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ATP citrate lyase inhibitor Bempedoic Acid alleviate long term HFD induced NASH through improvement in glycemic control, reduction of hepatic triglycerides & total cholesterol, modulation of inflammatory & fibrotic genes and improvement in NAS score

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) and Non-alcoholic steatohepatitis (NASH) are chronic liver disorders, the prevalence of which is increasing worldwide. Long term High Fat Diet (HFD) induced NASH animal models closely mimic the characteristics of human NASH and hence used by investigators as a model system for studying the mechanism of action of new drugs. Bempedoic acid (ETC-1002), a ATP citrate lyase (ACLY) inhibitor that lowers the LDL cholesterol was recently approved by US FDA for the treatment of heterozygous familial hypercholesterolemia (HeFH) and established atherosclerotic cardiovascular disease (ASCVD). ACLY is one of the genes modulated in NASH patients and hence we studied the effect of ACLY inhibitor Bempedoic acid in long term HFD induced NASH animal model to understand the pharmacological benefits and the associated mechanism of action of this newly approved drug in NASH. Mice fed with 60% Kcal High Fat Diet for 32 weeks were used for the study and the animals were given Bempedoic acid for 5 weeks at doses of 10 mg kg^{-1}, po, qd, and 30 mg kg^{-1}, po, qd. Bempedoic acid treatment resulted in inhibition of body weight gain and improved the glycemic control. Bempedoic acid treated group showed statistically significant reduction in plasma ALT, AST, hepatic triglycerides (TG) and total cholesterol (TC), along with statistically significant reduction in steatosis score by histological analysis. Hepatic gene expression analysis showed significant reduction in inflammatory and fibrotic genes such as *Mcp-1/Ccl2, Timp-1 & Col1α1*. Histological analysis showed significant improvement in NAS score. Overall, Bempedoic acid alleviated HFD induced Non-Alcoholic Steatohepatitis through inhibition of body weight gain, improvement in glycemic control, reduction of hepatic triglycerides & total cholesterol, modulation of inflammatory & fibrotic genes, and improvement in NAS score. Hence, Bempedoic acid can be a potential therapeutic option for metabolic syndrome and NASH.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) and Non-alcoholic steatohepatitis (NASH) are chronic liver disorders that is estimated to have worldwide prevalence ranging from 6.3% to 33% (Harrison et al., 2002; Chalasani et al., 2012). Around 20% of NAFLD patients were found to have NASH with excessive fat accumulation in hepatocytes along with the infiltrations of inflammatory cells, evidence of hepatocyte injury with ballooning, and deposition of fibrous tissue in liver (Chalasani et al., 2012). A "2-hit hypothesis" was proposed earlier to explain the development of NASH with Steatosis being the first hit followed by a second hit that includes inflammation, oxidative damage, and fibrosis (Harrison et al., 2002), which is now updated to "Multiple parallel hit hypothesis" as various parallel insults including dietary factors, epigenetic factors and gut-liver axis contribute to the development of steatosis and liver inflammation such as insulin resistance, obesity & adipose tissue dysfunction (Buzzetti et al., 2016).

Several animal models of NASH have been developed to screen for drugs that could be useful in NASH management. The currently available animal models of NASH can be classified into models with genetic

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manipulation, diet induced models and models from carcinogenic exposure. Diet induced NASH models include the proprietary NASH-HCC STAM™ mouse model (Stelic animal model) to methionine-choline deficient (MCD) model, high fat-cholesterol (HFC) diet model, choline-deficient high fat diet (CD-HFD) model, choline-deficient amino acid-defined (CDAD), and American sedentary lifestyle-induced obesity syndrome (ALIOS) model. In general, animals fed with diet rich in lipogenic nutrients, particularly simple sugars such as fructose or sucrose and/or saturated fats develop steatosis rather than steatohepatitis. In older animals, evidence of hepatocellular injury, focal lobular inflammation and even early pericellular fibrosis may be observed, particularly during prolonged intake of a lipogenic diet (Schultz et al., 2014). There is also evidence to suggest that steatotic hepatocytes activate the Kupffer cells and promote the inflammatory cascade observed in NASH in addition to expressing the inflammatory cytokines themselves (Pan et al., 2015).

Bempedoic acid (8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid, ETC-1002) is a drug developed for treatment of dyslipidemia & other cardio-metabolic disorders and was recently approved by US FDA for the treatment of heterozygous familial hypercholesterolemia (HeFH) and established atherosclerotic cardiovascular disease (ASCVD). Bempedoic acid is a prodrug which get converted to an active form ETC-1002-CoA by acyl-CoA synthetase and is known to modulate adenosine monophosphate-activated protein kinase (AMPK) activation and ATP citrate lyase (ACLY) inhibition (Pinkosky et al., 2013; Zagelbaum et al., 2019). ACLY is a cytoplasmic enzyme responsible for the generation of acetyl coenzyme A (acetyl-CoA) for the de novo synthesis of fatty acids (FAs) and cholesterol (Samsoondar et al., 2017; Lemus and Mendivil, 2015). ACLY is an enzyme located at the juncture of nutrient catabolism & cholesterol and fatty acid biosynthesis (Pinkosky et al., 2017). DNA methylation and mRNA expression profiling of liver samples from morbidly obese patients recognized ACLY as one of the genes to be methylated and increased in NAFLD patients (Pinkosky et al., 2017; Ahrens et al., 2013). There are reports that suggests the benefits associated with Bempedoic acid in reduction of plasma and hepatic TG and TC and plasma LDL and VLDL (Pinkosky et al., 2013). The objective of the current study is to test Bempedoic acid in long term (32 weeks) HFD induced NASH animal model to understand the pharmacological benefits and the associated mechanism of action of this newly approved drug in NASH.

