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DISCOVERY OF A NOVEL CYSTEINYL-LEUKOTRIENE-RECEPTOR 1 ANTAGONIST AND BILE ACID RECEPTOR GPBAR1 AGONIST THAT REDUCES INFLAMMATION IN A MOUSE MODEL OF COLITIS

Cristina Di Giorgio [1], Michele Biagioli [1], Silvia Marchianò [1], Martina Bordoni [1], Rachele Bellini [1], Rosalinda Roselli [1], Angela Zampella [2] and Stefano Fiorucci[1]

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INTRODUCTION

The cysteinyl leukotrienes (CysLTs), i.e. LTC4, LTD4 and LTE4, are a family of pro-inflammatory agents synthesized from the arachidonic acid. In target cells, these lipid mediators bind to the cysteinyl leukotriene receptors (CysLTR), a family of seven transmembrane G-protein coupled receptors. The CysLTR1 is a validated target for treatment of pulmonary diseases and several selective antagonists for this receptor, including montelukast, zafirlukast and pranlukast, have shown effective in the management of asthma. In a previous study we demonstrate that the CysLTR1 antagonist REV5901, interacts with GPBAR1, a well characterized cell membrane receptor for secondary bile acids which activation leads to inhibition of the immune response with a decrease in pro-inflammatory cytokines production and phagocytic capacity. In vivo, in contrast to montelukast, REV5901 attenuates inflammation and immune dysfunction in rodent models of colitis. In this background we have decided to explore the REV5901 scaffold with the aim to synthetize novel multi-target ligands acting as GPBAR1 agonists and CysLTR1 antagonists, such as CHIN117.

AIM

To investigate whether simultaneous modulation of CysTLR1 and GPBAR1 by CHIN117 protects against the development of colitis in mouse model.

METHOD

Colitis was induced in C57BL/6J mice by 2% Dextran sulfate sodium salt (DSS) administration for 8 consecutive days, in drinking water. CHIN117 was administrated at the dose of 5, 15 or 30 mg/kg, BAR501 (30 mg/Kg) and montelukast (30 mg/Kg) by oral gavage. The severity of disease was monitored daily by measuring body weight, CDAI score (body weight lost occult blood and stool consistency), endoscopy and survival. At the end of the experiment mice were sacrificed, blood samples collected, and the colon was excised, weighed, evaluated for macroscopic and histological scores (H&E) and used for cytokines assay (RT-PCR).

RESULTS

The novel class of quinolinic derivatives has been developed. The new synthetic compounds have a dual activity, binding simultaneously GPBAR1 and Cysltr1. The first in class of these agents, is CHIN117 (Figure 1A). It exerts a dual modulatory activity with an EC50 on human GPBAR1 of 4.6 μM and an IC50 on human CYSLTR1 of 5.7 μM (Figure 1B, C). Firstly, we have demonstrated that CHIN117 at the dosage of 30 mg/kg reversed the clinical signs and symptoms of DSS-induced colitis in mice. The dual compound acts with an improve effectiveness of both the singles BAR501 and montelukast as measured by body weight loss (Figure 2A) and CDAI score (Figure 2B), and attenuated the severity of the macroscopic features of colonic inflammation as shown by H&E staining of colonic tissue (Figure 2 E) and by endoscopy score (Figure 3). The pro-inflammatory cytokine profile was assessed on colonic tissue and measured by RT-PCR, CHIN117 downregulates the expression of proinflammatory cytokine in a dose-dependent manner (Figure 4). Montelukast had no effect on revert DSS-inflammation on mice confirming data reported in a previous study. Macrophages have a central role in regulating intestinal immunity. Therefore, we investigated the effect of CHIN117 on these cells. Particularly, the *in vitro* pharmacological effect of this compound was evaluated on U937 cell line, a line of human monocytes (Figure 5). The cells were stimulated by exposing them simultaneously for 24 h to TNF-α + LTD4 alone or treated with CHIN117 at 1, 5 or 10 μM. TNF-α + LTD4 induced pro-inflammatory cell polarization (M1 like) by up-regulating the expression of pro-inflammatory cytokines genes TNF-α and IL-1β (Figure 5C, D). CHIN117 counteracted this effect in a concentration-dependent manner by inducing a down-regulation of the pro-inflammatory cytokines, inducing Il-10 secreting M2 phenotype. (Figure 5E).

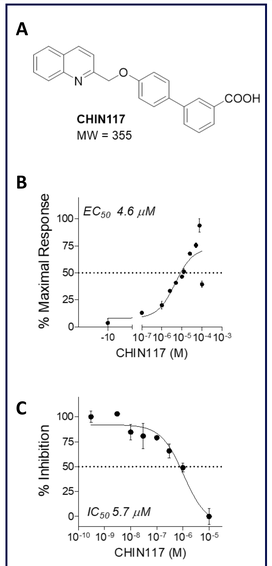

Figure 1. (A) Chemical structure CHIN117. In vitro pharmacological evaluation of CHIN117 on **(B)** Gpbar1 and **(C)** Cysltr1 activity.

