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ORAL ADMINISTRATION OF A NEW HRI ACTIVATOR AS A NEW STRATEGY TO IMPROVE HIGH-FAT-DIET-INDUCED GLUCOSE INTOLERANCE, HEPATIC STEATOSIS AND HYPERTRIGLYCERIDEMIA THROUGH FGF21

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Running title: An oral HRI activator prevents hepatic steatosis and hypertriglyceridemia

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BACKGROUND AND PURPOSE

Fibroblast growth factor 21 (FGF21) has emerged as a therapeutic strategy for treating type 2 diabetes mellitus due to its antidiabetic effects, and this has led to the development of FGF21 long-acting analogs. These compounds have some limitations, including requiring subcutaneous injection and their prolonged pharmacodynamic effect compared with native FGF21, which might be responsible for their reported side effects.

EXPERIMENTAL APPROACH

We have previously demonstrated that intraperitoneal administration of heme-regulated eukaryotic translation initiation factor 2α (eIF2α) kinase (HRI) activators increases hepatic and circulating levels of FGF21. In this study, we examined the effects of oral administration of a new HRI activator, EPB-53, on high-fat diet (HFD)-induced glucose intolerance, hepatic steatosis, and hypertriglyceridemia, compared with metformin.

KEY RESULTS

Administration of EPB-53 administration for the last two weeks, to mice fed a HFD for 10 weeks, reduced body weight gain, improved glucose intolerance, and prevented hepatic

steatosis and hypertriglyceridemia; whereas metformin only ameliorated glucose intolerance. Moreover, EPB-53, similarly to the reported effects of FGF21, reduced lipogenesis in cultured human hepatocytes and in the liver of mice fed a HFD. Administration of EPB-53 to *Fgf21*-knockout mice had no effects, demonstrating that its efficacy is dependent on this hormone.

CONCLUSIONS AND IMPLICATIONS

Overall, the findings of this study demonstrate that oral administration of HRI activators is a promising strategy for the treatment of type 2 diabetes mellitus and non-alcoholic fatty liver disease by increasing FGF21.

***Abbreviations*:** Acox, acyl-CoA oxidase; AMPK, AMP-activated protein kinase; ATF4, activating transcription factor 4; BTCtFPU, 1-(benzo[*d*][1,2,3]thiadiazol-6-yl)-3-(4-chloro-3-(trifluoromethyl)phenyl)urea; BTdCPU, 1-(benzo[*d*][1,2,3]thiadiazol-6-yl)-3-(3,4-dichlorophenyl)urea; Chop, C/EBP homologous protein; Cpt-1α, carnitine palmitoyl-transferase 1 α; eIF2α, eukaryotic translation initiation factor 2α; FGF21, fibroblast growth factor 21; HFD, high-fat diet; Hmgcs2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; HRI, heme-regulated eIF2α kinase; Hsd3b5, 3-β-hydroxysteroid dehydrogenase type 5; Mcad, medium-chain acyl-CoA dehydrogenase; Mup1, major urinary protein 1; NAFLD, non-alcoholic fatty liver disease; PGC-1α, PPARγ co-activator 1α; PPAR, peroxisome proliferator-activated receptor.

Keywords: HRI activator; FGF21 inducer; glucose intolerance; fatty liver.

Bullet point summary

What is already known: Targeting FGF21 is an emerging therapeutic strategy for treating type 2 diabetes mellitus.

What this study adds: Oral administration of HRI activators improved glucose intolerance and prevented hepatic steatosis by increasing FGF21.

Clinical significance: The use of an oral drug to induce endogenous FGF21 levels might have advantages over FGF21 analogs

Introduction

Fibroblast growth factor 21 (FGF21) is a secreted protein belonging to the FGF19 subfamily (Goetz *et al.*, 2007). It elicits actions through binding to a plasma membrane receptor complex consisting of the FGF receptor 1c isoform (FGFR1c) and β-klotho co-receptor (Goetz *et al.*, 2007; Ogawa *et al.*, 2007). Circulating FGF21 is liver-derived (Markan *et al.*, 2014), and serum FGF21 levels correlate with hepatic expression (Hale *et al*., 2012). FGF21 was originally identified as a fasting-induced hormone that promotes increased glucose uptake in adipocytes (Kharitonenkov *et al.*, 2005). Later studies demonstrated that pharmacological administration of FGF21 to animal models of obesity and/or diabetes improved glucose tolerance and insulin sensitivity, reduced hepatic and serum triglyceride levels, and caused weight loss (Kharitonenkov *et al.*, 2005; Ding *et al.*, 2012; Coskun *et al.*, 2008; Inagaki *et al.*, 2007; Xu *et al.*, 2009). Despite these pharmacological effects, serum FGF21 levels are paradoxically increased in obesity, both in rodents (Hale *et al.*, 2012; Zhang *et al.*, 2008; Muise *et al.*, 2008; Fisher *et al.*, 2010; Satapati *et al.*, 2008) and humans (Chavez *et al.*, 2009; Chen *et al.*, 2008; Mraz *et al.*, 2009), and especially in type 2 diabetes mellitus

(T2DM) (Badman *et al.*, 2009; Zhang *et al.*, 2008). The presence of high endogenous FGF21 levels in obesity has led to this condition being considered as an FGF21-resistant state (Fisher *et al.*, 2010). However, this assumption is controversial, since, as mentioned above, exogenous pharmacological administration of FGF21 is effective in genetic and diet-induced animal models of obesity (Hale *et al.*, 2012). The increase in circulating FGF21 levels in obesity and other metabolic alterations probably reflects deposition of fat in liver (Hale *et al.*, 2012; Maratos-Flier, 2017) and, consistent with this, serum FGF21 levels correlate with non-alcoholic fatty liver disease (NAFLD) in humans (Dushay *et al.*, 2010; Yilmaz *et al.*, 2010). This assumption is also supported by the fact that administration of exogenous FGF21 reduces its expression in liver as plasma and hepatic triglyceride levels decrease, or as adiposity and insulin resistance improve in animal models of obesity and insulin resistance (Hale *et al.*, 2012).

The beneficial effects of pharmacological administration of FGF21 have led to the establishment of FGF21 as a therapeutic target for the treatment of metabolic diseases (Gimeno and Moller, 2014; Kharitonenkov and DiMarchi, 2015). This has encouraged the development of FGF21 analogs to treat human metabolic disorders such as obesity, dyslipidemia, and T2DM (Reitman, 2013; Talukdar *et al.*, 2016; Kim *et al.*, 2017). However, because of their peptidic origin, these analogs require parenteral administration and, therefore, there is a need for more convenient orally available drugs targeting FGF21 to treat metabolic disorders. In addition, native FGF21 has a short half-life and, to overcome this issue, long-acting FGF21 analogs, with prolonged pharmacodynamics compared to native FGF21, have been designed (Huang *et al.*, 2007, Weng *et al.*, 2015). However, prolonged activation of the

FGF21 receptor by these long-acting FGF21 analogs might be responsible for their side effects, including bone loss (Talukdar *et al.*, 2016), and increases in blood pressure and heart rate (Kim *et al.*, 2017).

Recently, we reported that intraperitoneal administration of a heme-regulated eukaryotic translation initiation factor 2α (eIF2α) kinase (HRI) activator increases hepatic *Fgf21* expression and reduces lipid-induced hepatic steatosis and glucose intolerance in mice fed a high-fat diet (HFD) (Zarei *et al.*, 2016). Those effects were dependent on FGF21, since they were abolished in *Fgf21*-null mice (Zarei *et al.*, 2016). The activation of HRI resulted in the phosphorylation of eIF2α and the subsequent increase in the activity of activating transcription factor (ATF) 4, which is essential for *Fgf21*-induced expression (De Sousa-Coelho *et al.*, 2012). This converts HRI activators, which are small molecules, into potential candidates for an orally administered treatment of T2DM. In this study, we compare the oral effects of a new HRI activator, EPB-53 (Figure 1A), and metformin, on glucose tolerance, hepatic steatosis, and hypertriglyceridemia in mice fed a HFD. Our findings show that EPB-53 treatment reduces body weight gain, glucose intolerance, hepatic steatosis, and hypertriglyceridemia and that these effects are dependent on FGF21.