2. Results

2.1. Bempedoic acid inhibits body weight gain in long term HFD induced NASH model

The overall study design is shown in Fig. 1. 7 weeks old C57BL6/N mice of similar body weight were used for the study (n = 10). A group of animals designated as "lean control" were placed on regular diet and the rest were placed on high fat diet (HFD) as described in the methods section. The animals placed on HFD gained significant weight after 32 weeks (39 weeks of age) as compared to lean control. The animals placed on HFD diet were randomized as described in the methods section after 32 weeks and the treatment with Bempedoic acid was initiated. Bempedoic acid at doses of both 10 mg kg^{-1}, po, qd, and 30 mg kg^{-1}, po, qd. inhibited further body weight gain starting day 8 of treatment till end of the treatment period [5 weeks] (Fig. 2a and b). There was no significant change in energy intake of all groups (Fig. 2c and d). It suggests that the inhibition of body weight gain observed in the Bempedoic acid treatment group is driven by Bempedoic acid and not by reduction in energy intake.

2.2. Treatment with Bempedoic acid improves glycemic control in long term HFD induced NASH model

Oral Glucose Tolerance Test (OGTT) was performed after 4 weeks of treatment with Bempedoic acid at doses of 10 mg kg^{-1}, po, qd, and 30 mg kg^{-1}, po, qd. Glucose tolerance was markedly decreased in HFD fed animals as compared to control diet fed lean animals (Fig. 3a). Basal glucose at 0 min in HFD controls were significantly higher compared to lean control and the AUC showed impaired glucose response of HFD fed control animals (Fig. 3b). Bempedoic acid at both doses has shown statistically significant increase in glucose tolerance as seen by decrease in glucose levels at 15 & 30 min after glucose challenge (Fig. 3a), and reduction in AUC blood glucose levels during OGTT (Fig. 3b). Marked decrease in glucose levels were also observed in the blood samples of 6h fasted animals in the Bempedoic acid treatment group tested at 0 min during the OGTT (Fig. 3c).

2.3. Bempedoic acid treatment improves liver lipid profile in long term HFD induced NASH model

The changes in the lipid profile of animals put on HFD and the effect of Bempedoic acid is shown in Fig. 4. Liver triglycerides and total cholesterol levels were significantly increased in the HFD fed mice as compared to control diet fed lean mice. The reduction in liver triglycerides (Fig. 4a) & total cholesterol levels (Fig. 4b) upon the administration of Bempedoic acid at doses of 10 mg kg^{-1}, po, qd, and 30 mg kg^{-1}, po, qd were statistically significant. Terminal plasma ALT & AST levels were significantly increased in HFD fed animals which was reduced in a statistically significant manner by both doses of Bempedoic acid (Fig. 4c–f). Fig. 4e–f shows the plasma ALT and AST levels on day 0 and day 35 of the study. On day 0, there were marked difference between the AST and ALT levels of the lean control animals, and the HFD fed control animals/HFD fed animals grouped for Bempedoic acid treatment. On day 35 of Bempedoic acid treatment, there were

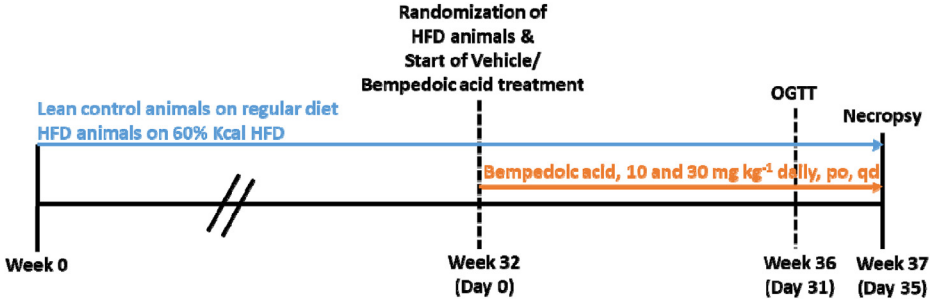

Fig. 1. Study Design- 7 weeks old C57BL6/N mice were given either the rodent chow diet (Teklad; Harlan) or HFD (60% Kcal; Research diet, D12492). The animals that were given the rodent chow diet constituted the lean control group and received the chow diet for the duration of the study (37 weeks). These control animals received the vehicle (0.5% w/v methyl cellulose and 0.5% v/v Tween 80) from week 32 till week 37. The animals that were given the HFD were randomized based on body weight & random blood glucose levels after 32 weeks and segregated into three groups. The animals received either the vehicle (0.5% w/v methyl cellulose and 0.5% v/v Tween 80) or Bempedoic acid at 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd for five weeks (till 37 weeks). Oral glucose tolerance test (OGTT) was performed in week 36 (day 31 of treatment). The study was terminated on day 35 of the treatment period and the biochemical, histological and biomarker analysis were performed.