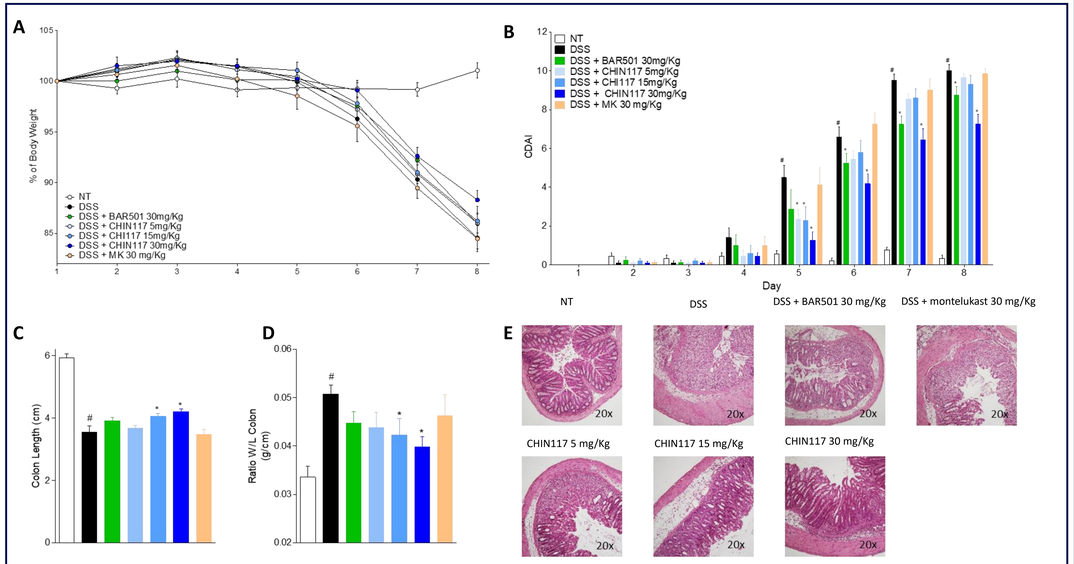

Figure 2. Administration of the dual-acting compound CHIN117 exerted a greater dose-dependent protective effect on DSS mouse model of colitis compared to BAR501 and montelukast. Colitis was induced in C57BL/6 male mice by oral administration of 2% DSS in drinking water alone or in combination with BAR501 (30mg/kg), CHIN117 5 mg/Kg, CHIN117 15 mg/Kg, CHIN117 30 mg/Kg and Montelukast 30 mg/kg. Data shown are: **(A)** Changes in body weight and **(B)** Colitis Disease Activity Index (CDAI) of mice during the course of DSS-induced colitis. **(C)** Colon length (cm) **(D)** Ratio weight (W)/ length (L) (g/cm) of colon. **(E)** Hematoxylin and eosin (H&E) staining of colon tissue (Magnification 20X). Results are the mean ± SEM of 7-10 mice per group; * represents statistical significance versus NT, and # versus DSS, $p < 0.05$.

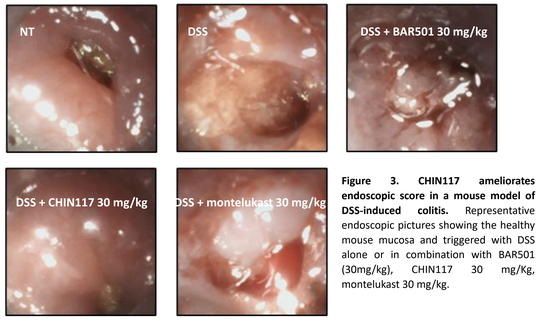

Figure 3. CHIN117 ameliorates endoscopic score in a mouse model of DSS-induced colitis. Representative endoscopic pictures showing the healthy mouse mucosa and triggered with DSS alone or in combination with BAR501 (30mg/kg), CHIN117 30 mg/Kg, montelukast 30 mg/kg.

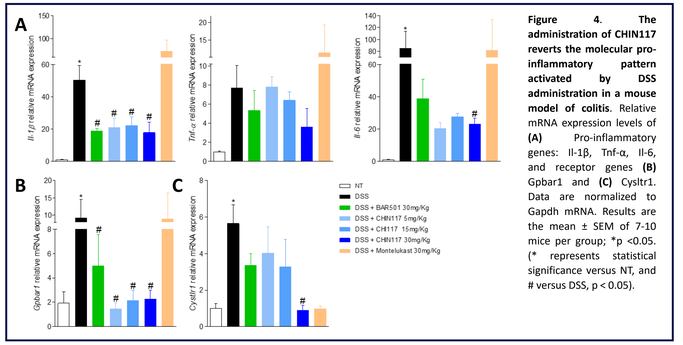

Figure 4. The administration of CHIN117 reverts the molecular pro-inflammatory pattern activated by DSS administration in a mouse model of colitis. Relative mRNA expression levels of **(A)** Pro-inflammatory genes: Il-1β, Tnf-α, Il-6, and receptor genes **(B)** Gpbar1 and **(C)** Cysltr1. Data are normalized to Gapdh mRNA. Results are the mean ± SEM of 7-10 mice per group; *p <0.05. (* represents statistical significance versus NT, and # versus DSS, $p < 0.05$).

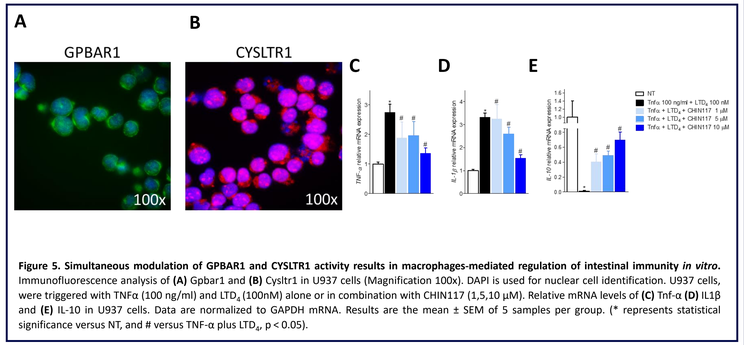

Figure 5. Simultaneous modulation of GPBAR1 and CYSLTR1 activity results in macrophages-mediated regulation of intestinal immunity *in vitro*. Immunofluorescence analysis of **(A)** Gpbar1 and **(B)** Cysltr1 in U937 cells (Magnification 100x). DAPI is used for nuclear cell identification. U937 cells, were triggered with TNFα (100 ng/ml) and LTD_4 (100nM) alone or in combination with CHIN117 (1,5,10 μM). Relative mRNA levels of **(C)** Tnf-α **(D)** IL1β and **(E)** IL-10 in U937 cells. Data are normalized to GAPDH mRNA. Results are the mean ± SEM of 5 samples per group. (* represents statistical significance versus NT, and # versus TNF-α plus LTD_4, $p < 0.05$).

