels compared to animals fed on a normal control diet (Fig. 1A & B).

Among all combinations, Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) showed significant decrease in plasma ALT level, and Pioglitazone (30 mg/kg) alone showed equivalent effect. AST levels were also decreased significantly by Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination, however Pioglitazone alone (30 mg/kg) did not show any marked decrease. ALP levels were not changed significantly in any of the groups, either treated with Pioglitazone and Ezetimibe combinations or Pioglitazone alone in this MASH mouse model (Fig. 1). Overall, pioglitazone at half dose in combination with ezetimibe showed similar effects as shown by pioglitazone alone at 30 mg/kg in STAM mouse model for MASH.

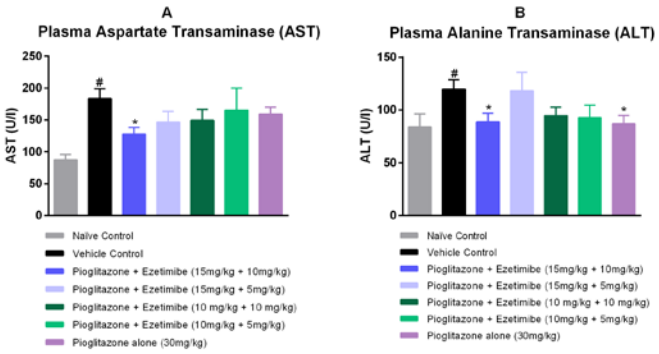

Figure 1. Effect of Pioglitazone and Ezetimibe combinations on the AST (A) and ALT (B) levels of streptozotocin and high fat diet induced MASH mouse model. One-way ANOVA followed by Dunnett's Multiple Comparison Test was used for statistical analysis. Data is shown as Mean ± S.E.M.(n=7-8), #Significant difference as compared to Naive Control group. * Significant difference as compared to Disease/Vehicle Control. */#P < 0.05.

Lipid parameters

Abnormal lipoprotein concentration in plasma reflects disturbances in homeostasis of major lipid components of lipoproteins, triglycerides, cholesterol, and cholesterol esters. Excessive accumulation of triglycerides in the liver is the hallmark of MASLD. Hence, Plasma and hepatic triglycerides (TGs) were also measured in STAM mice model for MASH and changes in TGs levels illustrated in Fig. 2A and B respectively. Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination showed significant decrease in plasma and hepatic TGs in comparison to disease control, however, no significant decrease was found in other Pioglitazone and Ezetimibe combinations. In contrast to Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination, Pioglitazone (30 mg/kg) alone showed marked decrease in plasma TGs levels, while hepatic TGs were not decreased at all (Fig. 2 A & B). Overall, Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination was found more effective in preventing the accumulation of both serum and liver TGs compared to Pioglitazone alone in STAM mouse model for MASH. All Pioglitazone and Ezetimibe combinations or Pioglitazone alone (30 mg/ml) did not show significant decrease in plasma LDL-cholesterol (Fig. 2C).

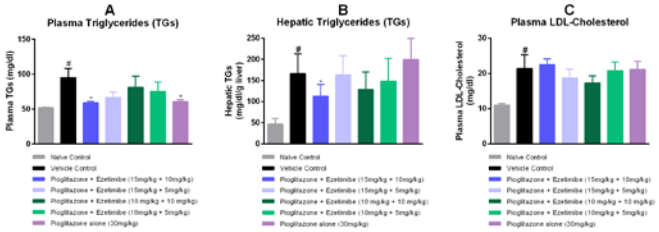

Figure 2. Effect of Pioglitazone and Ezetimibe combination on the plasma TGs (A), hepatic TGs (B) and LDL-Cholesterol (C) in streptozotocin and high fat diet induced MASH mouse model. One-way ANOVA followed by Dunnett's Multiple Comparison Test was used for statistical analysis. Data is shown as Mean ± S.E.M.(n=7-8), #Significant difference as compared to Naive Control group. *Significant difference as compared to Disease/Vehicle Control. */#P < 0.05.

Histopathological examination for NAFLD Activity Score (NAS) and Fibrosis

Histological analysis of STAM mice livers from all treatment and non-treatment groups was performed to measure steatosis, inflammation and fibrosis using hematoxylin and eosin (H&E) staining. NAFLD Activity Score (NAS) was calculated using steatosis, inflammation and fibrosis scores (H&E staining). Our results revealed a significant increase in NAS in disease control group as compared to naïve control mice. However, STAM mice

Table 1. Effect of pioglitazone and Ezetimibe combination on NAFLD Activity Score (NAS) in streptozotocin and high fat diet-induced MASH mouse model