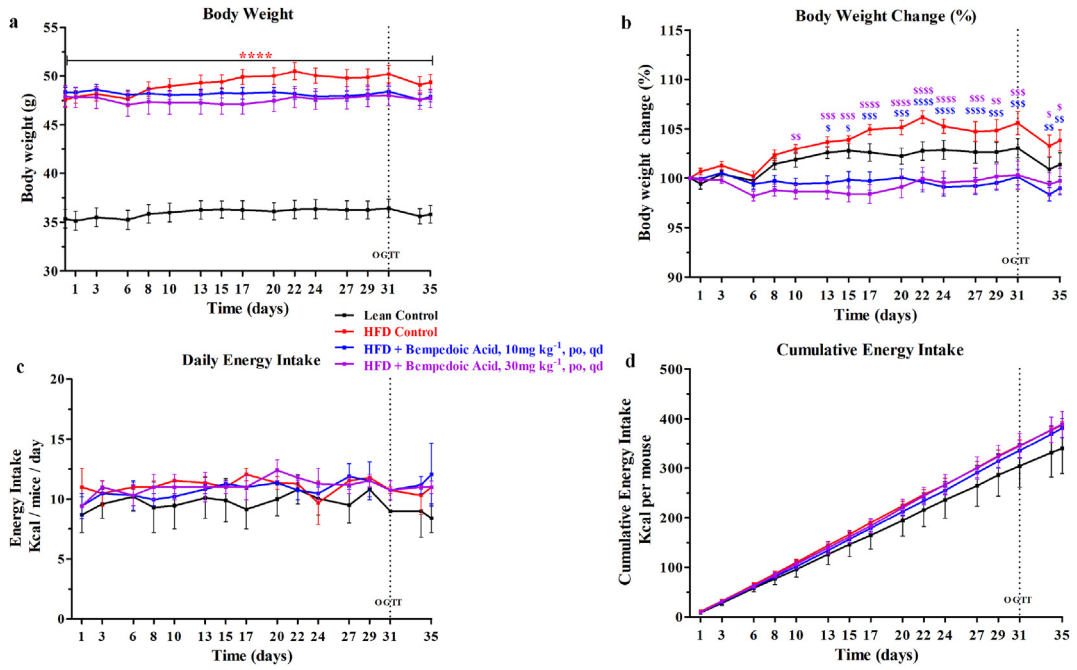

Fig. 2. Effect of Bempedoic acid on body weight gain in long term HFD induced NASH model – The body weight and food intake were measured during the course of the treatment period and represented. (a) Body weight in grams (b) % body weight change (c) Daily energy intake (d) Cumulative energy intake. ****p<0.0001 when HFD control was compared with Lean control and $ p<0.05, $$ p<0.01, $$$ p<0.001 and $$$$ p<0.0001 when HFD + Bempedoic acid, 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd group was compared with HFD control (Two-way ANOVA followed by Bonferroni's post-hoc test). Data shown as Mean ± SEM. n = 10. Bempedoic acid at doses of both 10 mg kg^{-1}, po, qd, and 30 mg kg^{-1}, po, qd. inhibited body weight gain starting day 8 of the treatment till end of the study as compared to the HFD control (a & b). There was no significant change in energy intake of all groups (c & d). OGTT was performed on day 31.

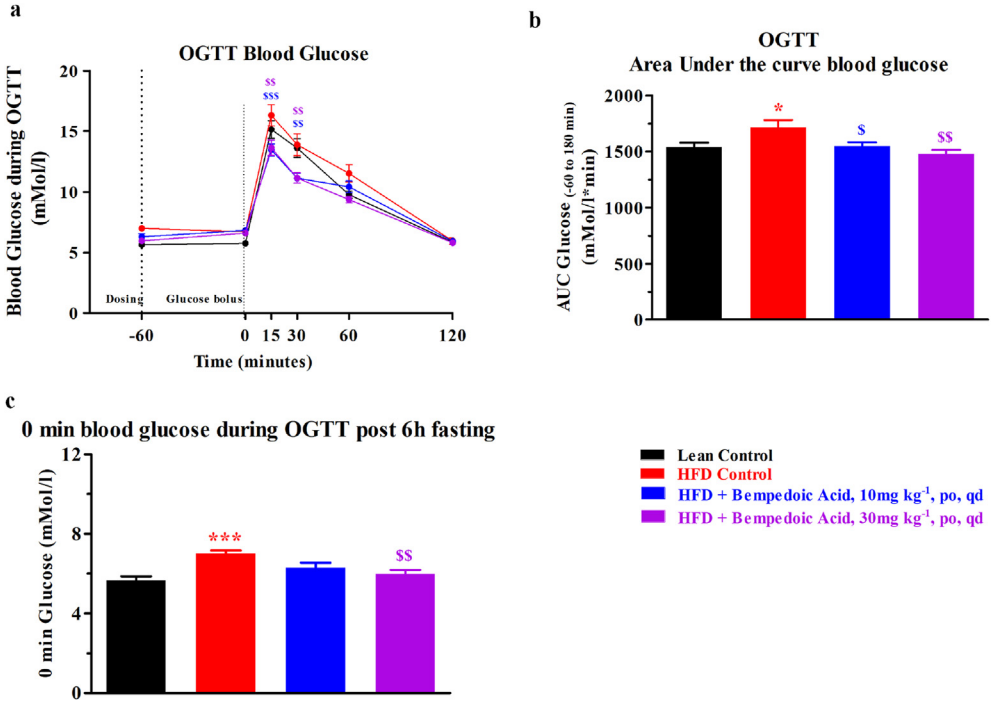

Fig. 3. Effect of Bempedoic acid on OGTT profile in long term HFD induced NASH model. (a) Blood glucose curve during OGTT (b) AUC glucose (c) 0 min blood glucose levels from fasted (6h) animal samples. $$ p<0.01, $$$ p<0.001 when HFD + Bempedoic acid, 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd group were compared with HFD control (Two-way ANOVA followed by Bonferroni's post-hoc test) *p<0.05 and ***p<0.001 when HFD control was compared with Lean control and $ p<0.05 and $$ p<0.01 when HFD + Bempedoic acid, 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd group were compared with HFD control (One-way ANOVA followed by Bonferroni's post-hoc test). Data shown as Mean ± SEM. n = 9–10. Bempedoic acid at both doses has shown statistically significant increase in glucose tolerance as seen by decrease in glucose levels at 15 & 30 min after glucose challenge (a), and reduction in AUC blood glucose levels during OGTT (b). Marked decrease in glucose levels were also observed in the blood samples of 6h fasted animals in the Bempedoic acid treatment group tested at 0 min during the OGTT (c).

statistically significant reduction in the AST and ALT levels in animals treated with both doses of Bempedoic acid. These results clearly demonstrate the role of Bempedoic acid in modulating the liver enzymes. The changes in the plasma TG and cholesterol profile of animals put on HFD and the effect of Bempedoic acid is shown in Fig. 4g and h. Plasma triglycerides and total cholesterol levels were significantly increased in the HFD fed mice as compared to control diet fed lean mice. However, there is no reduction in plasma triglycerides (Fig. 4g) & total cholesterol levels (Fig. 4h) upon the administration of Bempedoic acid. The results suggest that under the current experimental conditions, the lipid lowering effect seen by Bempedoic acid is limited to the liver.