Methods

Reagents

N,N'-diarylureas, 1-(benzo[d][1,2,3]thiadiazol-6-yl)-3-(3,4-dichlorophenyl)urea (BTdCPU) and 1-(benzo[d][1,2,3]thiadiazol-6-yl)-3-(4-chloro-3-(trifluoromethyl)phenyl)urea (BTCtFPU), were synthesized as previously described (Zarei *et al.*, 2016). Synthesis of EPB-

53 is included in Supplementary Materials and Methods. Triglyceride (Sigma-Aldrich, Madrid, Spain), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Spinreact, Girona, Spain) and FGF21 (Millipore, Bedford, MA) levels were measured using a commercial kit.

Mice

Male C57BL/6 mice (10-12 weeks old) (Harlan Ibérica S.A., Barcelona, Spain) were housed and maintained under a constant temperature (22 ± 2°C) and humidity (55%). The mice had free access to water and food and were subjected to 12 h light-dark cycles. After 1 week of acclimatization, mice were randomly distributed in two experimental groups (n=6 each) and either received one daily oral gavage of vehicle (2% w/v, (2-hydroxypropyl)-β-cyclodextrin) or one daily oral dose of 300 mg · kg^{-1} · day^{-1} of the HRI activator EPB-53 dissolved in the vehicle (volume administered 1 ml kg^{-1}) for 4 days. This high dose was selected because of the presence of two parameters that may lower efficacy, the poor solubility of the compound (ClogP=5.5) and its high plasma protein binding (99.85%). In a second study, male C57BL/6 mice (10-12 weeks old) were randomly distributed into four experimental groups (n=6 each) and fed either standard chow (one group) or HFD (45% fat mainly form hydrogenated coconut oil, Product D08061110, Research Diets Inc.) for ten weeks. Mice fed standard chow and one of the groups of mice fed the HFD received one daily oral gavage of vehicle (2% w/v, (2-hydroxypropyl)-β-cyclodextrin)), meanwhile the remaining two groups fed the HFD received one daily oral dose of either the HRI activator EPB-53 (300 mg · kg^{-1} · day^{-1}) or metformin (150 mg · kg^{-1} · day^{-1}) (YS Kim et al., 2017), for the last 2 weeks. In a third study, male *Fgf21* knockout (*Fgf21*$^{-/-}$) mice (8-10 weeks old) (B6N;129S5-Fgf21tm1Lex/Mmcd,

obtained from the Mutant Mouse Regional Resource Centre; MMRRC) and their wild-type littermates (*Fgf21*$^{+/+}$) were randomly distributed into three experimental groups (standard chow, HFD, and HFD+EPB-53; n=5 each) and fed the different diets for three weeks. The standard chow and HFD groups received one daily oral gavage of vehicle (2% w/v, (2-hydroxypropyl)-β-cyclodextrin)), whereas the HFD+EPB-53 group received one daily oral administration of the HRI activator EPB-53 (300 mg · kg^{-1} · day^{-1}) for the last week. These last conditions were reproduced for the dose-response study, where male C57BL/6 mice (10-12 weeks old) fed a HFD for three weeks were treated during the last week with EPB-53 (100, 200 and 300 mg · kg^{-1} · day^{-1}).

For the glucose tolerance test (GTT), animals received 2 g · kg^{-1} body weight of glucose by intraperitoneal injection, and blood was collected from the tail vein after 0, 15, 30, 60 and 120 min.

The research complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the University of Barcelona Bioethics Committee, as stated in Law 5/21 July 1995 passed by the Generalitat de Catalunya. The animals were treated humanely, and all efforts were made to minimize the animals' suffering and the animal numbers. Animal studies are reported in compliance with the ARRIVE guidelines (McGrath and Lilley, 2015).

Pharmacokinetics

CD-1 male mice were treated with EPB-53 via the oral route at a single dose of 20 mg · kg^{-1}. Plasma samples were obtained from cava vein at 0, 0.5, 1, 3, 5, and 24 hours post-

administration (3 mice/point). Analytical measurements were performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Pharmacokinetic parameters were calculated by means of non-compartmental analysis, Phoenix 7.0 (WinNonlin 6.3).

Cell culture

Human Huh-7 cells [RRID:CVCL_0336] (a kindly gift from Dr Mayka Sánchez, of the Josep Carreras Leukaemia Research Institute) were cultured in DMEM supplemented with 10% serum, at 37°C/5% CO_2. Hepatocytes were exposed to a concentration of 10 µM of each diarylurea, as previously reported (Chen *et al.*, 2011).

RNA preparation and quantitative RT-PCR

The relative levels of specific mRNAs were assessed by real-time RT-PCR, as previously described (Zarei *et al.*, 2016). The results for the expression of specific mRNAs were normalized to the expression of a control gene to avoid unwanted sources of variation. The primer sequences used are displayed in Supplementary Table 1.

Immunoblotting

Isolation of total protein extracts was performed as described elsewhere (M Zarei et al., 2016). Proteins (30 µg) were separated by SDS-PAGE on 10% acrylamide separation gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Western blot analysis was performed using antibodies against ATF4 (sc-390063), GAPDH (sc-32233), HRI (sc-365239) (RRID:AB_10843794), VLDLR (sc-18824) (Santa Cruz Inc., Heidelberg, Germany), VLDLR (AF2258) (R&D Systems, Minneapolis, MN), AMPK (2532), p-AMPK

Thr172 (2535), eIF2α (9722), phospho-eIF2α (Ser51) (9721) (Cell Signaling Technology Inc., Danvers, MA), β-actin (A5441), and tubulin (T9026) (Sigma-Aldrich). Detection was performed using the Western Lightning® Plus-ECL chemiluminescence kit (PerkinElmer, Waltham, MA). The equal loading of proteins was assessed by Ponceau S staining. The size of detected proteins was estimated using protein molecular mass standards (Bio-Rad, Barcelona, Spain). The results for protein quantification were normalized to the levels of a control protein to avoid unwanted sources of variation.

Hematoxylin-eosin and Oil Red O staining

We performed hematoxylin-eosin and Oil Red O (ORO) staining as previously reported (Zarei *et al.*, 2016).

Statistical Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015; Curtis *et al.*, 2018). For *in vivo* experiments animals were randomly distributed between groups and experimenters blinded for liver analysis purposes. Results are expressed as means ± S.D. Significant differences were established by ANOVA, using the GraphPad Prism program (V6.01) (RRID:SCR_002798) (GraphPad Software Inc., San Diego, CA). When significant variations were found by one-way ANOVA, the Tukey-Kramer multiple comparison post-test was performed only if F achieved $P<0.05$ and there was no significant variance inhomogeneity. Differences were considered significant at $p<0.05$.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

Results

EPB-53 increases FGF21 expression in human Huh-7 hepatocytes, and in liver and serum of mice

It has previously been reported that N,N'-diarylureas, including BTCtFPU and BTdCPU, are activators of HRI and induce eIF2α phosphorylation (Chen *et al.*, 2013). We demonstrated that intraperitoneal administration of both BTCtFPU and BTdCPU increased mouse hepatic and serum FGF21 levels (Zarei *et al.*, 2016). Then, we synthesized a series of compounds featuring a diarylurea scaffold and screened these new compounds for their capacity to increase *FGF21* expression in human Huh-7 hepatocytes. Of all these new compounds, we selected EPB-53 as the best candidate for *in vivo* studies, since it showed a significant increase in *FGF21* mRNA levels compared to the previous HRI activators BTCtFPU and BTdCPU (Figure 1B), and it displayed a good pharmacokinetic profile in mice (Supplementary Table 2). Notably, the half-life of the elimination phase of EPB-53 by oral administration was 5.4 h in mice, thus allowing for a substantial period of activity after administration.

As a first step to check the effects of EPB-53 on mice, we examined whether acute oral administration of EPB-53 for 4 days activated the HRI-eIF2α-ATF4 pathway and increased

hepatic expression and serum levels of FGF21. HRI is activated by autophosphorylation (Lu *et al.*, 2001) and EPB-53 increased hepatic phospho-levels of HRI, compared to those of mice treated with the vehicle alone (Figure 2A). Consistent with the eIF2α kinase role of HRI, EPB-53 significantly enhanced phospho-eIF2α and ATF4 levels in liver (Figure 2A). Likewise, EPB-53 increased the hepatic expression and serum levels of FGF21 (Figure 2B and C).