CONCLUSIONS

The hybrid ligands CHIN117 designed for simultaneous modulation of CysTLR1 and GPBAR1 attenuates intestinal inflammation in a mouse model of colitis, inducing Il-10-secreting-M2 phenotype *in vitro*.

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ROLE OF A DUAL GPBAR1/CYSLTR1 MODULATOR IN THE TREATMENT OF LIVER INJURY AND FIBROSIS IN A RODENT MODEL OF NON-ALCOHOLIC STEATOHEPATITIS (NASH)

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INTRODUCTION

Non alcoholic steatohepatitis (NASH) is a highly prevalent human disorder for which no approved treatment is currently available. Despite several hypotheses have been proposed, its mechanisms remain poorly understood. Cysteinyl leukotrienes are a family of pro-inflammatory lipid mediators, synthesized from arachidonic acid metabolism by a variety of cells, including mast cells, eosinophils, basophils and macrophages. They exert their effect by binding to Cysteinyl leukotriene receptors, a family of seven transmembrane G-protein coupled receptors. Cysteinyl leukotrienes have a significant role in inflammation processes working as pro-inflammatory mediators. Since their role in chronic asthma and allergic rhinitis was demonstrated, different Cysteinyl leukotrienes receptor antagonists were designed and some of them were approved in clinical practice. GPBAR1 is a metabotropic membrane G-protein receptor activated by secondary bile acids and highly expressed in intestine, adipose tissues, muscles, and immune cells. Although the liver parenchymal cells lack GPBAR1 expression, the receptor is highly represented in liver non parenchymal cells such as liver sinusoidal cells and liver resident macrophages. In metabolically active tissues such as adipose tissues and muscles, GPBAR1 regulates energy expenditure. Its activation is important for the maintenance of an immunological tolerance state in tissues, working as an anti-inflammatory and immunosuppressor agent.

AIM

The aim of the study was to explore the potential role of a dual modulation GPBAR1/CysLTR1 in a rodent model of NASH.

METHOD

CHIN117, a GPBAR1 agonist and CysLTR1 antagonist was synthetized. C57BL/6J mice were fed 61 days a high fat diet and fructose (HFD-F) alone or in combination with CHIN117 (30 mg/kg/day) and RNAseq analysis was performed on livers samples of each mice group.

RESULTS

CHIN117, a new compound derived from REV5901 scaffold is a dual CysLTR1 antagonist/GPBAR1 agonist (Fig.1) and was tested in this work in a rodent model of NASH.

Mice on a HFD-F developed an obesogenic phenotype gaining significantly more weight than naïve mice and becoming insulin resistant as demonstrated by OGTT results. The HFD-F increased plasma levels of AST, ALT, TG and cholesterol. Treating mice with CHIN117, reversed this pattern, not only reduced the body weight gain and body mass index (BMI) but improved insulin sensitivity, reduced AST and ALT plasma levels and partially reversed the proatherogenic lipid profile (Fig.2). HFD-F mice developed NASH-like features with microvescicular steatosis and ballooning of hepatocytes (H/E) and liver inflammation. Treating mice with CHIN117 completely reversed these patterns improving liver histopathology. Additionally, Sirius red stained of liver sections revealed that feeding an HFD-F caused the development of a mild liver fibrosis and increased liver collagen deposition, these histopathology findings were robustly attenuated by feeding HFD-F mice with CHIN117, which also reversed the expression of several pro-fibrogenic genes, αSma and Col1α1 (Fig. 3). The beneficial effects exerted on liver steatosis were manifested also in RNAseq analysis. Treating HDF-F mice with CHIN117 resulted in robust remodeling of the expression of genes involved in the uptake, synthesis and storage of lipids such as Fasn, Elov5, Mogat1, fabp2, Cd36, Me1, Cidea and Cidec. Furthermore CHIN117 reduced the expression of several chemokine, including Ccl3, Cxcl2, Ccl2 and Serpine1 (Fig.4).

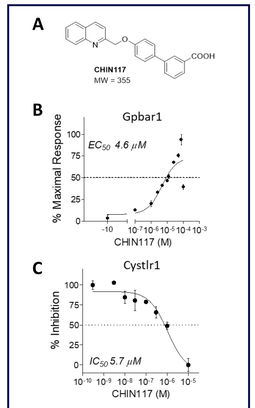

Figure1. (**A**) Chemical structure of new compound CHIN117. (**B**) GPBAR1 transactivation on Hek293T cells co-transfected with GPBAR1 and a reporter gene containing a cAMP responsive element in front of the luciferase gene. (**C**) In vitro pharmacological evaluation of CHIN117 on Cysltr1 activity. The results are expressed as a percent inhibition of the response to LTD4.