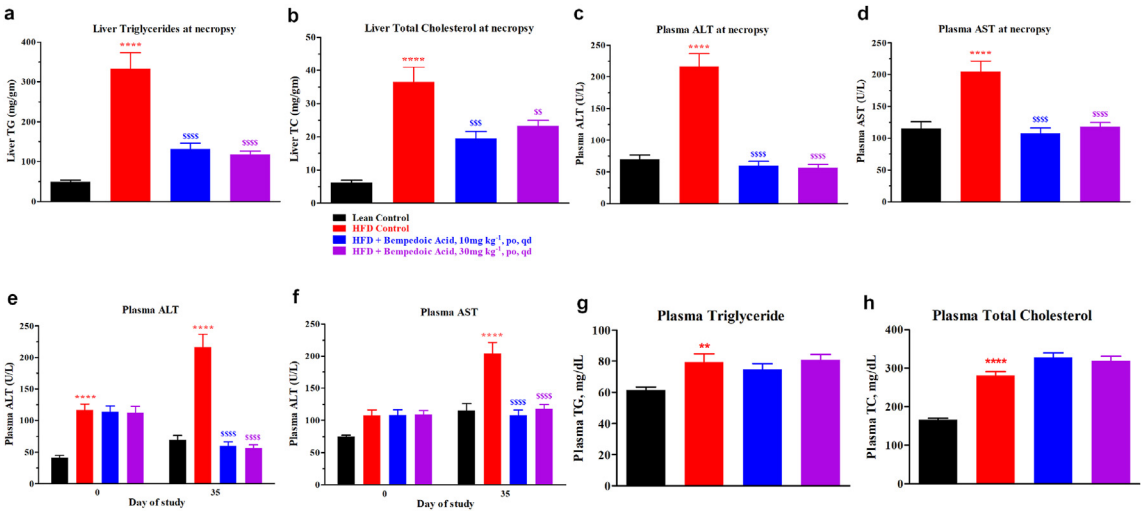

Fig. 4. Effect of Bempedoic acid on lipid profile in long term HFD induced NASH model. The plasma and liver samples were analyzed for ALT, AST, TG and total Cholesterol. (a) Liver Triglyceride levels at the end of the study (b) Liver total cholesterol levels at the end of the study (c) Plasma ALT levels at the end of the study (d) Plasma AST levels at the end of the study. (e) Plasma ALT levels at day 0 (before the start of Bempedoic acid treatment) and on day 35 (after 5 weeks of Bempedoic acid treatment). (f) Plasma AST levels at day 0 (before the start of Bempedoic acid treatment) and on day 35 (after 5 weeks of Bempedoic acid treatment) (g) Plasma Triglycerides at the end of the study (h) Plasma total cholesterol levels at the end of the study. **p<0.01 and ****p<0.0001 when HFD control was compared with Lean control and $$ p<0.01, $$$ p<0.001 and $$$$ p<0.0001 when HFD + Bempedoic acid, 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd group was compared with HFD control (One-way ANOVA followed by Bonferroni's post-hoc test). Data Shown as Mean ± SEM. n = 9–10. The liver triglycerides (a) and total cholesterol levels (b) were significantly reduced upon treatment with Bempedoic acid. Plasma ALT & AST levels were significantly reduced by both doses of Bempedoic acid (c–f). Plasma Triglycerides and plasma total cholesterol were increased in HFD control group (g, h), however, treatment with Bempedoic acid did not show any modulation of plasma triglycerides and plasma total cholesterol.

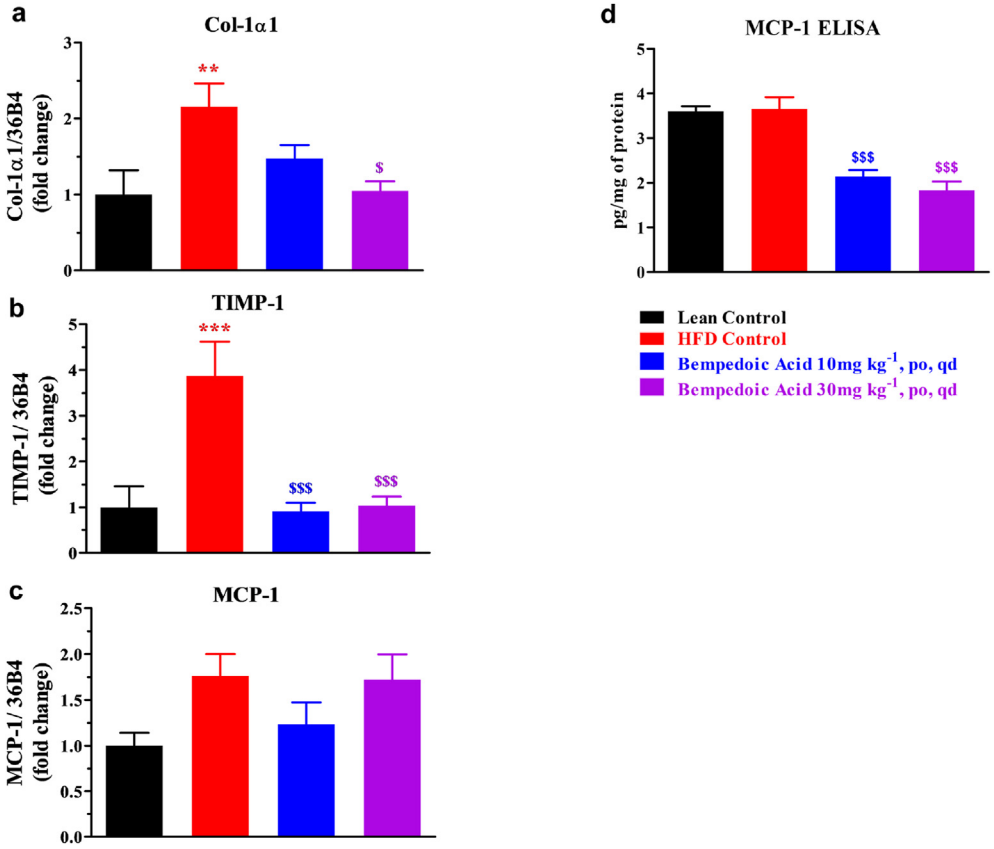

Fig. 5. Effect of Bempedoic acid on the liver fibrotic and inflammation gene/ protein expression profile in long term HFD induced NASH model. Expression profile of liver inflammation and fibrotic genes/proteins were analyzed at the end of Bempedoic acid treatment. (a–c) mRNA analysis, (a) *Col1α1* (b) *Timp-1* (c) *Mcp-1*. (d) MCP-1 protein analysis using ELISA. **p<0.01 and ***p<0.001 when HFD control was compared with Lean control and $ p<0.05 and $$$ p<0.001 when HFD + Bempedoic acid, 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd group were compared with HFD control (One-way ANOVA followed by Bonferroni's post-hoc test). Data Shown as Mean ± SEM. n = 9–10. Treatment with Bempedoic acid significantly down regulated the mRNA expression of *Col1α1* (a), *Timp-1* (b) and protein levels of MCP-1 (d). Modulation of *Mcp*-1 mRNA levels upon Bempedoic acid was moderate and was observed only in animals treated with Bempedoic acid at 10 mg kg-1 dose (c).