Next, we analyzed the expression of two additional ATF4-target genes: very-low-density lipoprotein receptor (VLDLR) (Jo *et al.*, 2013) and CCAAT/enhancer-binding protein homologous protein (CHOP). VLDLR binds apolipoprotein E (apoE) triglyceride-rich lipoproteins such as chylomicrons, VLDL, and intermediate-density lipoproteins, leading to lipid entry into the cell. Notably, hepatic VLDLR upregulation plays an essential role in the triglyceride-lowering effect of fenofibrate (Gao *et al.*, 2014). Meanwhile CHOP induces cell cycle arrest and apoptosis (Zinszner *et al.*, 1998). EPB-53 treatment significantly increased the expression of *Vldlr*, but it did not affect *Chop* mRNA levels (Figure 2D).

It has been suggested that acute administration of FGF21 upregulates the expression of the transcriptional co-activator PGC-1α (Fisher *et al.*, 2011), which in turn controls the activity of the master regulator of hepatic fatty acid oxidation, PPARα (Kersten and Stienstra, 2017). Consistent with the increase in FGF21 levels following EPB-53 treatment, *Pgc-1α* expression tended towards upregulation, but the differences did not reach statistical significance. Although expression of *Pparα* was not affected, the increase in the expression of its target genes, *Cpt-1α* and *Mcad*, suggested an increase in its activity (Figure 2E). Moreover, it has been reported that FGF21 controls hepatic triglyceride content in liver by reducing *de novo* lipogenesis (Xu *et al.*, 2009; Zhang *et al.*, 2011). Consistent with the increase in FGF21

levels, the expression of the lipogenic gene fatty acid synthase (*Fas)* was downregulated (Figure 2E).

EPB-53 administration improves glucose intolerance, hepatic steatosis and hypertriglyceridemia in mice fed a HFD

Next, we examined the effects of EPB-53 on mice fed a HFD, a model of diet-induced obesity and T2DM. First, mice were fed the HFD for 10 weeks and the last 2 weeks they were treated with either the vehicle alone, EPB-53 or metformin. Mice treated with EPB-53 did not show any sign of discomfort or toxicity. We compared the effects of EPB-53 with metformin, the first-line drug treatment for T2DM. Mice fed the HFD for 10 weeks showed an increase of 17.2±1.6 g in body weight, compared to the 7.6±0.8 g observed in mice fed the standard diet (Figure 3A). EPB-53 treatment significantly reduced body weight gain (10.9±1.3 g); whereas the reduction observed with metformin was of lower intensity (14.3±1.8 g) (Figure 3A). Drug treatment did not affect food intake (Supplementary Figure 1A). HFD feeding also increased basal glucose levels and this was prevented by EPB-53 and metformin (Figure 3B). In addition, glucose intolerance caused by the HFD was prevented by EPB-53 and by metformin (Figure 3C).

Interestingly, EPB-53 administration abolished the hepatic steatosis caused by HFD feeding, as demonstrated by ORO and hematoxylin-eosin staining, and quantification of hepatic triglyceride levels (Figures 4A and B). In contrast, the trend towards a reduction in the accumulation of hepatic triglycerides caused by metformin did not reach statistical significance. Likewise, the 106% increase in serum triglyceride levels caused by the HFD was

nearly completely abolished by treatment with EPB-53; whereas metformin merely tended towards a slight reduction which was not significant ($p<0.05$ *vs.* mice fed the standard diet). Consistent with the reduction in hepatic triglyceride levels caused by EPB-53, this compound also prevented the increase in serum ALT and AST caused by the HFD, whereas metformin only significantly reduced ALT (Figures 4D and E).

When we examined the HRI-eIF2α pathway, we observed that EPB-53 increased the levels of phospho-HRI and phospho-eIF2α, indicating that this compound activated this pathway, whereas metformin did not (Figure 5A). As expected, feeding mice a HFD increased the expression and serum levels of FGF21, and this was exacerbated in mice fed the HFD and treated with metformin (Figures 5B and C), which is consistent with the reported effects of metformin on FGF21 in liver and plasma (Kim *et al.*, 2013). Surprisingly, mice fed the HFD and treated with EPB-53 showed *Fgf21* mRNA and serum levels similar to those present in the control group (Figures 5B and C). However, this is consistent with the lack of hepatic steatosis in both groups, since FGF21 levels reflect deposition of fat in liver (Hale *et al.*, 2012; Maratos-Flier, 2017). Moreover, when we assessed the expression of two FGF21 target genes negatively regulated by this hormone, 3-β-hydroxysteroid dehydrogenase type 5 (*Hsd3b5*), and major urinary protein 1 (*Mup1*) (Inagaki *et al.*, 2008), we observed that the expression of these genes was reduced in the liver of mice fed the HFD and treated with EPB-53 (Figures 5D and E), suggesting a previous increase in the activity of FGF21. In contrast, the increase in FGF21 in mice fed the HFD or the higher increase in mice fed the HFD and treated with metformin did not reduce the expression of these genes, suggesting the presence of FGF21 resistance.

Metformin acts via AMP-activated protein kinase (AMPK)-dependent mechanisms, although additional mechanisms have been reported (Rena *et al.*, 2017). Consistent with this, treatment with this drug increased hepatic phospho-AMPK levels (Figure 5F). EPB-53 did not significantly affect AMPK phosphorylation, but it increased the protein levels of the ATF4 target gene VLDLR (Figure 5F). Neither HFD feeding nor drug treatment affected the expression of the ATF4 target genes *Trb3* or *β-klotho* (Supplementary Figure 1B and C). In contrast, EBP-53 significantly reduced the mRNA levels of *Chop* and both the HFD and EPB-53 reduced the expression of *Fgfr1c* (Supplementary Figure 1D and E, respectively). When we examined the expression of genes involved in fatty acid oxidation, the mRNA levels of *Pparα* and *Cpt-1α* were upregulated by the HFD, whereas the expression of the latter gene was not further increased by either EPB-53 or metformin treatment (Figure 6A). These findings suggest that an increase in fatty acid oxidation is not involved in the effects of EPB-53 in hepatic steatosis. When we examined the expression of lipogenic genes, we observed that mice fed the HFD and those fed the HFD and also treated with metformin showed an increase in the mRNA levels of the lipogenic transcription factor *Srebp1c* (Figure 6B); whereas this was not observed in mice treated with EPB-53. Interestingly, EPB-53 treatment reduced the expression of the lipogenic genes stearoyl-CoA desaturase 1 (*Scd1),* *Fas* and glycerol phosphate acyltransferase (*Gpat)* (Figure 6B). EPB-53 also reduced the expression of the transcription factor carbohydrate-responsive element-binding protein (ChREBP): a major mediator of glucose action on lipogenic gene expression and a key determinant of lipid synthesis *in vivo* (Postic *et al.*, 2007). Consistent with the increase in hepatic triglycerides, levels of the protein FAS were increased in the liver of mice fed the HFD, but this increase was attenuated by EPB-53 (Figure 6C). Moreover, the protein levels of PPARγ and its target

gene *CD36*, both involved in lipid accumulation, were increased in the livers of mice fed the HFD, and this increase was prevented by EPB-53, but not by metformin. The effects of EPB-53 seemed not to be mediated by the reduction in body weight, since when we treated cultured human Huh-7 hepatocytes with EPB-53, a strong reduction in *FAS* and *CD36* expression was observed (Figure 6D), which is consistent with the strong increase in FGF21 observed in these cells following exposure to EPB-53 (Figure 1B). Exposing hepatocytes to EPB-53 also increased expression of *VLDLR* (Figure 6D).

Effects of EPB-53 on glucose intolerance, hepatic steatosis and hypertriglyceridemia are dependent on FGF21.

Next, to examine whether EPB-53 displayed dose-response behavior, we fed mice with a HFD for three weeks and the last week mice were treated with three different doses of EPB-53 (100, 200 or 300 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}). EPB-53 showed a dose-response trend in the parameters assessed (glucose intolerance, liver triglyceride content, hepatic *Fgf21* and *Fas* expression) (Figure 7A-D).

Finally, we examined whether the effects of EPB-53 were dependent on FGF21 by taking advantage of the use of *Fgf21*-knockout mice. In this experiment both wild-type and *Fgf21*-knockout mice were fed a HFD for 3 weeks, the last week of which they were treated with either the vehicle alone or EPB-53. This shorter period of treatment was selected to examine whether EPB-53 increased the expression and serum levels of FGF21 in mice fed a HFD. In fact, the HFD increased the mRNA levels of FGF21 in the liver of wild-type mice and also the serum levels of this hormone (Figure 8A), but these changes were even higher in mice fed the HFD and treated with EPB-53. When we examined glucose intolerance (Figure 8B) and

hepatic steatosis by quantification of hepatic triglyceride levels and ORO and hematoxylin-eosin staining (Figures 8C and D), we observed that EPB-53 ameliorated these conditions in wild-type mice fed a HFD, but not in *Fgf21*-knockout mice. Similarly, the reduction observed in serum triglycerides in wild-type mice fed the HFD and treated with EPB-53 compared with mice fed the HFD and treated with the vehicle alone, disappeared in *Fgf21*-knockout mice (Figure 8E). Finally, EPB-53 attenuated the increase in serum levels of ALT and AST (Figure 8F and G) caused by the HFD in wild-type mice; but this effect was absent in mice lacking FGF21.