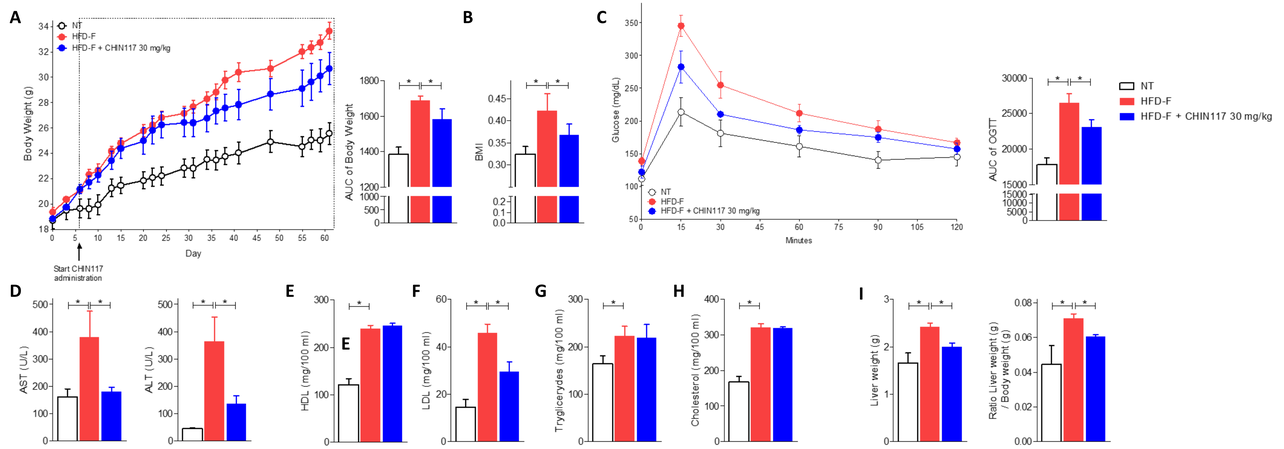

Figure2. C57BL/6J male mice were fed a high fat diet and fructose (HFD-F) or normal chow diet for 61 days. From day 7, CHIN117 was administered by oral gavage at the dose of 30 mg/kg/daily. (**A**) Changes in body weight (%) and AUCs of body weight expressed in arbitrary units. (**B**) Body Mass Index (BMI) at the end of the experiment. (**C**) Glucose plasma levels in response to OGTT and AUCs of glucose plasma levels expressed in arbitrary units. Results are the mean ± SEM of 5-7 mice per group; *p <0.05. Plasmatic levels of (**D**) AST, ALT, (**E**) HDL , (**F**) LDL ,(**G**) Triglycerides, (**H**) Cholesterol, at the end of the study. Macroscopic features of the liver: (**I**) liver weight (g) and ratio between liver weight (g) and body weight (g).

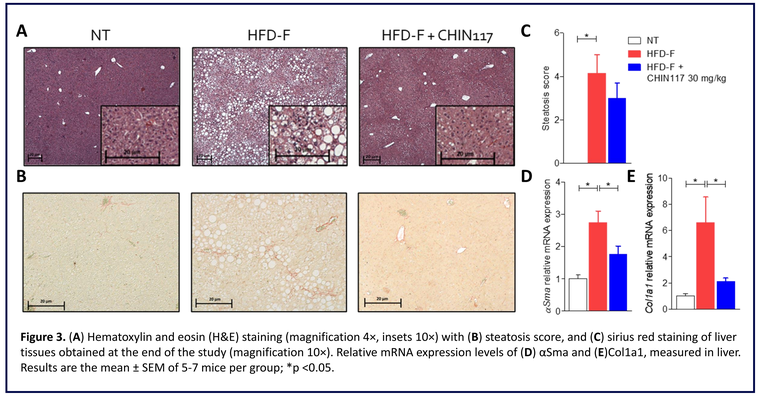

Figure 3. (**A**) Hematoxylin and eosin (H&E) staining (magnification 4×, insets 10×) with (**B**) steatosis score, and (**C**) sirius red staining of liver tissues obtained at the end of the study (magnification 10×). Relative mRNA expression levels of (**D**) αSma and (**E**)Col1a1, measured in liver. Results are the mean ± SEM of 5-7 mice per group; *p <0.05.

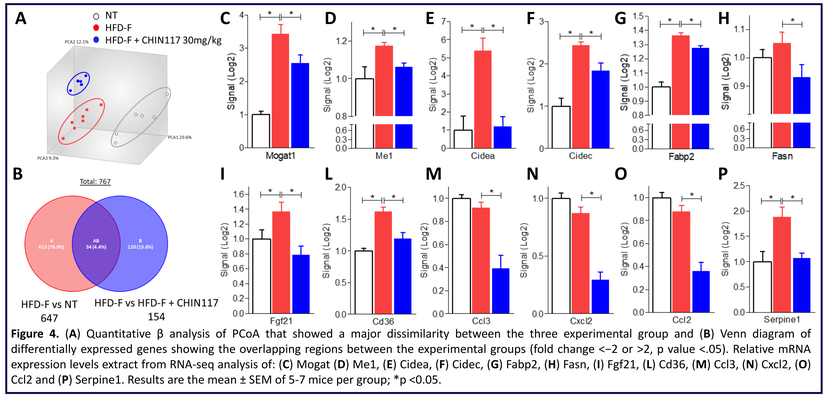

Figure 4. (**A**) Quantitative β analysis of PCoA that showed a major dissimilarity between the three experimental group and (**B**) Venn diagram of differentially expressed genes showing the overlapping regions between the experimental groups (fold change <–2 or >2, p value <.05). Relative mRNA expression levels extract from RNA-seq analysis of: (**C**) Mogat (**D**) Me1, (**E**) Cidea, (**F**) Cidec, (**G**) Fabp2, (**H**) Fasn, (**I**) Fgf21, (**L**) Cd36, (**M**) Ccl3, (**N**) Cxcl2, (**O**) Ccl2 and (**P**) Serpine1. Results are the mean ± SEM of 5-7 mice per group; *p <0.05.

CONCLUSIONS

CHIN117, a dual GPBAR1 agonists and CysLTR1 antagonists, reverses liver steatosis and ameliorates insulin resistance in a mouse model of NASH and might represent a novel therapeutic approach in the pharmacological treatment of metabolic disorders of the liver.