2.4. Bempedoic acid treatment modulates inflammation and fibrotic gene expression and MCP-1 protein levels in long term HFD induced NASH model

The gene expression profile of inflammation and the fibrotic markers such as Collagen, type I, alpha 1 (*Col1α1),* Tissue inhibitor of metalloproteinase-1 *(Timp-1),* and Monocyte Chemoattractant Protein-1 (*Mcp-1)* were investigated. The expression of all three genes were elevated in HFD fed animals compared to control diet fed lean animals. Treatment with Bempedoic acid down regulated the expression of *Col1α1* (Fig. 5a) & *Timp-1* (Fig. 5b) significantly compared to HFD fed control animals indicating reduction in hepatic fibrosis. However, the modulation of *Mcp*-1 mRNA levels upon Bempedoic acid was moderate and was observed only in animals treated with Bempedoic acid at 10 mg kg^{-1} dose (Fig. 5c). Hence, we also analyzed the MCP-1 protein levels using the samples from this experiment to understand the overall modulation of MCP-1 by Bempedoic acid. While the MCP-1 protein levels were almost comparable between the lean controls and the HFD fed controls, statistically significant reduction in MCP-1 protein levels were observed with Bempedoic acid treatment (Fig. 5d). Collectively, the data from mRNA and protein analysis of inflammation and fibrotic genes suggests that Bempedoic acid plays a role in reduction of hepatic fibrosis by modulating the expression of these genes.

2.5. Bempedoic acid treatment improves the NAFLD activity score (NAS score) in long term HFD induced NASH model

The control diet fed lean animals have shown minimal/no signs of hepatocellular ballooning degeneration, lobular inflammation & steatosis, whereas the animals fed with HFD for prolonged period of 37 weeks induced many characteristics of NASH including hepatocellular ballooning, lobular inflammation & steatosis (Fig. 6a, b, 6c – black versus red bar; Fig. 6d versus 6e [in 10X]; Fig. 6h versus 6i [in 20X]). Bempedoic acid at 10 and 30 mg kg^{-1} have shown significant reduction of lobular inflammation, hepatocellular ballooning degeneration and the NAS scores induced by HFD diet (Fig. 6a, b, 6c – red versus blue & purple bar; Fig. 6e versus 6f & 6g). Bempedoic acid at both 10 and 30 mg kg^{-1} doses demonstrated statistically significant inhibition in steatosis (Fig. 6a), and NAS scores (Fig. 6c). The reduction in lobular inflammation which was not statistically significant (Fig. 6b). We also analyzed the collagen deposition in liver at the end of the study. The Sirius red stained liver sections from the lean controls, HFD controls, and Bempedoic acid treatment groups are shown in Fig. 7a–d. Steatosis characterized by large vacuoles that occupy the whole cytoplasm was markedly seen in the sections from HFD control group (Fig. 7b). The scoring for collagen deposition was carried out using Sirius red staining of the liver sections and represented in a graph (Fig. 7e). Moderate increase in Sirius red staining was observed in HFD controls as compared to the lean controls and this increase was abrogated in the Bempedoic acid treated groups (Fig. 7e). However, neither the moderate increase of Sirius red staining observed in the HFD controls (as compared to lean controls) nor the abrogation of the increase in Sirius red staining (as compared to HFD controls) observed in Bempedoic treatment groups were statistically significant.

3. Discussion

Bempedoic acid (ETC-1002) is an orally administered small molecule prodrug which gets converted to its active form ETC-1002-CoA in liver by Acyl CoA synthetase (Bilen and Ballantyne, 2016). The active form inhibits ACLY enzyme that catalyzes the reaction that links cellular glucose catabolism and lipogenesis by converting cytosolic citrate to acetyl-coenzyme A (CoA), which is further converted to malonyl-CoA, the precursor for fatty acid biosynthesis. (Zagelbaum et al., 2019; Bilen