Discussion

FGF21 is a potential new target for obesity, T2DM and associated co-morbidities (Gimeno and Moller, 2014). This has raised interest in the potential of FGF21 to treat metabolic diseases. However, the use of wild-type native FGF21 is challenging as it has several limitations. One of these limitations is its short half-life (Kharitonenkov *et al.*, 2007), which has led to the development of long-acting FGF21 analogs with prolonged pharmacodynamic effects compared to native FGF21 (Huang *et al.*, 2013; Hecht *et al.*, 2013). However, this increase in the potency of long-acting FGF21 analogs may exacerbate some of the unwanted effects of FGF21, such as the reported increase in bone loss caused by this hormone (Wei *et al.*, 2012). In fact, concerns raised following treatment with potent long-acting FGF21 analogs in humans include changes in multiple markers of bone turnover (Talukdar *et al.*, 2016) and an increase in both blood pressure and heart rate (Kim *et al.*, 2017). Another limitation to the use of FGF21 in humans is the need for subcutaneous administration, because, due to its simplicity and convenience, oral administration improves patient

compliance. Orally bioavailable drugs that increase the levels of native FGF21 might overcome all these limitations. We have previously reported that intraperitoneal administration of HRI activators increases hepatic and plasma levels of FGF21 through activation of the eIF2α-ATF4 pathway (Zarei *et al.*, 2016). Based on those findings, we have developed new orally bioavailable HRI activators with enhanced potency, to increase FGF21 expression in human hepatocytes; and we selected EPB-53 for an *in vivo* proof of concept. Administration of EPB-53 for 4 days to normal mice increased the hepatic expression and circulating levels of FGF21, and slightly upregulated the expression of genes involved in fatty acid oxidation; whereas the expression of the lipogenic gene *Fas* was markedly downregulated. In mice fed a HFD for 10 weeks, administration of EPB-53 for the last 2 weeks attenuated body weight gain caused by the HFD. This effect is consistent with the well-known reported effect of FGF21 administration on body weight (Xu *et al.*, 2009).

Treatment with EPB-53 also reduced the serum levels of these lipids, whereas metformin did not. The reduction in serum triglycerides caused by EPB-53 might be dependent, at least partially, on the increase in the levels of the ATF4-target gene VLDLR (Jo *et al.*, 2013). In fact, the upregulation of hepatic VLDLR via PPARα is required for the triglyceride-lowering effect of fenofibrate (Gao *et al.*, 2014). Thus, we can envisage that the upregulation of hepatic VLDLR by EPB-53 increases the delivery of triglycerides transported by VLDL to the liver, reducing the availability of these lipids to be delivered to peripheral tissues, such as white adipose tissue. This action may contribute to the reduction in body weight. Notably, hepatic VLDLR upregulation following EPB-53 treatment does not result in hepatic steatosis,

suggesting that the uptake of triglycerides from plasma cannot compensate for the reduction in lipogenesis.

Both EPB-53 and metformin also reduced glucose intolerance in mice fed a HFD. However, whereas metformin did not prevented either hepatic steatosis or hypertriglyceridemia, EPB-53 prevented both alterations completely. The reduction in hepatic steatosis in these mice seems to be the result of inhibition of hepatic lipogenesis, which is consistent with the effects observed following FGF21 administration (Wei *et al.*, 2012). This effect of EPB-53 was also observed *in vitro* in human hepatocytes. This finding rules out the possibility that the reduction in lipogenesis observed *in vivo* is secondary to body weight reduction. However, when we examined the levels of hepatic *Fgf21* expression and serum levels in EPB-53-treated mice, no changes were observed compared to control mice, although the phosphorylated levels of HRI and eIF2α were increased. The lack of an increase in FGF21 in mice treated with EPB-53 for 2 weeks might be explained by the following mechanism. As mentioned above, administration of FGF21 reduces its expression in liver, as plasma and hepatic triglyceride levels decrease (Hanle *et al.*, 2012). This suggests that a similar mechanism might also operate with EPB-53. Since EPB-53 reduced hepatic triglyceride accumulation to values similar to those present in control mice, once the normal hepatic lipid content is achieved, the effect of EPB-53 on FGF21 upregulation would be attenuated. In fact, a similar effect has been reported with inhibitors of fibroblast activation protein (FAP) (Sánchez-Garrido *et al.*, 2016). This enzyme cleaves FGF21 and its inhibition increases FGF21 levels, thereby lowering body weight and improving glucose tolerance in mice fed a HFD; but these effects are much less intense in lean mice (Sánchez-Garrido *et al.*, 2016). Moreover, the reduction in

the hepatic expression and serum levels of FGF21 might be explained by the presence of negative feedback by which enhanced levels of FGF21 inhibit the eIF2α-ATF4 pathway (Jiang *et al.*, 2014). This negative feedback mechanism would control FGF21 levels thereby avoiding excessive production of this hormone following EPB-53 administration when plasma and hepatic triglyceride levels reach normal values. Notably, this feedback mechanism might avoid the development of side effects (Talukdar *et al.*, 2016; Kim *et al.*, 2017) reported with long-acting FGF21 analogs due to overactivation of the FGF21 receptor. Surprisingly, and in contrast to EPB-53, metformin treatment was accompanied by increased hepatic and circulating levels of FGF21; although this increase resulted neither in an amelioration of hepatic steatosis nor affected the expression of genes negatively regulated by FGF21, such as *Hsd3b5* and *Mup1*, which were downregulated by EPB-53. The increase in FGF21 levels following metformin treatment has been demonstrated to be dependent on the inhibition of mitochondrial complex I activity and the subsequent activation of the PKR-like endoplasmic reticulum (ER) kinase (PERK)-eIF2α-ATF4 pathway (Kim *et al.*, 2013). We currently have no explanation for the lack of effect of the increase in FGF21 levels caused by metformin compared to EPB-53, although changes in the levels of total and intact serum FGF21 caused by differences in the activity of FAP might be implicated (Sánchez-Garrido *et al.*, 2016).

Confirmation of the dependence of EPB-53 effects on FGF21 was obtained using *Fgf21*-knockout mice. In mice deficient in FGF21, the effects of EPB-53 were abolished. Oral administration of EPB-53 for 1 week upregulated hepatic expression and serum levels of FGF21, supporting the notion that in shorter treatments EBP-53 increases the levels of FGF21, whereas in longer treatments (2 weeks), upregulation of FGF21 is attenuated.

Overall, the findings of this study demonstrate that oral administration of HRI activators is a potential strategy for the treatment of T2DM and NAFLD since it increases FGF21. It remains to be studied via long-term treatment whether the use of HRI activators to increase FGF21 levels avoids the side effects reported with long-acting FGF21 analogs. In the clinical setting, the use of an oral drug to induce endogenous FGF21 levels might have advantages over FGF21 analogs for the treatment of insulin resistance, type 2 diabetes mellitus and NAFLD.

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Author's contributions: MZ, EB, XP, TQL, JPD, and MVC performed the experiments; EP, and SV synthesized the compounds; MVC and FV analyzed the data and revised the results; MZ and MVC designed the experiments and revised the results; MVC was primarily responsible for writing the manuscript. All authors contributed to manuscript editing and approved the final version.