Histological feature	Naïve Control	Vehicle Control	Pioglitazone + Ezetimibe (15mg/kg + 10mg/kg)	Pioglitazone + Ezetimibe (15mg/kg + 5mg/kg)	Pioglitazone + Ezetimibe (10mg/kg + 10mg/kg)	Pioglitazone + Ezetimibe (10mg/kg + 5mg/kg)	Pioglitazone alone (30mg/kg)
Steatosis (Scale of 0-3)	0.00 ± 0.00	3.00 ± 0.00	1.25 ± 0.16	1.38 ± 0.18	1.29 ± 0.29	1.29 ± 0.18	1.25 ± 0.16
Ballooning of Hepatocytes (Scale of 0-2)	0.00 ± 0.00	2.00 ± 0.00	0.63 ± 0.18	0.63 ± 0.18	1.14 ± 0.26	1.29 ± 0.18	1.00 ± 0.00
Lobular Inflammation (Scale of 0-3)	0.00 ± 0.00	0.50 ± 0.30	0.13 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
H & E (NAFLD Activity Score)	0.00 ± 0.00	5.50 ± 0.30	2.00 ± 0.20	2.00 ± 0.20	2.10 ± 0.50	2.30 ± 0.50	2.30 ± 0.20
Fibrosis score- Ishak Scoring system (MT staining)	0.00 ± 0.00	3.80 ± 0.40	0.40 ± 0.30	0.90 ± 0.40	0.70 ± 0.40	1.00 ± 0.60	0.50 ± 0.30

treated with all combinations of Pioglitazone and Ezetimibe as well as Pioglitazone alone showed significant improvement in MASH compared to disease control (Table 1 and Fig. 3A).

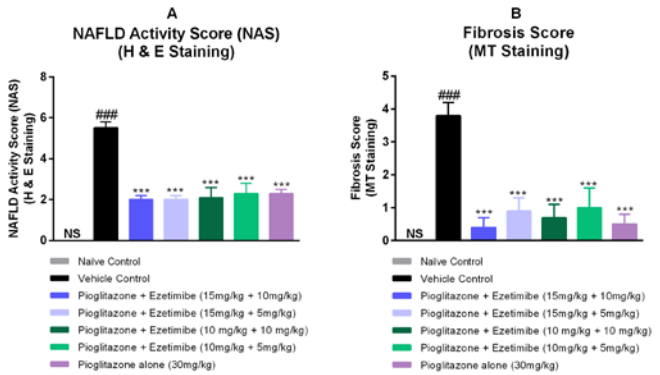

Figure 3. Effect of Pioglitazone and Ezetimibe combinations on NAS (A) as well as Fibrosis (B) in streptozotocin and high fat diet-induced MASH mouse model. One-way ANOVA followed by Dunnett's Multiple Comparison Test was used for statistical analysis. Data is shown as Mean ± S.E.M.(n=7-8), #Significant difference as compared to Naive Control group. *Significant difference as compared to Disease/Vehicle Control. */#P < 0.05 and ***P < 0.001.

Similarly, histopathology analysis was performed by Massons's trichrome (MT) staining of liver sections to measure fibrosis score using Ishak scoring system. All combinations of Pioglitazone and Ezetimibe as well as Pioglitazone alone (30 mg/kg) showed significant improvement in fibrosis score compared to disease control (Table 1 and Fig. 3B). Representative photomicrographs of liver sections are described in Fig. 4A (H&E staining) and Fig. 4B (MT staining). Overall, a dose dependent improvement in the NAS (H&E staining) was observed with combinations of Pioglitazone and Ezetimibe. Among all combinations, Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination showed highest MASH resolution and improvement in fibrosis in comparison to disease control. Pioglitazone (30 mg/kg) alone showed similar improvement as of Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination. Our results showed that decreasing Pioglitazone dose to half in combination to Ezetimibe showed similar efficacy as of Pioglitazone alone at higher dose (30 mg/kg).

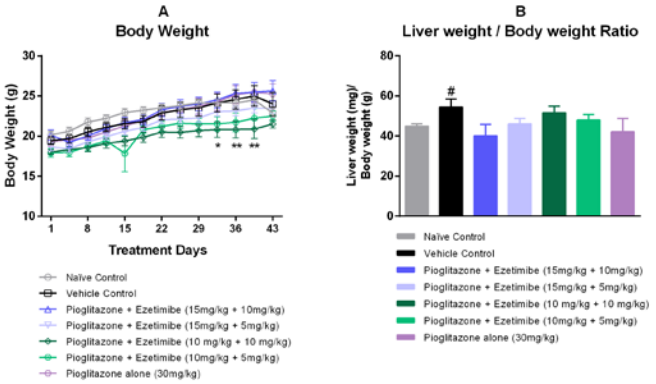

Figure 5. Effect of Pioglitazone and Ezetimibe combinations on body weight (A) and Liver weight/body weight ratio (B) in streptozotocin and high fat diet-induced MASH mouse model. One-way ANOVA followed by Dunnett's Multiple Comparison Test was used for statistical analysis. Data is shown as Mean ± S.E.M.(n=7-8), #Significant difference as compared to Naive Control group. *Significant difference as compared to Disease/Vehicle Control. */#P < 0.05, **P < 0.01 and ***P < 0.001.