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REGULATION OF INTESTINAL ACE2 EXPRESSION BY THE BILE ACID RECEPTOR GPBAR1 IS MEDIATED BY A GPBAR1/GLP-1/GLP-1R AXIS

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INTRODUCTION

ACE2 is a carboxypeptidase homolog to the dipeptidase ACE but with different substrate specificity; while ACE principally acts as a carboxydipeptidase (peptidyldipeptidase) removing the C-terminal dipeptide from Ang I to form Ang II, ACE2 functions exclusively as a carboxypeptidase removing a single C terminal amino acid from Ang II generating Ang-(1-7) or, much less efficiently, from Ang I forming Ang-(1-9). ACE and ACE2 than playing a key role in regulating the renin–angiotensin–aldosterone system (RAAS). In the normal lung, ACE2 mRNA is mainly expressed by type II alveolar epithelial cells and endothelial cells, but the level of expression increases in response to inflammation while is downregulated in response to SARS-CoV infection. ACE2, mRNA and protein, is highly expressed in the gastrointestinal tract, with the higher expression detected in epithelial cells of the ileum and the colon where mediates the absorption of amino acids. ACE2 expression in the intestine undergoes regulation in response to a variety of factors including intestinal microbiota and inflammation. Furthermore, previous studies have suggested that insulinotropic factor glucagon like peptide (GLP)-1 might regulate ACE2 expression in the heart, suggesting a potential interaction of GLP1 with ACE2. GPBAR1, G Protein Bile Acid Receptor, is robustly expressed in the gastrointestinal tract and its activation in the intestine promotes the release of GLP-1.

AIM

Tha aim of the study was to investigate the possible interaction between bile acids via GPBAR1 and the expression of ACE2 in the gastrointestinal tract.

METHOD

HT29 cells treated with TNF-α + IL-1β and mouse models of colitis were used to assess ACE2 expression and treatment with BAR501, a GPBAR1 agonist, was used to investigate its modulation.

RESULTS

First we investigated the expression levels of ACE2 mRNA in biopsy samples obtained from colon specimens from seven CD patients. qPCR analysis demonstrated that colon expression of ACE2 mRNA is upregulated in response to inflammation in CD patients such as two inflammatory biomarkers (TNF-α and IL-6). These findings were confirmed by immunohistochemical analysis with anti-ACE2 antibody (Fig.1). Because the immunohistochemistry data shown that in the colon ACE2 is mostly expressed in epithelial cells, we then investigated the regulation of the expression of this receptor in vitro on HT29 cells, a colon epithelial cell line. Exposure of HT29 cells to inflammatory stimuli resulted in a significant upregulation of ACE2 mRNA (≈8 times) and protein (≈1.5 times). This effect was reversed by simultaneous activation of the GPBAR1 receptor by BAR501, a selective synthetic agonist, in a concentration dependent manner. We further dissected these signaling pathways using a mouse model of oxazolone induced colitis. Results demonstrated that BAR501 protects against development of signs and symptoms of colitis caused by oxazolone, as shown by assessing the CDAI, the colonic macroscopic features, endoscopy and colon expression of cytokine. Contrary to what we expected, the treatment with BAR501, while reducing intestinal inflammation, induced an up-regulation of Ace2 of about 30%. Because these findings demonstrate that GPBAR1 functions as positive regulator of Ace2 expression in vivo, we examined whether a correlation exists between the levels of expression of Ace2 and Gpbar1 and its intestinal target gene Glp-1, that regulates Ace2 expression in the heart. The results of these assays demonstrate that BAR501 significantly increases the expression of Gcg, the gene encoding for GLP-1 in mice, and that a direct correlation exists between Gpbar1 and Gcg expression in the colon of mice with colitis. We then investigated whether liraglutide, a GLP-1 receptor agonist, reverses the in vitro inhibitory effects exerted by the GPBAR1 agonist on ACE2 expression. Results, demonstrated that adding liraglutide to the incubation mixture on HT29 cells produced a shift to the right of the concentration–response curve exerted by BAR501 and partially reduced the downregulation exerted by the GPBAR1 agonist on ACE2 mRNA, while it did not interfere with the anti-inflammatory effects exerted by BAR501 as measured by assessing the expression levels of IL-8, IL-1β and CCL2 mRNA. Furthermore, in vivo we demonstrated that while BAR501 reverts the inflammation and upregulates the expression of Ace2 mRNA, these beneficial effects were completed reversed by administration of Exendina-3, a GLP-1R antagonist, demonstrating that the GLP-1/GLP-1R axis is involved in the protective effects exerted by the GPBAR1 agonist and regulated the colonic Ace2 expression.

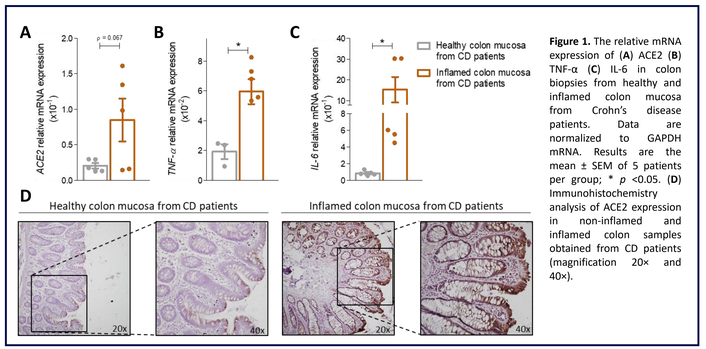


Figure 1. The relative mRNA expression of (**A**) ACE2 (**B**) TNF-α (**C**) IL-6 in colon biopsies from healthy and inflamed colon mucosa from Crohn's disease patients. Data are normalized to GAPDH mRNA. Results are the mean ± SEM of 5 patients per group; * p <0.05. (**D**) Immunohistochemistry analysis of ACE2 expression in non-inflamed and inflamed colon samples obtained from CD patients (magnification 20× and 40×).

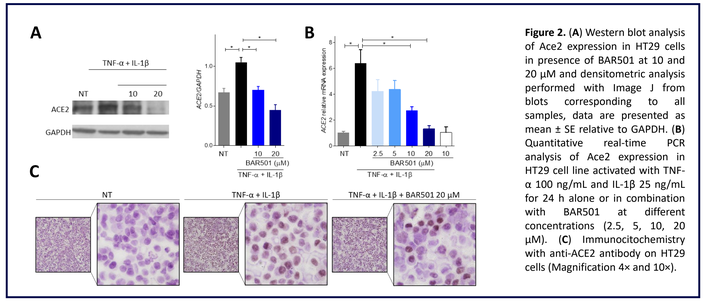


Figure 2. (**A**) Western blot analysis of Ace2 expression in HT29 cells in presence of BAR501 at 10 and 20 μM and densitometric analysis performed with Image J from blots corresponding to all samples, data are presented as mean ± SE relative to GAPDH. (**B**) Quantitative real-time PCR analysis of Ace2 expression in HT29 cell line activated with TNF-α 100 ng/mL and IL-1β 25 ng/mL for 24 h alone or in combination with BAR501 at different concentrations (2.5, 5, 10, 20 μM). (**C**) Immunocitochemistry with anti-ACE2 antibody on HT29 cells (Magnification 4× and 10×).