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and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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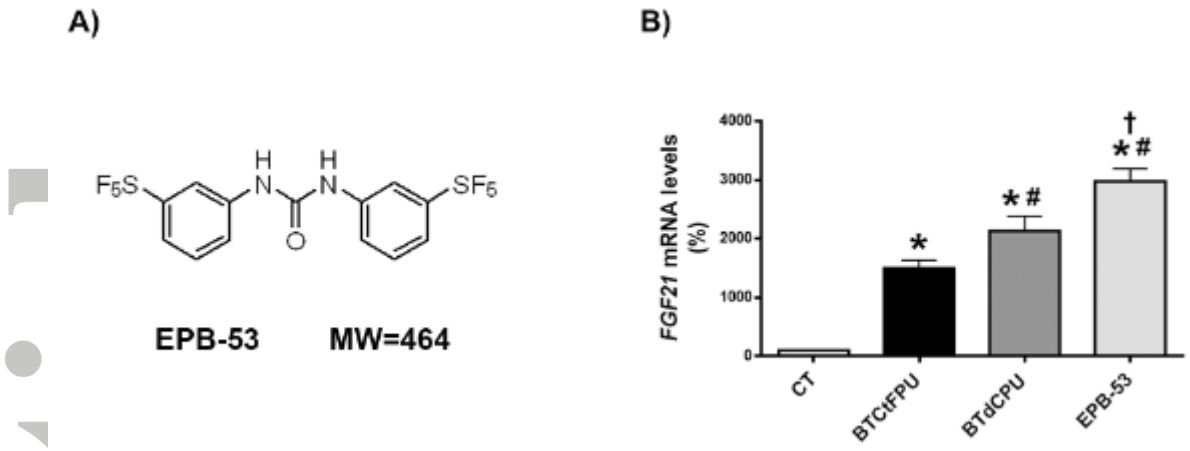

Fig. 1. **EPB-53 increases the expression of FGF21 in human Huh-7 hepatocytes.** A, molecular structure of EPB-53. B, *FGF21* mRNA abundance in human Huh-7 hepatocytes exposed to 10 µM of BTCtFPU, CTdCPU, and EPB-53 for 24 h. mRNA levels are presented as the mean ± S.D. (n=6 per group). *$p<0.05$ vs. control (CT). $^{\#}p<0.05$ *vs.* BTCtFPU-treated cells. $^{\dagger}p<0.05$ *vs.* c BTdCPU-treated cells.

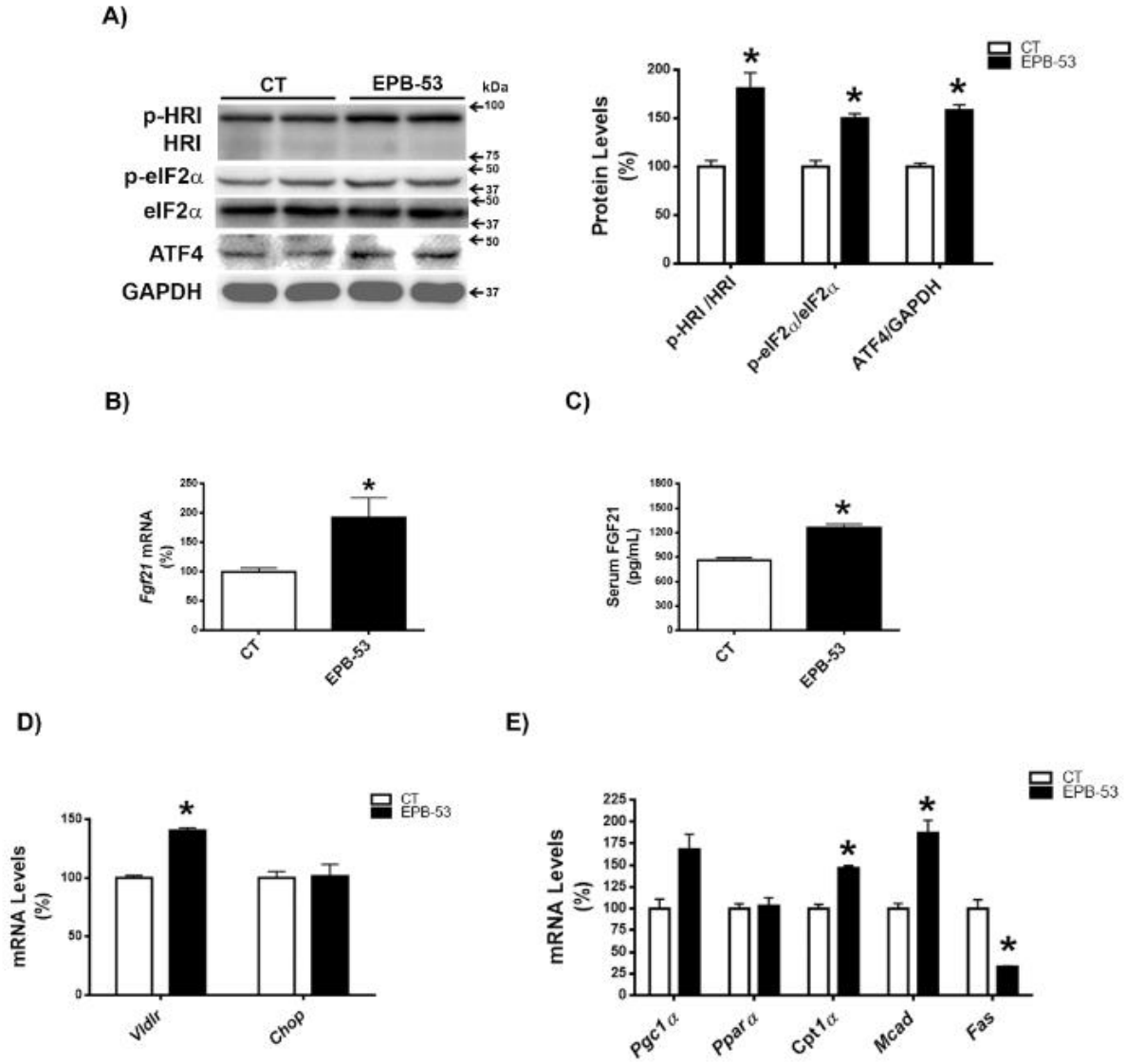

Fig. 2. Oral administration of the HRI activator EPB-53 for 4 days increases hepatic expression and serum FGF21 levels in mice. Mice received one daily oral gavage of vehicle (2% w/v cyclodextrin) or one daily oral dose of the HRI activator EPB-53 for 4 days. A, liver cell lysate extracts were assayed via Western blot analysis with antibodies against total and phospho-HRI, total and phospho-eIF2α, and ATF4. B, *Fgf21* mRNA abundance in the liver. C, serum FGF21 levels. D, *Vldlr* and *Chop* mRNA abundance in the liver. E, *Pgc-1α*, *Pparα*, *Cpt-1α*, *Mcad*, and *Fas* mRNA abundance in the liver. Data are presented as the mean ± S.D. (n=6 per group) relative to control mice. *p<0.05 *vs.* control mice (CT).