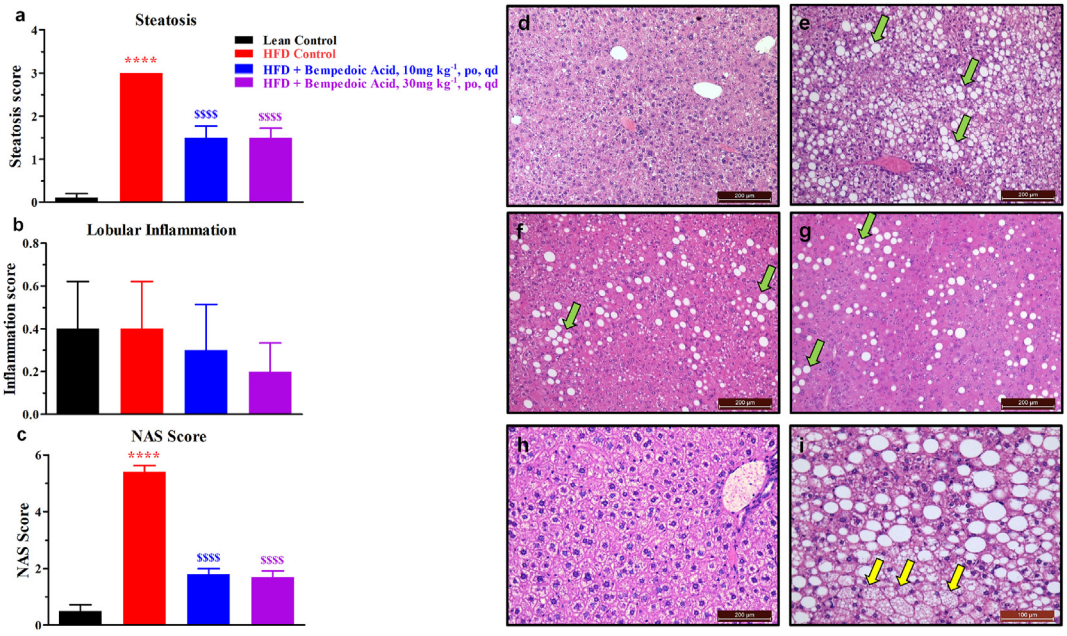

Fig. 6. Effect of Bempedoic acid on the NAS score in long term HFD induced NASH model. Histological assessment of liver was performed at the end of Bempedoic acid treatment and the data is provided. (a) Steatosis score (b) Lobular inflammation score (c) NAS score. Representative H&E stained liver microscopic images are provided. (d) Lean control (10X), (e) HFD control (10X), (f) HFD + Bempedoic acid 10 mg kg^{-1}, po, qd (10X), (g) HFD + Bempedoic acid 30 mg kg^{-1}, po, qd (10X). For better visualization, 20X images are provided for lean control and HFD control. (h) Lean control (20X) and (i) HFD control (20X). Green arrow shows steatosis characterized by large vacuoles that occupy the whole cytoplasm and push the nucleus to one side of the cell, and yellow arrows shows hepatocellular ballooning degeneration characterized by cells with swollen and rarefied cytoplasm. ****$p<0.0001$ when HFD control is compared with Lean control and $$$$ $p<0.0001$ when HFD + Bempedoic acid, 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd group is compared with HFD Control (One-way ANOVA followed by Bonferroni's post-hoc test). Data Shown as Mean ± SEM. n = 10. Treatment with Bempedoic acid showed significant reduction of lobular inflammation, hepatocellular ballooning degeneration and NAS scores as compared to the untreated HFD controls. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

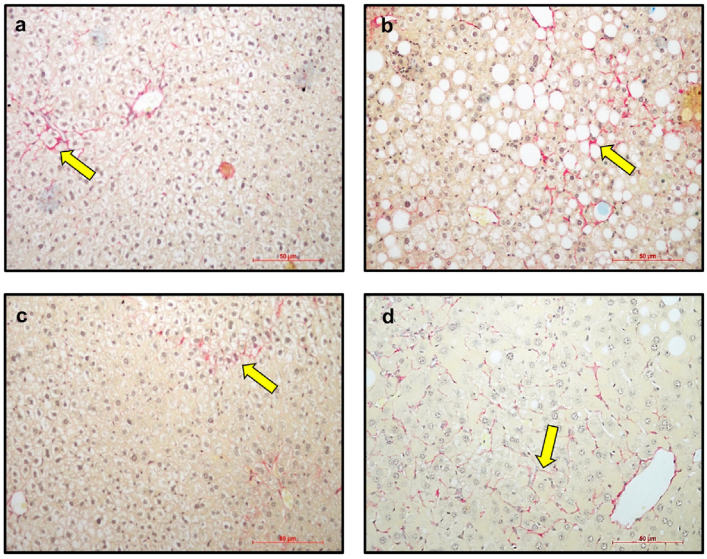

Fig. 7. Effect of Bempedoic acid on collagen deposition and fibrosis score as measured by Sirius red staining in long term HFD induced NASH model. Histological assessment of liver was performed at the end of Bempedoic acid treatment to study the collagen deposition and measure the fibrosis score. Representative Sirius red stained liver microscopic images are provided. (a) Lean control (20X), (b) HFD control (20X), (c) HFD + Bempedoic acid 10 mg kg^{-1}, po, qd (20X), (d) HFD + Bempedoic acid 30 mg kg^{-1}, po, qd (20X). Yellow arrow shows fibrosis (a, b, c, d). Steatosis characterized by large vacuoles that occupy the whole cytoplasm was markedly seen in the sections from HFD control group (b). The scoring for collagen deposition was carried out using Sirius red staining of the liver sections as described in the methods section and represented in a graph (e). Moderate increase in Sirius red staining was observed in HFD controls as compared to the lean controls and this increase was abrogated in the Bempedoic acid treated groups (e). Data was analyzed by One-way ANOVA followed by Bonferroni's post-hoc test. Data Shown as Mean ± SEM. n = 10. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and Ballantyne, 2016). ACLY is up-regulated in individuals with NAFLD as well in livers of obese and diabetic db/db mice (Guo et al., 2019; Wang et al., 2009). Abrogation of ACLY in liver of db/db mice using RNA interference resulted in reduced acetyl-CoA and malonyl-CoA levels, inhibited hepatic lipogenesis, and protected against hepatic steatosis (Wang et al., 2009). Dietary factors and metabolic disorders are still the leading causes of NAFLD and therefore investigation of ACLY inhibitor Bempedoic acid in HFD induced NASH animal model is warranted to understand the mechanism of action and the pharmacological benefits.

Different diet induced animal models are available to study NAFLD induction like the CDAA and MCD models but most of them involve diet patterns that are quite different from human diet (Nakamura and Terauchi, 2013; Zhong et al., 2020). HFD-induced hepatic steatosis model in male C57BL/6 mice is time consuming. However, it is considered as the closest model that mimics many features of human NASH and is widely used in NAFLD studies (Zhong et al., 2020; Stephenson et al., 2018). We investigated the mechanism of action of ACLY inhibitor Bempedoic acid and its pharmacological benefits in long term HFD induced NASH animal model.

The body weight of HFD fed animals were significantly higher than lean control animals after 32 weeks HFD feeding and the animals were insulin resistant. Plasma ALT and AST levels were significantly elevated after 32 weeks of HFD feeding which indicated poor liver health. Insulin resistance is known to lead to an abnormal response in liver wherein elevated blood insulin levels don't efficiently reduce gluconeogenesis but instead activates hepatic De Novo Lipogenesis. In insulin resistant liver, activated De Novo Lipogenesis together with increased influx of fatty acids from adipose tissue lipolysis leads to triglycerides and lipotoxic metabolites accumulation (Siculella et al., 2020). Long term dietary cholesterol is known to cause profound liver damage, evident by significant accumulation of both hepatic TG and cholesterol, marked decrease in liver density, accompanied by increased plasma levels of ALT and AST, indicating impaired liver function (Jensen et al., 2018). The liver triglycerides and cholesterol were markedly elevated in animals fed with long tern HFD diet in our study.