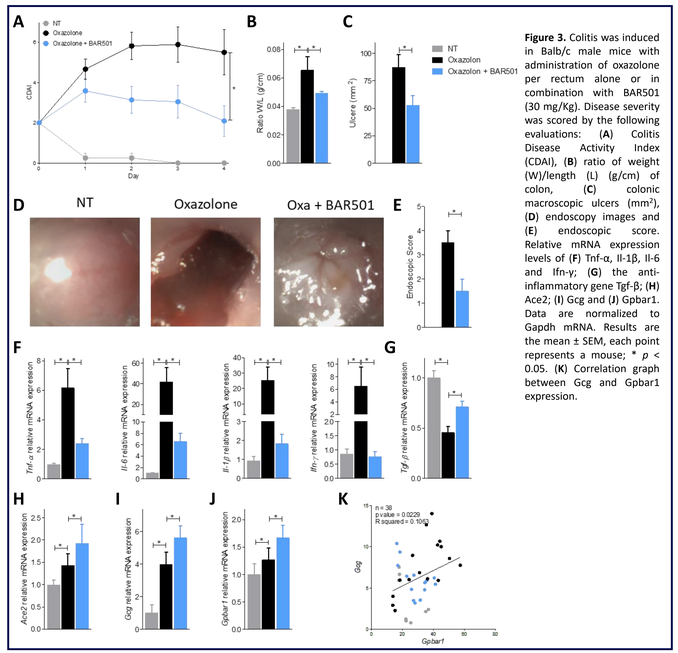


Figure 3. Colitis was induced in Balb/c male mice with administration of oxazolone per rectum alone or in combination with BAR501 (30 mg/Kg). Disease severity was scored by the following evaluations: (**A**) Colitis Disease Activity Index (CDAI), (**B**) ratio of weight (W)/length (L) (g/cm) of colon, (**C**) colonic macroscopic ulcers (mm²), (**D**) endoscopy images and (**E**) endoscopic score. Relative mRNA expression levels of (**F**) Tnf-α, Il-1β, Il-6 and Ifn-γ; (**G**) the anti-inflammatory gene Tgf-β; (**H**) Ace2; (**I**) Gcg and (**J**) Gpbar1. Data are normalized to Gapdh mRNA. Results are the mean ± SEM, each point represents a mouse; * p < 0.05. (**K**) Correlation graph between Gcg and Gpbar1 expression.

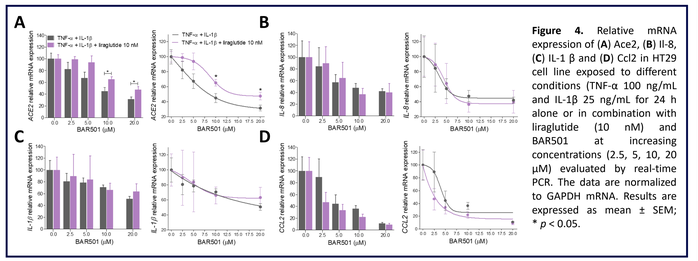
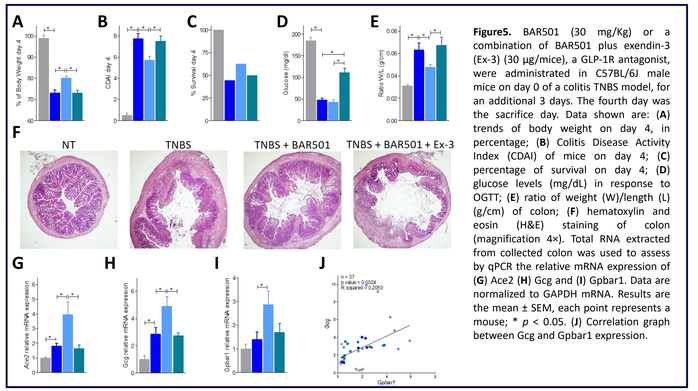


Figure 4. Relative mRNA expression of (**A**) Ace2, (**B**) Il-8, (**C**) IL-1 β and (**D**) Ccl2 in HT29 cell line exposed to different conditions (TNF-α 100 ng/mL and IL-1β 25 ng/mL for 24 h alone or in combination with liraglutide (10 nM) and BAR501 at increasing concentrations (2.5, 5, 10, 20 μM) evaluated by real-time PCR. The data are normalized to GAPDH mRNA. Results are expressed as mean ± SEM; * p < 0.05.



FigureS. BAR501 (30 mg/Kg) or a combination of BAR501 plus exendin-3 (Ex-3) (30 μg/mice), a GLP-1R antagonist, were administrated in C57BL/6J male mice on day 0 of a colitis TNBS model, for an additional 3 days. The fourth day was the sacrifice day. Data shown are: (**A**) trends of body weight on day 4, in percentage; (**B**) Colitis Disease Activity Index (CDAI) of mice on day 4; (**C**) percentage of survival on day 4; (**D**) glucose levels (mg/dL) in response to OGTT; (**E**) ratio of weight (W)/length (L) (g/cm) of colon; (**F**) hematoxylin and eosin (H&E) staining of colon (magnification 4×). Total RNA extracted from collected colon was used to assess by qPCR the relative mRNA expression of (**G**) Ace2 (**H**) Gcg and (**I**) Gpbar1. Data are normalized to GAPDH mRNA. Results are the mean ± SEM, each point represents a mouse; * p < 0.05. (**J**) Correlation graph between Gcg and Gpbar1 expression.