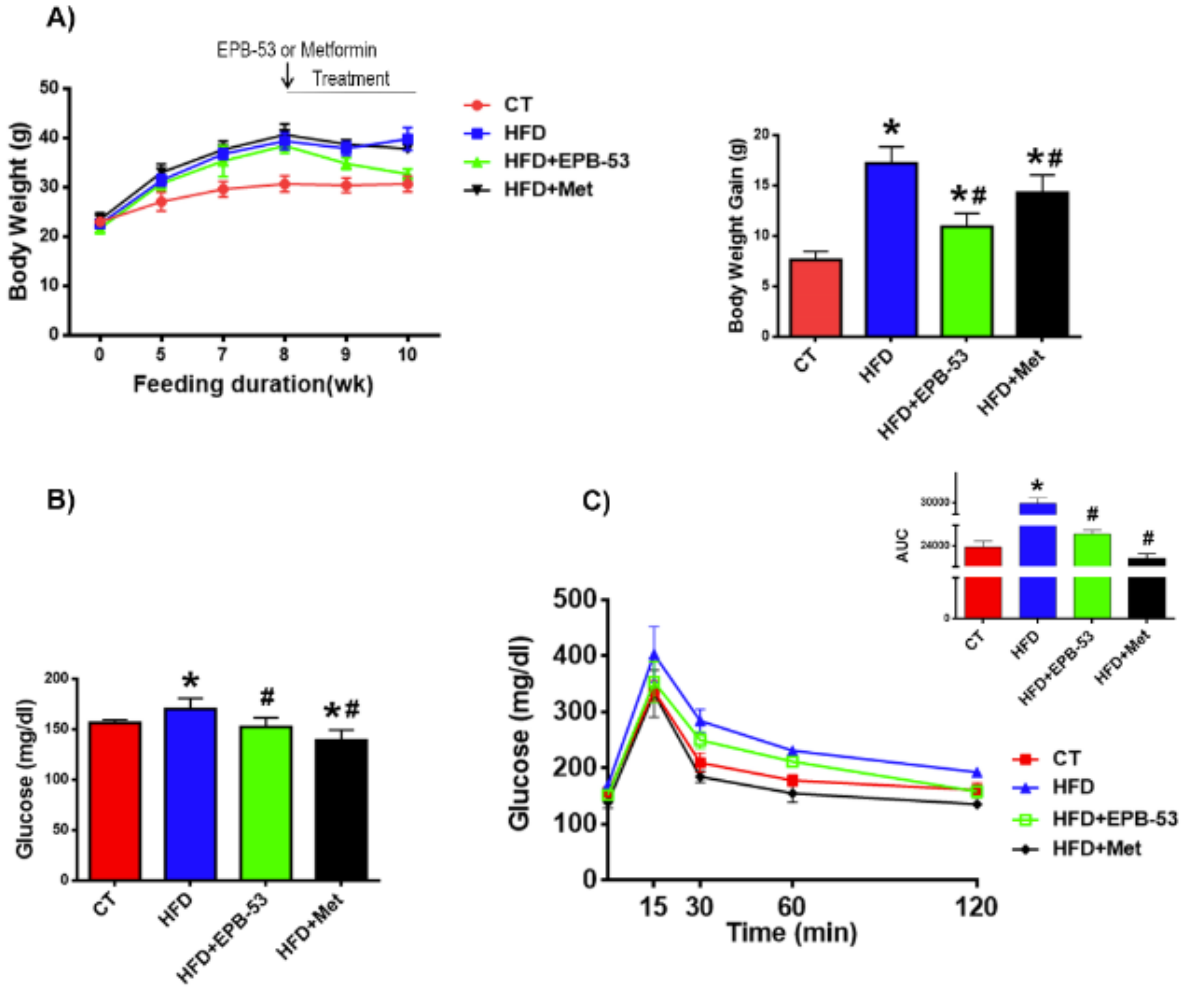

Fig. 3. Oral administration of EPB-53 reduces body weight gain, and improves glucose intolerance in mice fed a HFD. Mice were fed standard chow, a HFD for ten weeks or a HFD for 10 weeks plus EPB-53 or metformin during the last two weeks. A, Body weight and body weight gain. B, basal glucose levels. C, glucose tolerance test and area under the curve (AUC). Data are presented as the mean ± S.D. (n=6 per group). *p<0.05 *vs.* control (CT) mice treated with the vehicle alone. #p<0.05 *vs.* mice fed a HFD and treated with the vehicle alone.

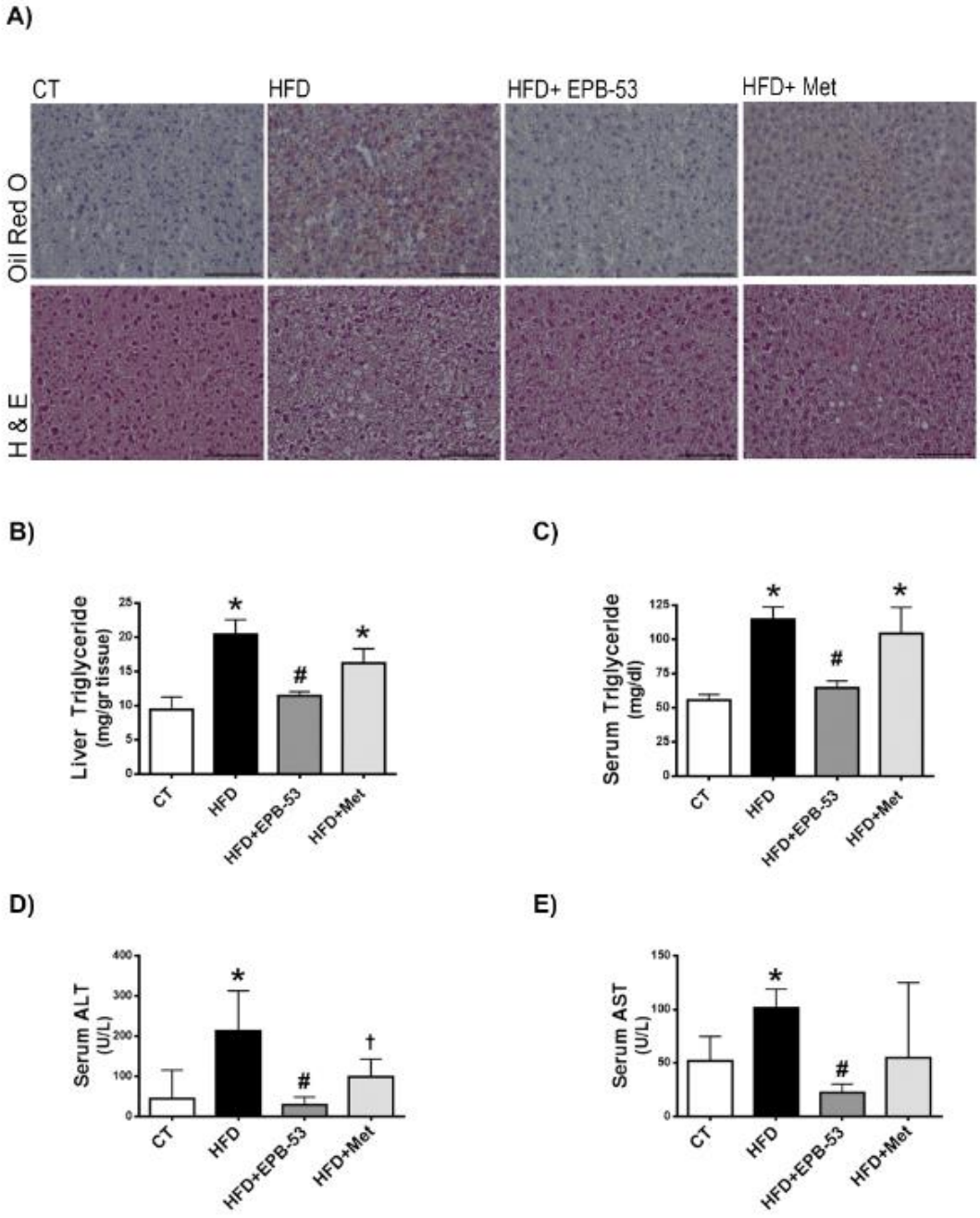

Fig. 4. **Oral administration of EPB-53 prevents fatty liver in mice fed a HFD.** Mice were fed standard chow, a HFD for 10 weeks or a HFD for 10 weeks plus EPB-53 or metformin during the last 2 weeks (n=6 per group). A, H&E and Oil Red O staining of livers. Scale bar: 100 µm. B, liver triglyceride levels. C, serum triglyceride levels. D, serum ALT levels. E, serum AST levels. *$p<0.05$ *vs.* control (CT) mice treated with the vehicle alone. $^{\#}p<0.05$ *vs.* mice fed a HFD and treated with the vehicle alone. $^{\dagger}p<0.05$ *vs.* mice fed a HFD and treated with EPB-53.