Body weight and liver weight/body weight ratio

During the study, the body weight of animals was recorded twice a week to find treatments related body weight changes. There was no significant reduction in body weight observed in all treatment groups except Pioglitazone and Ezetimibe (10 mg/kg and 10 mg/kg) combination group, where significant decrease in body weight was found on day-32, day-36 and day-39 of treatment (Fig. 5A). Liver weight/body weight ratio for all treatments groups and disease control group was also measured at the end of the study. Disease control showed significant increase in liver weight/body weight ratio vs. Naïve control, whereas treatment groups exhibited decrease in liver

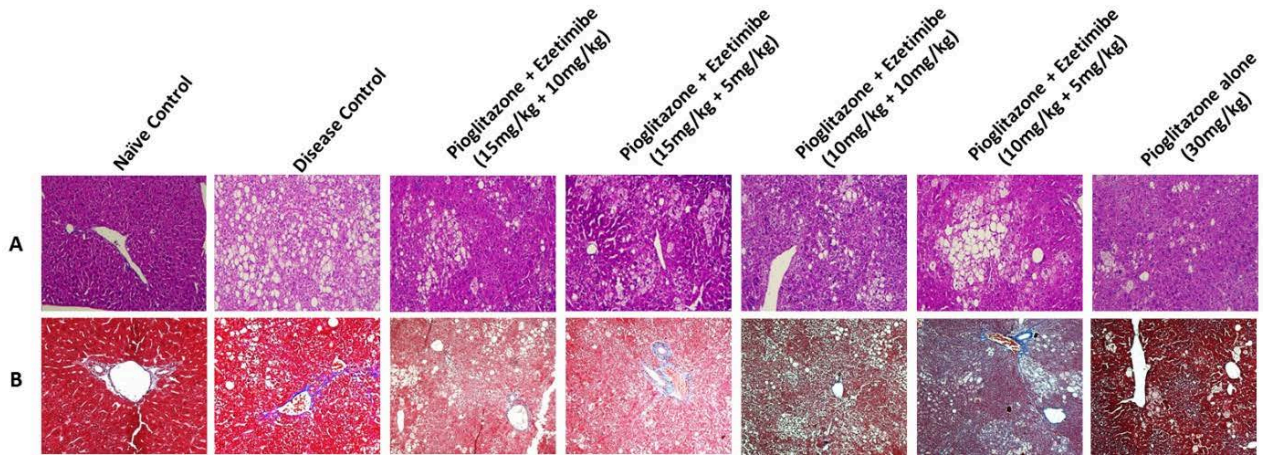

Figure 4. Effect of Pioglitazone and Ezetimibe combinations on liver histology in a streptozotocin and high fat diet-induced NASH model. Representative liver sections stained with hematoxylin-eosin (A) and Masson's trichrome (B)

weight/body weight ratio although the decrease was non-significant. (Fig. 5B).

Pioglitazone and ezetimibe combination reverses MASH and improves fibrosis in WDF/CCl4 induced mouse model

In the present study we have used another MASH mouse model induced with CCl4 and fed on Western Diet along with fructose (WDF). WDF enhances obesity and hepatosteatosis, as well as induces moderate fibrosis in mice, while administration of CCl4 (i.p., once weekly) accelerated liver fibrosis with increased bridging and liver hydroxyproline contents. C57BL/6 mice except naive control, were fed with western diet along with daily high sugar solution (23.1g/L d-fructose and 18.9 g/L d-glucose), and 0.2 ml/kg of CCl4 (i.p., once weekly) from day 1 to 12 weeks. On 4th week mice were divided into different groups and treated with Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) or Pioglitazone and Ezetimibe (15 mg/kg and 5 mg/kg) or Pioglitazone alone (30 mg/kg) or Saroglitazar (4 mg/kg) or vehicle (0.5% v/v Tween-80 and 0.5% Na-CMC in a ratio 0.5:99.5) for the 8 following weeks. At the end of 12 weeks, serum markers of liver damage ALT, AST, ALP, Glucose, cholesterol and triglycerides were estimated. We have tested two combinations, Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) or Pioglitazone and Ezetimibe (15 mg/kg and 5 mg/kg) in WDF/CCl4 induced mouse model, since other two combinations were not found effective in streptozotocin and high fat diet-induced MASH mouse model.