The progression of NASH pathology in long term high fat diet model in our study was confirmed using histopathological analysis where the features of NASH like steatosis, hepatocellular ballooning and lobular inflammation were established along with elevation in fibrotic and inflammatory markers *Mcp-1, Timp-1* and *Col1α1*. Hepatic infiltration of macrophages is stimulated by MCP-1 as these cells express CCR2, whereas hepatic recruitment of $CCR2^{+}$ myeloid cells stimulate hepatic steatosis. The MCP-1-CCR2 pathway is known to be upregulated in the livers of NASH animals and is critical for development of hepatic steatosis and fibrosis by stimulating the migration of hepatic stellate cells (Kitade et al., 2017; Miura et al., 2012; Seki et al., 2009). Activated hepatic stellate cells contribute towards liver fibrogenesis through proliferation, chemotaxis, extra cellular matrix synthesis and contractility (Robert et al., 2016). The profibrotic cytokine TGF-β activates the hepatic stellate cells, induces expression of matrix producing genes and inhibits degradation of extra cellular matrix by downregulating MMP expression. It also promotes TIMP-1 expression leading to excessive deposition of collagen and promotes liver fibrosis (Robert et al., 2016; Liu et al., 2006; Cui et al., 2011). We found a significant increase in fibrotic genes *Col1α1* and *Timp-1* in animals fed with HFD in our study.

Treatment of the HFD fed animals with Bempedoic acid at doses of 10 mg kg^{-1}, po, qd, and 30 mg kg^{-1}, po, qd from week 32 to week 37 significantly inhibited body weight gain as compared to HFD control animals without altering energy intake. Animals fed with long term HFD had impaired response to glucose challenge and treatment with Bempedoic acid at doses of 10 mg kg^{-1}, po, qd, and 30 mg kg^{-1}, po, qd showed significant improvements in glucose sensitivity. Bempedoic acid treatment also resulted in significant reduction of liver TG, total cholesterol, plasma ALT & AST levels. Histopathological analysis revealed that Bempedoic acid treatment significantly reduced steatosis, hepatocellular ballooning, lobular inflammation, along with reduction in fibrotic & inflammatory genes *Mcp-1, Timp-1* and *Col1α1*, leading to improvements in NAS score. The reduction in lobular inflammation seen with Bempedoic acid treatment was not statistically significant.

In studies using mouse carbon tetrachloride (CCl4) induced fibrosis

model, the animals treated with AMPK agonist 5-Aminoimidazole-4-carboxamide1-β-D-ribofuranoside (AICAR) had reduced Hepatic stellate cells (HSC) proliferation, collagen-α1 expression which correlated with attenuated hepatic fibrosis and improved liver function (Liang et al., 2017). Therefore, it is possible that Bempedoic acid by virtue of its role in AMPK activation may regulate the process of hepatic fibrogenesis by modulating the expression of pro-fibrotic genes including *Col1α1* in our experimental model resulting in attenuated hepatic fibrosis and improved liver function.

Collectively, the data from biochemical, histological and gene expression analysis of long term HFD fed controls are in line with multiple hit theory of NASH development, and ACLY inhibitor Bempedoic acid (ETC-1002) alleviated long term HFD induced NASH through inhibition of body weight gain, improvement in glycemic control, reduction of hepatic triglycerides & total cholesterol, modulation of inflammatory & fibrotic genes and improvement in NAS score. Our findings with the ACLY inhibitor Bempedoic acid (ETC-1002) is in line with the reported literature on liver-specific ACLY abrogation in db/db mice and its effect on inhibition of hepatic de novo lipogenesis, and protection against hepatic steatosis (Wang et al., 2009). In conclusion, our study demonstrates a promising role for Bempedoic acid in the amelioration of metabolic disorders and NASH. Further work is needed to investigate its potential for the treatment of NASH.

4. Materials & methods

4.1. Chemicals

Bempedoic acid (Cat No. 4100302568) was purchased from Aaron Pharmtech Ltd. It was stored at 4 °C and protected from light. Biochemical assay kits (Triglyceride, Cat # TR 210; Total Cholesterol, Cat # CH200; ALT, Cat # AL1205; and AST, Cat # AS1204) were procured from Randox, India. MCP-1 ELISA kit, Cat # DY479 was procured from R&D Systems. Picro sirius red stain kit, Cat # ab150681 was procured from Abcam. Rodent chow diet (Teklad) was purchased from Harlan and HFD (60% Kcal; D12492) from Research diet Inc. USA. cDNA conversion kit (I script cDNA synthesis kit, Cat # 170-8891) and I taq Universal SYBR green Mix (Cat # 172-5120) were procured from Biorad. Primers for *Col1α1, Mcp-1 and Timp-1* were obtained from Eurofins, India.

4.2. Animals

Male C57BL6/N mice were purchased from Vivo Bio Tech Ltd, India. Mice were housed in individually ventilated cages (n = 5/cage) under standard laboratory conditions. The study room was maintained at a temperature of 21- 24 °C and, relative humidity at 40–70%. The animal rooms were maintained under 12h light and dark cycle. Animals were provided with ad libitum access to water and food (except where noted otherwise, e.g. during fasting prior for glucose tolerance tests, fasting prior to terminal sacrifice). Animals were acclimatized for 1 week before initiation of the studies. All the experiments were approved by Institutional Animal Ethics Committee (IAEC) of Jubilant Biosys Ltd, Protocol No: IAEC-JDC-2018-163.

4.3. Study design and Bempedoic acid treatment plan

The overall study design is shown in Fig. 1. 7 weeks old C57BL6/N mice were given either the rodent chow diet (Teklad; Harlan) or HFD (60% Kcal; Research diet, D12492). The animals that were given the rodent chow diet constituted the lean control group and they continued to receive the chow diet for the duration of the study (37 weeks). The animals that were given HFD were randomized after 32 weeks and the animals were grouped to receive either the vehicle or Bempedoic acid via oral gavage and they continued to receive the HFD for the duration of the study (37 weeks). The treatment started after 32 weeks and continued for 5 weeks (till 37 weeks). The lean, non-diabetic mice comprised the control group (n = 10) and received the vehicle (0.5% w/v methyl cellulose and 0.5% v/v Tween 80). The HFD fed mice were randomized based on the body weight & random blood glucose levels and assigned to three groups (n = 10). They received either the vehicle (0.5% w/v methyl cellulose and 0.5% v/v Tween 80) or Bempedoic acid 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd for five weeks (35 days). The dosage of Bempedoic acid was chosen based on the literature information (Pinkosky et al., 2013, 2017; Filippov et al., 2013). Individual animal body weights and food consumption were recorded twice weekly during the study period.