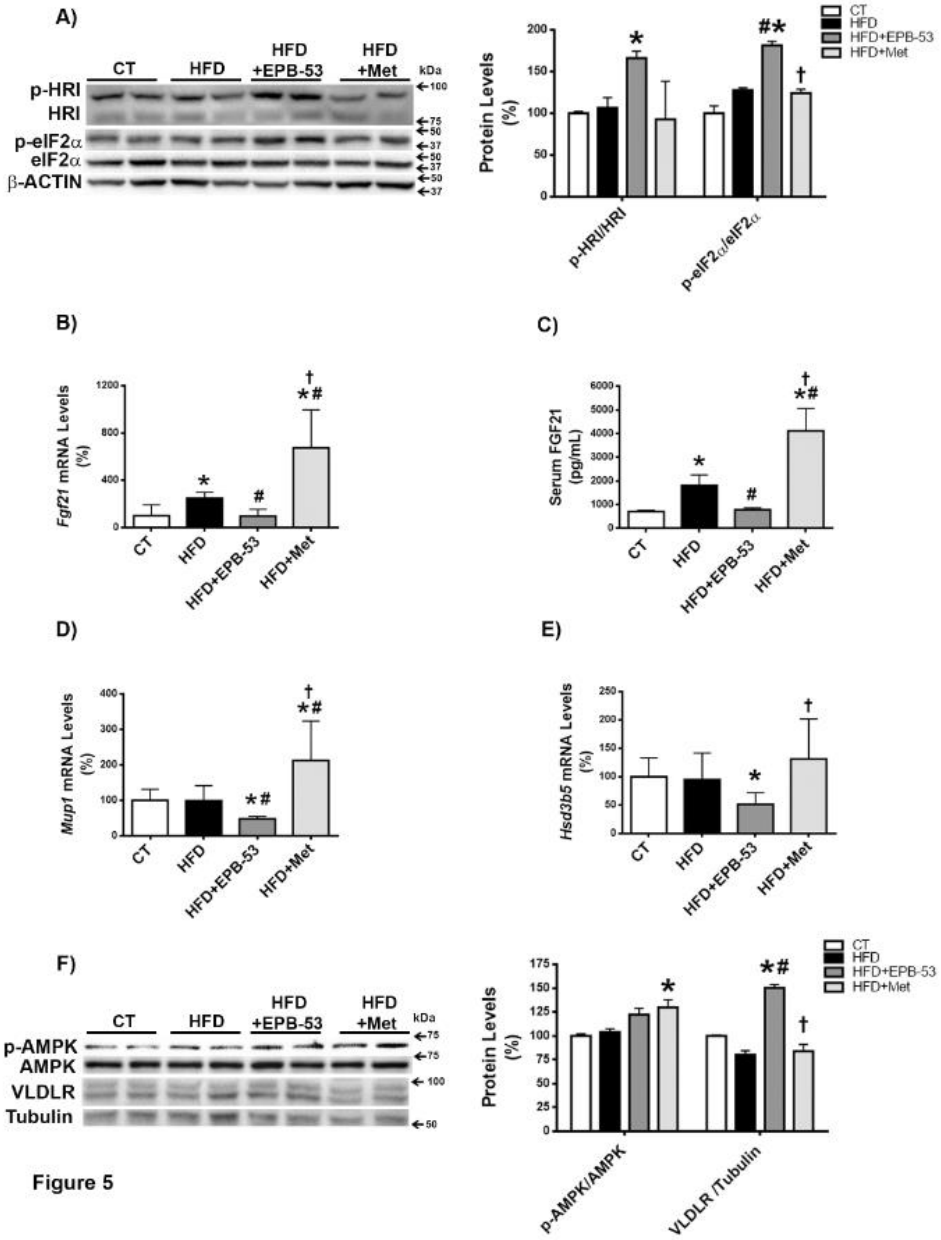

Figure 5

Fig. 5. **Oral administration of EPB-53 increases the hepatic levels of phospho-HRI and phospho-eIF2α in mice fed a HFD.** Mice were fed a standard chow, a HFD for 10 weeks or a HFD for 10 weeks plus EPB-53 or metformin during the last 2 weeks. A, liver cell lysates extracts were assayed via Western blot analysis with antibodies against total and phospho-HRI, and total and phospho-eIF2α. B, *Fgf21* mRNA abundance in the liver. C, serum FGF21 levels. D, *Mup1* mRNA abundance in the liver. E, *Hsd3b5* mRNA abundance in the liver. F, liver cell lysates extracts were assayed via Western blot analysis with antibodies against total and phospho-AMPK, and VLDLR. Data are presented as the mean ± S.D. (n=6 per group) relative to control mice. *$p<0.05$ *vs.* control (CT) mice treated with the vehicle alone. $^{\#}p<0.05$ *vs.* mice exposed to a HFD and treated with the vehicle alone. $^{\dagger}p<0.05$ *vs.* mice fed a HFD and treated with EPB-53.

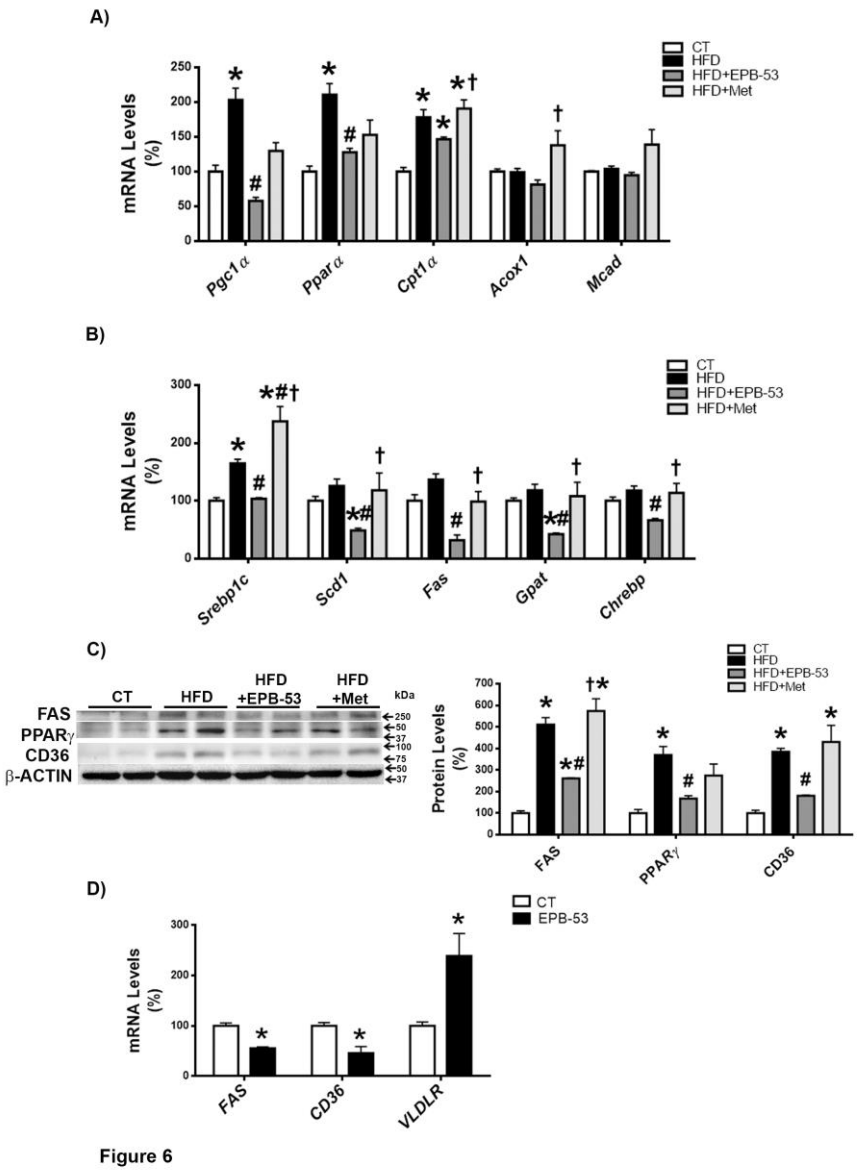

Figure 6

Fig. 6. Oral administration of EPB-53 decreases hepatic lipogenesis in mice fed a HFD. Mice were fed standard chow, a HFD for 10 weeks or a HFD for 10 weeks plus EPB-53 or metformin during the last 2 weeks. A, *Pgc-1α, Pparα, Cpt-1α, Acox1, and Mcad* mRNA abundance in the liver. B, *Srebp1c, Scd1, Fas, Gpat,* and *Chrebp* mRNA abundance in the liver. C, liver cell lysate extracts were assayed via Western blot analysis with antibodies against FAS, PPARγ, and CD36. Data are presented as the mean ± S.D. (n=6 per group) relative to control mice. D, *FAS, CD36* and *VLDLR* mRNA abundance in human Huh-7 hepatocytes exposed to 10 µM EPB-53 for 24 h. mRNA levels are presented as the mean ± S.D. (n=5 per group). *p<0.05 *vs.* control (CT) mice or control cells treated with the vehicle alone. $^{\#}$p<0.05 *vs.* mice fed a HFD and treated with the vehicle alone. †p<0.05 *vs.* mice fed a HFD and treated with EPB-53.