Serum AST, ALT, and ALP

Similar to STAM mouse model, disease control animals in WDF/CCl4 induced mouse model which were fed only on WDF exhibited significant increase in ALT and AST in comparison to animals fed on normal chow diet (Figure 6). Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination showed significant decrease in serum AST and ALT levels in comparison to disease control, however, Pioglitazone and Ezetimibe (15 mg/kg and 5 mg/kg) combination did not show any significant decrease. Pioglitazone alone (30mg/kg) showed similar decrease in ALT level, but no marked decrease in AST level was observed as found in STAM mouse model. Saroglitazar (4 mg/kg) showed significant decrease in ALT and AST levels compared to disease control and the decrease found was similar to Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination. As observed in STAM mouse model, ALP levels were not changed significantly in any of the test groups or in the disease control.

Lipid parameters

Higher levels of lipoproteins, triglycerides, cholesterol, and cholesterol esters in serum or excessive accumulation of triglycerides in the liver is associated with MASLD. Plasma and hepatic triglycerides (TGs) as well as plasma cholesterol were also measured in WDF/CCl4 induced mouse model for MASH and changes in their levels illustrated in Fig 7A & B, respectively. Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg), Pioglitazone alone (30 mg/kg) and Saroglitazar showed significant reduction in plasma triglycerides levels compared to disease control (Fig 7A). Hepatic triglycerides (TG) were significantly decreased in both combinations of Pioglitazone and Ezetimibe in comparison to disease control (Fig 7B), however, Pioglitazone alone (30 mg/kg) and Saroglitazar did not show any significant decrease in hepatic triglycerides. Serum cholesterol measured exhibited significant decrease in mice treated with both combinations of Pioglitazone and Ezetimibe, however, no reduction was detected in Pioglitazone alone and Saroglitazar treated groups. Overall, Pioglitazone (half dose) and Ezetimibe combinations exhibited better efficacy in restoring hepatic triglycerides and cholesterol.

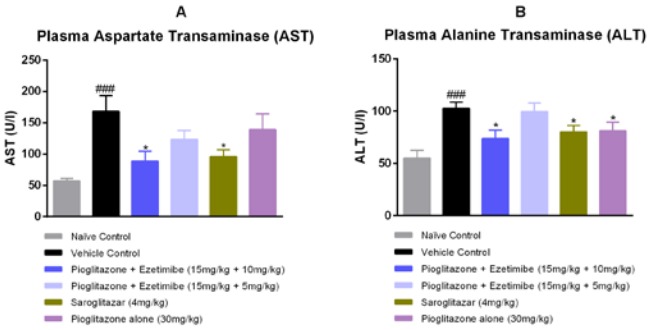

Figure 6. Effect of Pioglitazone and Ezetimibe combinations plasma parameters, ALT (A) and AST (B) in WDF/CCl4 induced mouse model for MASH. One-way ANOVA followed by Dunnett's Multiple Comparison Test was used for statistical analysis. Data is shown as Mean ± S.E.M.(n=7-8), #Significant difference as compared to Naive Control group. *Significant difference as compared to Disease/Vehicle Control. ###P < 0.001 and *P < 0.05.

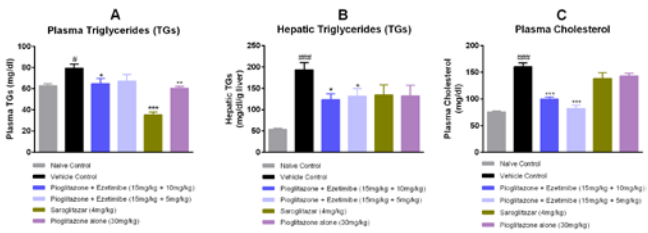

Figure 7. Effect of Pioglitazone and Ezetimibe combination on the plasma TGs (A), hepatic TGs (B) and cholesterol (C) in WDF/CCl4 induced mouse model for MASH. One-way ANOVA followed by Dunnett's Multiple Comparison Test was used for statistical analysis. Data is shown as Mean ± S.E.M.(n=7-8), #Significant difference as compared to Naive Control group. *Significant difference as compared to Disease/Vehicle Control. */#P < 0.05, **/##P < 0.01 and ***/###P < 0.001.

Histopathological examination for NAFLD Activity Score (NAS) and Fibrosis

Histopathological analysis of liver sections was performed in WDF/CCl4 induced mouse model for MASH. Microscopic examination of the H&E stained liver sections revealed that the treatment with Pioglitazone and Ezetimibe combinations, Pioglitazone (30 mg/kg) and Saroglitazar (4 mg/kg) led to the reversal of hepatic steatosis, reduced vacuolation and ballooning and significant reduction in the severity of inflammation (Table 2 and Fig. 8A).