CONCLUSIONS

Our results demonstrate that both in vivo and in vitro activation of GPBAR1 by a selective agonist exerts an anti-inflammatory effect. On the other hand, in vivo activation of GPBAR1 in the colon induces the release of GLP-1, which mediates some of the anti-inflammatory effects exerted by the bile acid receptor and further up-regulates Ace2 expression. Thus we have shown that a GPBAR1/GLP-1/GLP-1R axis regulates intestinal ACE2 expression.

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DEVELOPMENT OF A DUAL GPBAR1 AND CYSLTR1 MODULATOR TO PREVENT HEPATIC DAMAGE AND LIVER FIBROSIS

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INTRODUCTION

Hepatic disease due to non-treated acute Drug induced liver injury (DILI) or chronic liver disease can lead to liver fibrosis, which is a main challenge for global health. It is widely reported montelukast, a CysLTR1 antagonist attenuates hepatic damages, thanks to its anti-inflammatory and antioxidant property but the mechanism is yet unknown. GPBAR1, is a bile acid receptor activated receptors for secondary bile acids, expressed in monocyte and macrophages, that promotes a down-regulation of inflammatory response. Thus, a series of novel chemicals endowed with CysLTR1 antagonism and GPBAR1 agonism has been generated. The most potent of this agent, CHIN117, was tested in mice model of liver inflammation and fibrosis.

AIM

To investigate CHIN117 potential protective action in the development of hepatic inflammation and fibrosis in mice models of liver injury caused by acethaminophen (APAP) or CCL4.

METHOD

Hepatic acute damage was induced in GPBAR1 wild type on C57BL/6NCrl background by APAP (500 mg/kg) administered by oral gavage alone or in combination with BAR501 (30 mg/kg), CHIN117 (30 mg/kg) or Montelukast (30 mg/kg). Fibrosis was induced in C57BL76J by intraperitoneally administration of CCl4 (0.5 ml/kg), dissolved in vegetable oil, and injected into mice 2 times per one week.

RESULTS

The novel compound CHIN117, belonging to the class of novel quinolinic derivatives, has been developed (Figure 1A). It simultaneously exerts a dual modulatory activity with an EC50 on human GPBAR1 of 4.6 µM and an IC50 on human CYSLTR1 of 5.7 µM (Figure 1B, C). Firstly, we demonstrate that CHIN117 attenuates acute APAP-induced hepatitis with an improved efficacy of both BAR501 and montelukast as shown by AST (Figure 2A) and ALT (Figure 2B) plasma levels and confirmed H&E liver section staining (Figure 2C), the Area of necrosis (Figure 2D) and hepatic biochemical dosages (Figure 2E-F-G). RNAseq analysis of livers samples showed that the effect on the modulation of gene expression of CHIN117 overlaps more with the effect exerted by montelukast than with BAR501 (Figure 3). To further investigate the action mechanism of CHIN117, analysis on endothelial human cell line LSEC and monocyte human cell line U937 were performed. Both LSEC and U937 cells express high levels of GPBAR1 (Figure 4A) and CYSLTR1 (Figure 4B). Since this underlies how these cells represent an interesting therapeutic target of CHIN117, we have co-cultured human LSEC with U937 cells (Figure 4C). In this system, APAP increased the expression of pro-inflammatory markers on LSEC and U937 cells (Figure 4D-E) and CHIN117 reverted this effect in a concentration-dependent manner (Figure 4D-E). Furthermore, CHIN117 protects mice from the development of liver fibrosis induced by CCl4 trigger on C57 mice, as shown by biochemical analysis: AST (Figure 5A) and ALT (Figure 5B) plasma levels, cystenyl leucotrienes (pg/mg) liver homogenate concentration (Figure 5C), Sirius Red staining (Figure 5D) and Fibrosis score (Figure 5E). Furthermore, CHIN117 reverted the CCl4-induced pattern, downregulating Tnf-α and Il1-β (Figure 5F). Therefore, we investigated the effect of CHIN117 on hepatic stellate cell line (Figure 6), which express both target receptors of the new compound (Figure 6 A-B). In vitro stimulation of LX2 cells with TNF-α + LTD4 induced a bias towards pro-fibrotic phenotype of stellate cells (Figure 6C). CHIN117 counteracted this effect in a concentration-dependent manner reducing the activation of stellate cells thus exerting a potentially direct antifibrotic effect.

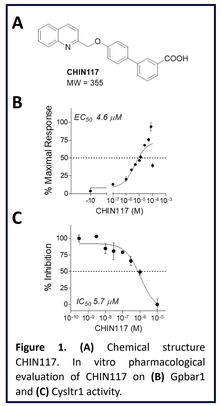

Figure 1. (A) Chemical structure CHIN117. In vitro pharmacological evaluation of CHIN117 on **(B)** Gpbar1 and **(C)** Cysltr1 activity.