Lean control + Vehicle (G1), and HFD control + Vehicle Control group (G2) mice were administered with vehicle. Dose formulations of HFD + Bempedoic acid, 10 mg kg^{-1}, po, qd (G3), and HFD + Bempedoic acid, 30 mg kg^{-1}, po, qd (G4) mice were administered at a dose volume of 5 mL kg^{-1} body weight. The dose volume for individual animals was calculated based on the most recently recorded body weight during the study period. Throughout the study period, the animals were observed for mortality/morbidity. Cage side observations of animals for visible clinical signs were performed once daily throughout the study period. The body weight and food intake were measured during the course of the treatment period and represented as body weight in grams, % body weight change, daily energy intake and cumulative energy intake.

4.4. OGTT

Glucose tolerance of the vehicle/Bempedoic acid treated animals was assessed in an oral glucose tolerance test (OGTT) on day 31 of treatment period. Animals were fasted for 6h followed by an oral administration of glucose (2 g kg^{-1}). One hour prior to glucose administration, mice were dosed with the vehicle or Bempedoic acid 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd. Blood glucose measurements from tail snips were performed at −60 min (prior to drug administration), 0 min (just before glucose administration), and 15, 30, 60, 120 and 180 min after glucose administration. Animals were re-fed after the last time point of blood glucose and dosing was continued till termination of the study on day 35.

4.5. Study termination

On day 35 of the treatment, animals were fasted for 4h and sacrificed by CO_2 asphyxiation. One hour prior to sacrifice, the mice were given the last dose of vehicle or Bempedoic acid 10 mg kg^{-1}, po, qd or 30 mg kg^{-1}, po, qd. Blood and tissue samples (liver) were collected from each animal. Plasma and tissue samples were stored at −80 °C until analysis.

4.6. Biochemical analysis

Plasma was collected from blood by centrifugation in Na-EDTA containing tubes. Plasma TG, TC, HDL and LDL were determined by colorimetric methods using commercially available Randox assay kits (Randox Lab., Ltd, UK). MCP-1 protein levels in liver tissue lysate was analyzed using commercially available ELISA Kit from R&D Systems as per kit insert. For Whole blood glucose estimation, blood was collected by tail-tip amputation method and estimated using Glucometer (Contour TS, Bayer). Total liver lipids were extracted by the Folch method (Folch et al., 1957). Hepatic triglycerides and total cholesterol were quantified using Randox assay kits.

4.7. Gene expression analysis

Total RNA was extracted from each liver using Krishgen RNA extraction kit (FastPrep-R Mini, Cat No. K305-100A). RNA purity and quantity was measured by nano-drop spectrophotometry, and 1 μg total RNA used to synthesize cDNA using I Script™ cDNA Synthesis Kit (Bio-rad). RT-qPCR assays were run in 384-well plates over 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s after the initial denaturation of 95 °C for 2 min. Threshold cycle (Ct) values were acquired by the accompanying software. Gene expression of the target gene was

normalized in relation to the expression of an endogenous control *36B4* using "2^-dCt" formula. Primer sets for *Col1α1, Timp-1, Mcp-1 and 36B4* were as follows:

Primer Name	Forward	Reverse
Col 1α1	TGGTCTCCAGGGTCCTCCT	AGAACCAGCAGAGCCAGGG
Timp-1	TGAGCCCTGCTCAGCAAAGA	GAGGACCTGATCCGTCCACAA
Mcp-1	ACTGAAGCCAGCTCTCTCTTCCTC	TTCCTTCTTGGGGTCAGCACAGAC
36B4	TTCCAGGCTTTGGGCATCA	ATGTTCAGCATGTTCAGCAGTGTG

4.8. Histopathology

10% neutral buffered formalin fixed liver tissues were paraffin embedded and sliced at 5 μm sections on a rotatory microtome and standard H&E staining was performed. The photomicrographs were taken at the magnification of 10X and 20X. Slides were scored for hepatocellular steatosis, ballooning lobular inflammation severity according to the published methods. Steatosis: Grade 0 - less than 5%; Grade 1 - between 5 and 33%; Grade 2 - between 33 and 66%; and Grade 3 - more than 66%. Cell ballooning was scored as none (0), mild (1- few swelled cells) and severe (2 - many swelled cells) based on its severity. For lobular inflammation, minimal or absence of inflammatory cells accumulation (infiltration) was scored as Grade 0, mild infiltration (Grade 1), moderate to severe infiltration (Grade 2) and severe inflammatory cells accumulation (Grade 3). NAS score were calculated by summation of the scores of hepatocellular steatosis, ballooning and lobular inflammation (Savari et al., 2019). Sirius red staining was performed on liver sections as per manufacturer's instructions for staining of collagen. The photomicrographs were taken at the magnification of 20X. Slides were scored for fibrosis with following scoring criteria. Grade 0 – None; Grade 1 – mild perisinusoidal; Grade 2- moderate, perisinusoidal; Grade 3 - moderate portal/periportal; Grade 4 – bridging fibrosis (Maciejewska et al., 2019).

4.9. Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.04 software. Two-way ANOVA followed by Bonferroni's post-test was used for change in body weight, cumulative energy intake and OGTT. One-way ANOVA followed by Bonferroni's post-test was used to analyze AUC blood glucose in OGTT, 0 min blood glucose, mRNA expression, biochemical analysis and histopathological analysis. The sample size was kept as 9–10 animals in each group. Data is reported as the mean ± SEM throughout, and p value of <0.05 was used as the threshold for statistical significance.

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Author contribution

BRZ, SKV, SV, SD: Conceptualization and Manuscript preparation; BRZ, VSM: Design and execution of pharmacology study; SKV: Pharmacology study oversight; SM: Execution of Histology Work; SV, SD: Supervision.

Institutional review board statement

Animal studies were carried out in compliance with the institutional guidelines of Jubilant Biosys Ltd. Protocol No: IAEC-JDC-2018-163.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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