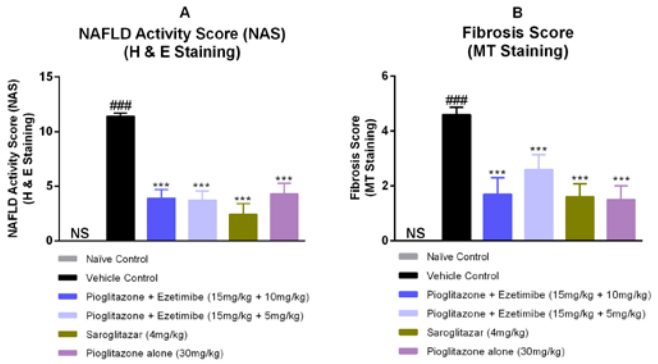

Figure 8. Effect of Pioglitazone and Ezetimibe combinations on NAFLD Activity Score (NAS) (A) and Fibrosis (MT staining) (B) in WDF/CCl4 induced mouse model for NASH. One-way ANOVA followed by Dunnett's Multiple Comparison Test was used for statistical analysis. Data is shown as Mean ± S.E.M.(n=7-8), #Significant difference as compared to Naive Control group. *Significant difference as compared to Disease/Vehicle Control. ***/###P < 0.001.

Similarly, Massons's Trichrome (MT) staining of liver sections was performed to measure fibrosis using Ishak Scoring system. Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) and Pioglitazone and Ezetimibe (15 mg/kg and 5 mg/kg) combinations exhibited significant improvement in fibrosis score compared to disease control (Table 2 and Fig. 8B) and reduction observed was dose dependent. Pioglitazone alone (30 mg/kg) and Saroglitazar (4 mg/kg) showed similar effects as exhibited by Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination.

The haematoxylin & Eosin and Masson's Trichrome-stained liver tissues of animals revealed that treatments with Pioglitazone and Ezetimibe combinations resolved MASH and protected mice from CCl4-induced fibrosis in same way as found at higher dose of Pioglitazone alone (30mg/kg) and Saroglitazar (4mg/kg). Representative photomicrographs of liver sections are described in Fig. 9A (H&E staining) and Fig. 9B (MT staining).

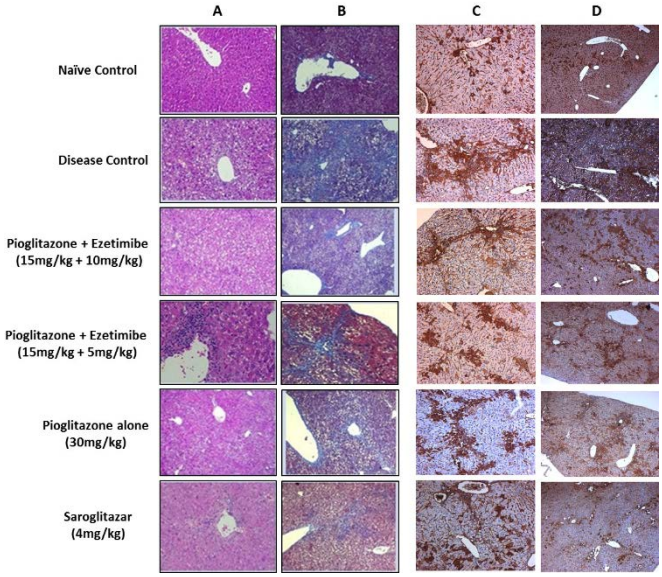

Figure 9. Effect of Pioglitazone and Ezetimibe combinations on liver histology in WDF/CCl4 induced mouse model for NASH. Representative liver sections stained with hematoxylin-eosin (A) and Masson's trichrome (B). Effect of Pioglitazone and Ezetimibe combinations on TNF-α (C) and TGF-β (D) expression in liver tissues by immune-histochemistry.

Table 2. Effect of Pioglitazone and Ezetimibe combinations on NAFLD Activity Score (NAS) and Fibrosis (MT staining) in WDF/CCl4 induced mouse model for MASH