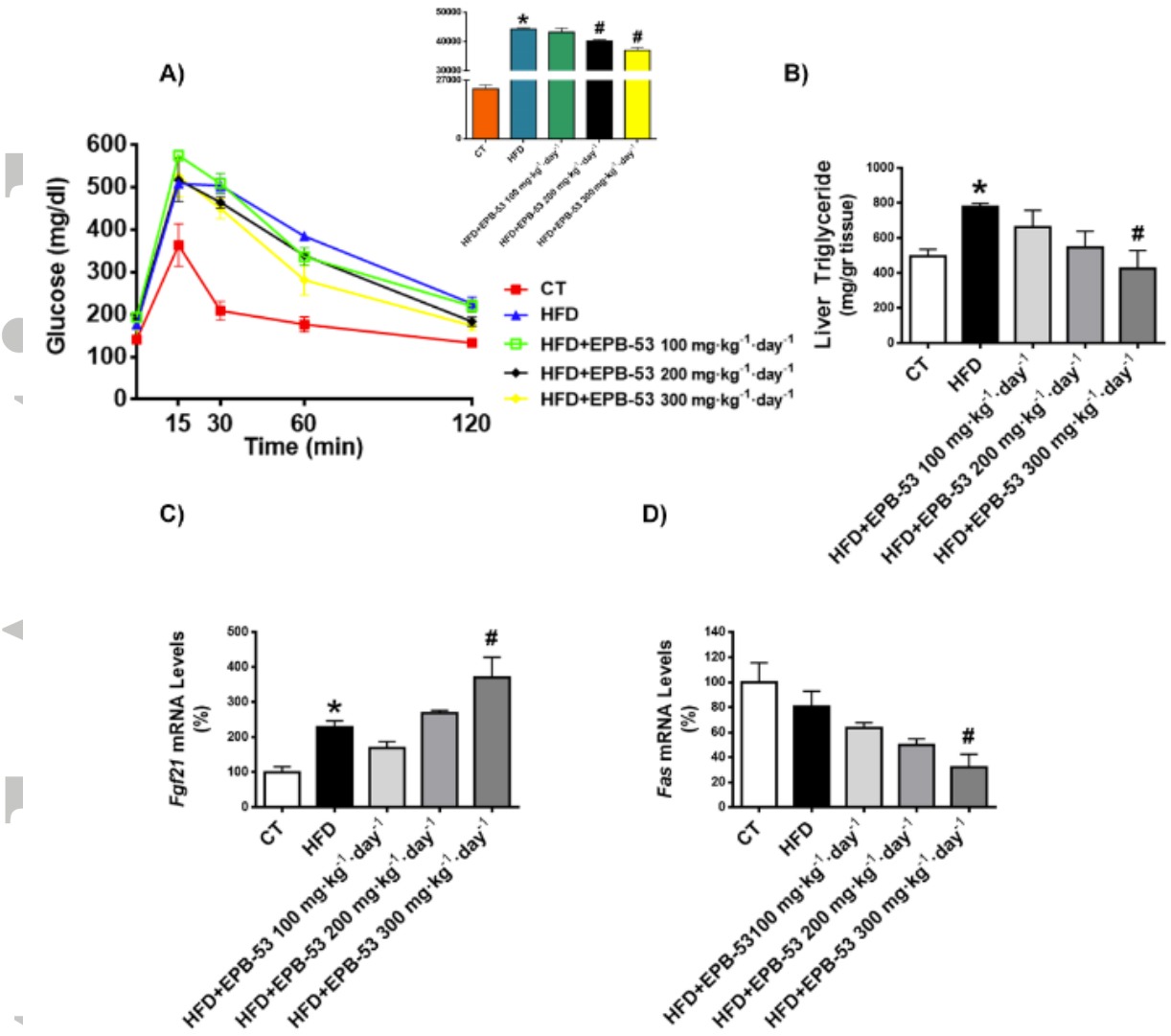

Fig. 7. EPB-53 shows a dose-response relationship. Mice were fed a standard chow or a HFD for 3 weeks, and the last week they received one daily oral gavage of the vehicle or three different doses of EPB-53. A, glucose tolerance test and area under the curve (AUC). B, liver triglyceride levels. *Fgf21* (C) and *Fas* (D) mRNA abundance in the liver. Data are presented as the mean ± S.D. (n=5 per group). *p<0.05 vs. mice fed a standard diet and treated with the vehicle alone. #p<0.05 vs. mice fed a HFD and treated with EPB-53.

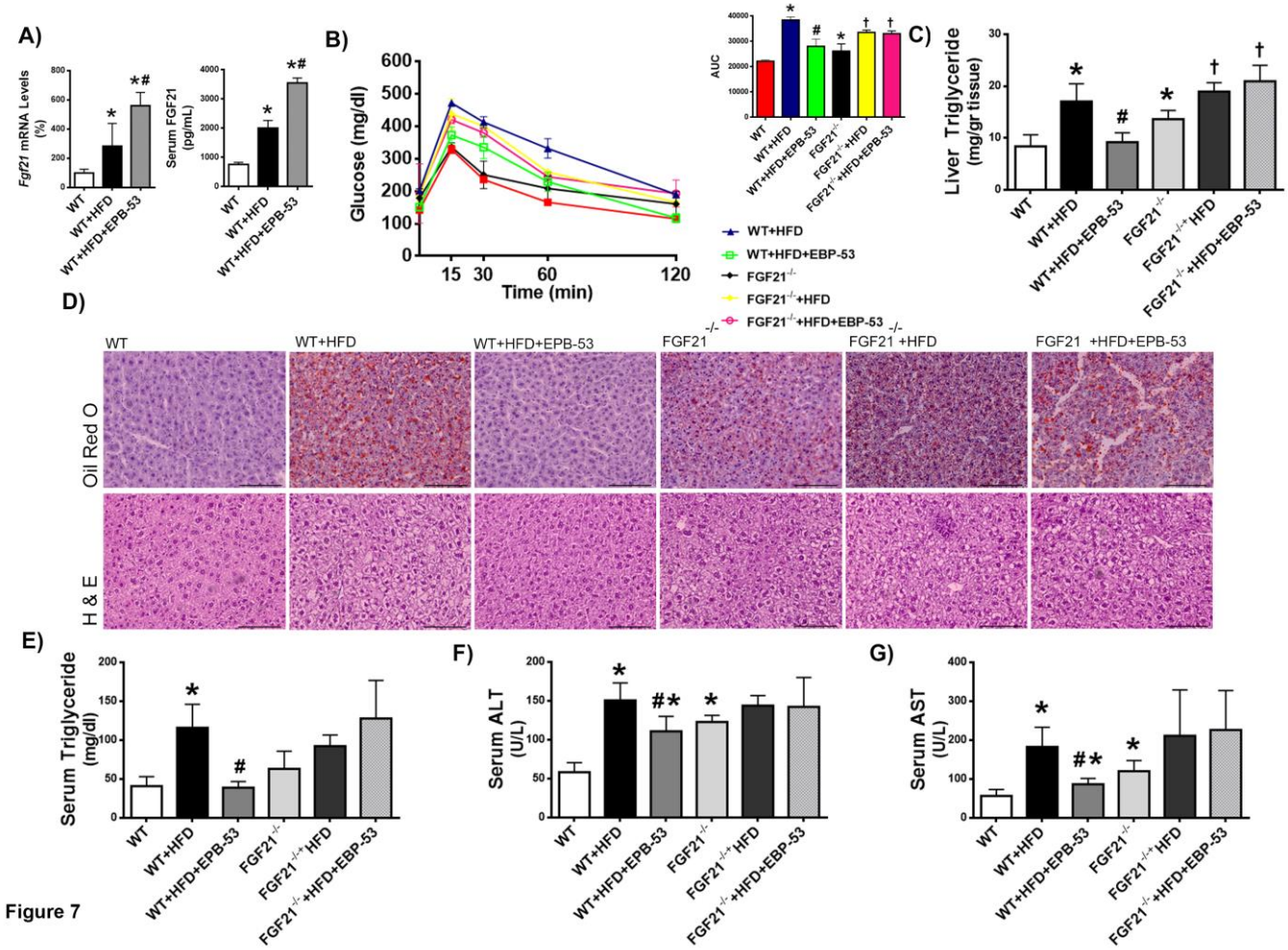

Figure 7

Fig. 8. The effects of the HRI activator on HFD-induced glucose intolerance and hepatic steatosis are dependent on FGF21. *Fgf21*$^{-/-}$ mice and their wild-type (WT) littermates (*Fgf21*$^{+/+}$) were fed a standard chow or a HFD for 3 weeks, and the last week they received one daily oral gavage of the vehicle or EPB-53. A, *Fgf21* mRNA abundance in the liver and serum FGF21 levels. B, glucose tolerance test and area under the curve (AUC). C, liver triglyceride levels. D, H&E and Oil Red O staining of livers. Scale bar: 100 µm. E, serum triglyceride levels. Serum ALT (F) and AST (G) levels. Data are presented as the mean ± S.D. (n=5 per group). *p<0.05 *vs.* WT mice fed a standard diet and treated with the vehicle alone. $^{\#}$p<0.05 *vs.* WT mice fed a HFD and treated with the vehicle alone. †p<0.05 *vs. Fgf21*-null mice fed a standard diet and treated with the vehicle alone.