Parameter	**Naïve Control**	**Vehicle Control**	**Pioglitazone + Ezetimibe (15mg/kg + 10mg/kg)**	**Pioglitazone + Ezetimibe (15mg/kg + 5mg/kg)**	**Saroglitazar (4mg/kg)**	**Pioglitazone alone (30mg/kg)**
Steatosis [Micro vesicular/ macro vesicular vacuolar/ Fatty degeneration] - Scale of 0-3	0.00 ± 0.00	3.00 ± 0.00	0.30 ± 0.30	0.40 ± 0.27	0.60 ± 0.40	0.90 ± 0.38
Ballooning of Hepatocytes - Scale of 0-2	0.00 ± 0.00	2.00 ± 0.00	1.00 ± 0.33	1.10 ± 0.38	0.20 ± 0.13	1.50 ± 0.34
Lobular Inflammation - Scale of 0-3	0.00 ± 0.00	2.00 ± 0.00	0.60 ± 0.22	0.30 ± 0.21	0.60 ± 0.34	0.40 ± 0.27
Portal / peri biliary infiltration inflammatory cells / inflammation - Scale of 0-3	0.00 ± 0.00	2.00 ± 0.00	0.70 ± 0.21	0.60 ± 0.27	0.50 ± 0.27	0.60 ± 0.31
Fibrosis (H&E staining) - Scale of 0-3	0.00 ± 0.00	2.40 ± 0.31	1.30 ± 0.37	1.30 ± 0.37	0.50 ± 0.34	0.90 ± 0.41
H & E (NAFLD Activity Score)	0.00 ± 0.00	11.40 ± 0.31	3.90 ± 0.78	3.70 ± 0.86	2.40 ± 0.99	4.30 ± 0.96
Fibrosis score– Ishak Scoring system (MT staining)	0.00 ± 0.00	4.60 ± 0.27	1.70 ± 0.60	2.60 ± 0.54	1.60 ± 0.48	1.50 ± 0.50

Histochemical analysis of liver samples was also performed to quantify TNF-α and TGF-β, which are inflammatory markers for fibrosis. Both combinations of Pioglitazone and Ezetimibe as well as Pioglitazone alone (30mg/kg) and Saroglitazar (4mg/kg) showed significant reduction in TNF-α and TGF-β levels in comparison to disease control, however, no significant difference was found within treatment groups (Fig. 9C & D).

Body weight and liver weight/body weight ratio

Throughout the study, body weight of animals was recorded twice a week to observe treatment related toxicity. No significant change in body weights was observed across all the treatment groups in comparison to disease control as well as naïve control (Fig. 10A). Liver weight/body weight ratio for all treatments groups and disease control group was also measured at the end of the study. Disease control showed significant increase in liver weight/body weight ratio vs. Naïve control. Both combinations of Pioglitazone and Ezetimibe as well as Pioglitazone alone (30mg/kg) significantly decreased liver weight/body weight ratio back to normal as compared to disease control, however, Saroglitazar (4 mg/kg) did not exhibit any decrease compared to disease control (Fig 10B).

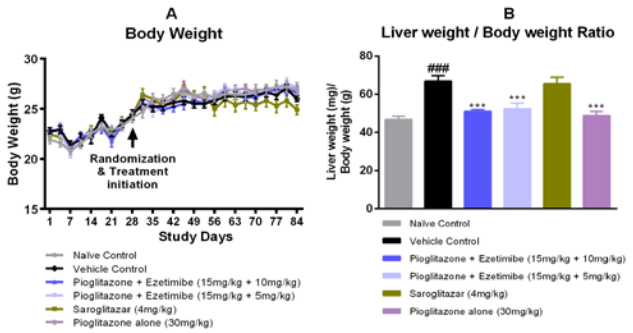

Figure 10. Effect of Pioglitazone and Ezetimibe combinations on body weight (A) and Liver weight/body weight ratio (B) in WDF/CCl4 induced mouse model for MASH. One-way ANOVA followed by Dunnett's Multiple Comparison Test was used for statistical analysis. Data is shown as Mean ± S.E.M.(n=7-8), #Significant difference as compared to Naive Control group. *Significant difference as compared to Disease/Vehicle Control. ***/###P < 0.001.

Pioglitazone and ezetimibe do not show any drug-drug interaction

In-vitro data reported for Pioglitazone demonstrated that multiple CYP isoforms are involved in its metabolism. Major cytochrome P450 isoforms involved in Pioglitazone metabolism are CYP2C8 and to a lesser degree CYP3A4, with additional contributions from a variety of other isoforms including mainly extrahepatic CYP1A1.

In vivo study of pioglitazone in combination with gemfibrozil, a strong CYP2C8 inhibitor showed that pioglitazone is a CYP2C8 substrate. While, ezetimibe does not undergo CYP-mediated metabolism to any appreciable extent (<4.1%) in vivo, nor does it affect the pharmacokinetics of drugs known to be metabolised via CYP3A4 (dextromethorphan, midazolam, atorvastatin, lovastatin and simvastatin) or CYP2C8 (tolbutamide). Therefore, ezetimibe is unlikely to cause any significant drug interaction when co-administered with pioglitazone in humans.[24] We have also evaluated the drug-drug interaction between pioglitazone and ezetimibe in human liver Microsomes (HLM) and mouse hepatocytes. Our results in HLM and mouse hepatocytes showed that metabolism of pioglitazone alone or ezetimibe alone was not affected in Pioglitazone and Ezetimibe combination (Table 3).

Table 3. Drug-drug interaction analysis between Pioglitazone and Ezetimibe in human liver microsomes and mouse hepatocytes

Compound	Human Liver Microsomes (HLM)		Mouse Hepatocytes	
	$T_{1/2}$ (in min)	Clearance (µl/min)	$T_{1/2}$ (in min)	Clearance (µl/min)
Pioglitazone alone	245	5.7	210	6.6
Ezetimibe alone	15	92.0	15	92.2
Pioglitazone (+ Ezetimibe)	223	6.2	196	7.1
Ezetimibe (+ Pioglitazone)	15	92.0	15	92.2

DISCUSSION

Tremendous efforts have been invested towards development of a monotherapy for the treatment of MASH, however, significant and approximately equal number of combination strategies have also been proposed and currently assessed in the clinical trials. One possible reason for the emergence of combination strategy for MASH treatment being the FDA and EMA guidelines, which indicate to include patients who have significantly higher risk of progression to cirrhosis and hepatic decompensation, and have biopsy-proven MASH with stage 2 fibrosis or higher.[25] As part of the subpart H approval process, clinical endpoints include either one-stage improvement in liver fibrosis or resolution of MASH. Several monotherapies were assessed for clinical endpoints for liver histological improvement, however, majority of the monotherapies with different mechanisms of action did not exceed 32% efficacy over placebo in different phases of clinical trials.[26] Though MASH is a liver centric disease, but there are extra hepatic factors such as endocrine organs - pancreas and adipose tissue,[27] gut and immune cells, which influence MASH regulation or progression. This complexity presents a challenge for monotherapies to demonstrate robust clinical efficacy in improving liver-related outcomes and ultimately obtaining regulatory approval for MASH treatment.

PPARs regulate many of the pathologically affected pathways in MASH, making these nuclear receptors attractive therapeutic targets. Although, activation of single PPARs (PPARα, PPARδ and PPARγ) showed efficacy in MASH patients, but due to limited effect single PPARs agonists failed in late stage of development. Pioglitazone - a PPAR γ/α agonist, exerts its mechanism of action by improving insulin sensitivity through its action at PPARγ and affects lipid sensitivity through action at PPARα. Functional analysis of Pioglitazone showed that it

activates PPARγ with higher efficacy and PPARα at much lesser efficacy,[28] thus, Pioglitazone is actually considered a PPARγ-selective agonist.

The beneficial attributes of pioglitazone demonstrated in clinical trials in terms of regulating the metabolic load on liver as well as regulating hepatic inflammation made it as a recommended drug for the pharmacological treatment of MASH in patients with T2DM.[29] An 18-month proof-of-concept study of the combination therapy of vitamin E and pioglitazone 30-45 mg in patients with T2DM showed improvement in NAS, and MASH resolution occurred more in the combination than the placebo group.[30] Several trials of pioglitazone alone or in combination for MASLD treatment are currently ongoing.[31] Considering dose associated adverse effects, a long term administration of pioglitazone at higher doses may pose a challenge for MASH patients.[32] In spite of associated drawbacks, pioglitazone is recommended to clinicians by regulatory bodies as there is a dearth of treatment options for MASH treatment.

Given the pathophysiology of MASH, data from ongoing clinical studies suggest that dual and pan-PPAR agonists have a broader and more potent therapeutic effect on MASH by simultaneously engaging different targets and pathways.[33] Saroglitazar is the first glitazar developed by Zydus Therapeutics (India), which has been granted marketing authorization in India for treating diabetic dyslipidemia with its potent PPARα and moderate PPARγ activities.[34] In the phase 2 clinical trial (NCT03061721), Saroglitazar (4 mg/day) significantly improved blood ALT levels (the primary endpoint), hepatic fat content, insulin resistance, and atherogenic dyslipidemia (the secondary endpoints) in MASLD/MASH patients without worsening of fibrosis.[7] The phase 2b clinical trial (NCT05011305) is currently recruiting US participants with the primary endpoint of resolution of MASH without worsening of fibrosis after 76 weeks of treatment with 2 and 4 mg/day doses.[35]

Based on available literature, we hypothesized that combining a PPARγ agonist (pioglitazone) with a lipid lowering drug (ezetimibe) could have similar or better effects compared to PPARα/γ dual agonists. Due to safety concern of higher doses of pioglitazone (30 mg and 45 mg),[30,36-38] we tested pioglitazone at reduced clinical dose (10mg/day or 15 mg/day) in combination with Ezetimibe (10mg/kg). We anticipated that proposed combination might improve effectiveness by complementary or synergistic effects on MASLD/MASH patients, and improve tolerability by using lower doses.

The hallmark of MASLD is triglyceride accumulation in the cytoplasm of hepatocytes,[39,40] which arises due to an imbalance between lipid acquisition (i.e., fatty acid uptake and de novo lipogenesis) and removal (i.e., mitochondrial fatty acid oxidation and export as a component of VLDL particles). Ezetimibe inhibits the absorption of cholesterol from the small intestine by blocking the Niemann-Pick C1-like 1 (NPC1L1) protein present on the gastrointestinal tract epithelial cells as well as in hepatocytes. It also inhibits aminopeptidase N and interrupts a caveolin 1–annexin A2 complex involved in cholesterol trafficking. The decreased levels of cholesterol in the liver cells leads them to absorb more cholesterol and thus lowering the levels of circulating cholesterol.[12] Moreover, ezetimibe has also been shown to improve major clinical parameters, histological observations and decrease NAS in MASH clinical trials, but hepatic inflammation and fibrosis were not improved.[41-44] In our MASH animal models, Pioglitazone and Ezetimibe combination (15mg/kg and 10mg/kg), Pioglitazone alone (30 mg/kg) and Saroglitazar (4 mg/kg) showed significant reduction in plasma triglycerides levels compared to disease control, whereas hepatic triglycerides were significantly reduced only in Pioglitazone and Ezetimibe combination (15 mg/kg and 10 mg/kg) in both mice model for MASH, which confirmed our hypothesis.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are liver enzymes which are elevated in about 90% of people with MASLD, which includes MASH. High levels of ALT have been linked to a higher risk of MASH. Pioglitazone and Ezetimibe (15 mg/kg and 10 mg/kg) combination showed superior effects compared to Pioglitazone alone and Saroglitazar in regulating AST and ALT levels. No change in plasma cholesterol was observed in STAM mice model in any of the treatment groups. However, Pioglitazone and Ezetimibe combinations (15 mg/kg and 10 mg/kg & 15 mg/kg and 5 mg/kg) exhibited significant decrease in plasma cholesterol in WDS/CCl4 model. Decrease in plasma cholesterol in Pioglitazone and Ezetimibe combinations could be attributed to anti-cholesterol absorption effect of Ezetimibe.

Histological findings are considered as a gold standard to rank order the efficacy of a therapeutic options for MASH treatment. Therefore, the histological features of livers from treatments or vehicle groups were evaluated for steatosis, ballooning of hepatocytes and lobular inflammation. Pioglitazone and Ezetimibe combinations showed MASH resolution comparable to Pioglitazone alone (30mg/kg) and Saroglitazar (4mg/kg) in both MASH models. Massons's Trichrome (MT) staining of liver sections was performed to measure fibrosis using Ishak Scoring system. Combination of Pioglitazone and Ezetimibe showed significant improvement in fibrosis score in both MASH models compared to disease control, and the effect found was dose dependent.

Histochemical analysis of liver sample was done for TGF-β and TNF-α, which are inflammatory markers for fibrosis. Pioglitazone and Ezetimibe combinations as well as Pioglitazone alone (30mg/kg) and Saroglitazar (4mg/kg) showed significant reduction in TGF-β and TNF-α levels.

In-vitro drug-drug interaction studies in human liver microsomes and mouse hepatocytes showed no interaction between Pioglitazone and Ezetimibe. Thus, our novel combination is safe for concomitant usage.

Our study has confirmed that Pioglitazone and Ezetimibe combinations showed superior MASH resolution and improvement in fibrosis with respect to disease control. Overall, biochemical and histological findings revealed that reduced dose of Pioglitazone (15 mg/kg) in combination with Ezetimibe (10 mg/kg) either produced similar effects or superior effects compared to Pioglitazone alone (30 mg/kg) in two different MASH animal models. Hence, present study for the first time established the proof of concept in preclinical MASH models that

a reduction of Pioglitazone dose by half, in combination with Ezetimibe could able to regulate the steatosis, hepatocyte inflammation and ballooning which ultimately resulted in MASH resolution and improvement in fibrosis score. Based on the preclinical studies, patent (WO2023026130 A1) has been granted for the combination of pioglitazone with ezetimibe.[45]

CONCLUSION

Pioglitazone and Ezetimibe combinations improved plasma and liver markers in mice models for MASH. Our combination either showed equivalent or numerically superior efficacy in terms of NAS as well as fibrosis score in comparison Pioglitazone or Saroglitazar alone in streptozotocin and high fat diet induced MASH mouse model and WDF/CCl4 induced MASH mouse model. Overall, results from this combination study further warrants the evaluation of Pioglitazone and Ezetimibe combination in clinical settings.

CONFLICT OF INTEREST

Authors do not have conflict of interest for this work.

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