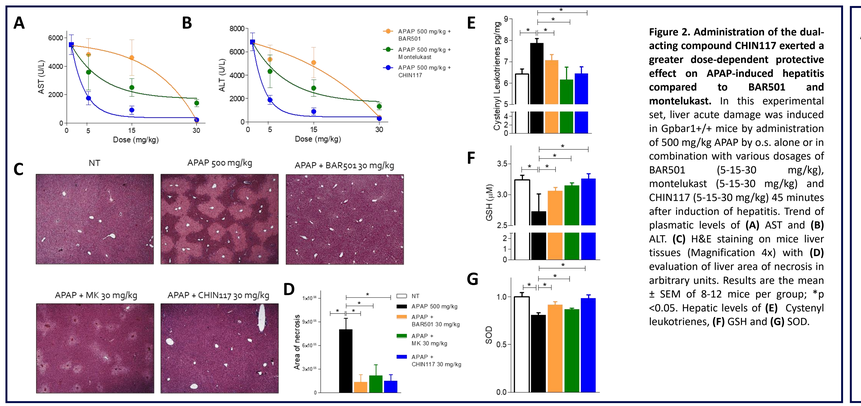

Figure 2. Administration of the dual-acting compound CHIN117 exerted a greater dose-dependent protective effect on APAP-induced hepatitis compared to BAR501 and montelukast. In this experimental set, liver acute damage was induced in Gpbar1+/+ mice by administration of 500 mg/kg APAP by o.s. alone or in combination with various dosages of BAR501 (5-15-30 mg/kg), montelukast (5-15-30 mg/kg) and CHIN117 (5-15-30 mg/kg) 45 minutes after induction of hepatitis. Trend of plasmatic levels of **(A)** AST and **(B)** ALT. **(C)** H&E staining on mice liver tissues (Magnification 4x) with **(D)** evaluation of liver area of necrosis in arbitrary units. Results are the mean ± SEM of 8-12 mice per group; *p <0.05. Hepatic levels of **(E)** Cystenyl leukotrienes, **(F)** GSH and **(G)** SOD.

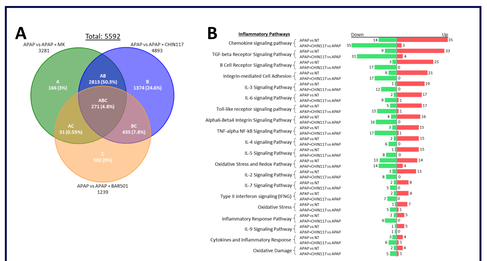

Figure 3. Analysis of the BAR501, montelukast and CHIN117 effects on APAP-induced hepatitis by RNAseq. Hepatitis was induced in C57BL6 mice, through oral administration (o.s.) of acetaminophen (APAP) at 500 mg/kg alone or in combination with BAR501 (15 mg/kg), montelukast (15 mg/kg) and CHIN117 (15 mg/kg) 45 minutes after APAP administration. **(A)** Heterogeneity characterization of the five experimental group showed by principal component analysis (PCA) plot. **(B)** Venn diagram of differentially expressed gene showing the overlapping regions (identified as ABC, AC, AB and BC sets) between the three comparison groups of genes (Fold Change <-2 or>2, *p value < 0.05). **(C)** Inflammatory pathways analysis on subset of genes included in region AB of the corresponding Venn diagram.

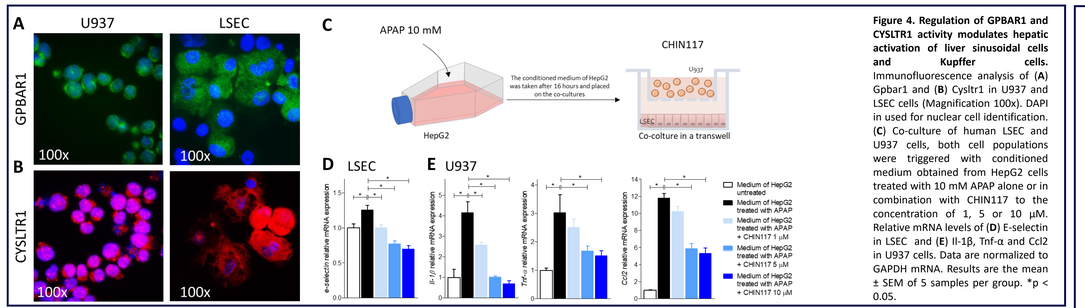

Figure 4. Regulation of GPBAR1 and CYSLTR1 activity modulates hepatic activation of liver sinusoidal cells and Kupffer cells. Immunofluorescence analysis of (**A**) Gpbar1 and (**B**) Cysltr1 in U937 and LSEC cells (Magnification 100x). DAPI in used for nuclear cell identification. (**C**) Co-culture of human LSEC and U937 cells, both cell populations were triggered with conditioned medium obtained from HepG2 cells treated with 10 mM APAP alone or in combination with CHIN117 to the concentration of 1, 5 or 10 µM. Relative mRNA levels of **(D)** E-selectin in LSEC and **(E)** Il-1β, Tnf-α and Ccl2 in U937 cells. Data are normalized to GAPDH mRNA. Results are the mean ± SEM of 5 samples per group. *p < 0.05.

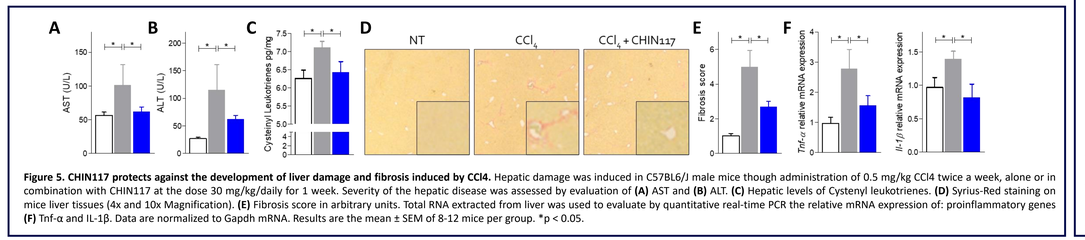

Figure 5. CHIN117 protects against the development of liver damage and fibrosis induced by CCl4. Hepatic damage was induced in C57BL6/J male mice though administration of 0.5 mg/kg CCl4 twice a week, alone or in combination with CHIN117 at the dose 30 mg/kg/daily for 1 week. Severity of the hepatic disease was assessed by evaluation of **(A)** AST and **(B)** ALT. **(C)** Hepatic levels of Cystenyl leukotrienes. **(D)** Syrius-Red staining on mice liver tissues (4x and 10x Magnification). **(E)** Fibrosis score in arbitrary units. Total RNA extracted from liver was used to evaluate by quantitative real-time PCR the relative mRNA expression of: proinflammatory genes **(F)** Tnf-α and IL-1β. Data are normalized to Gapdh mRNA. Results are the mean ± SEM of 8-12 mice per group. *p < 0.05.

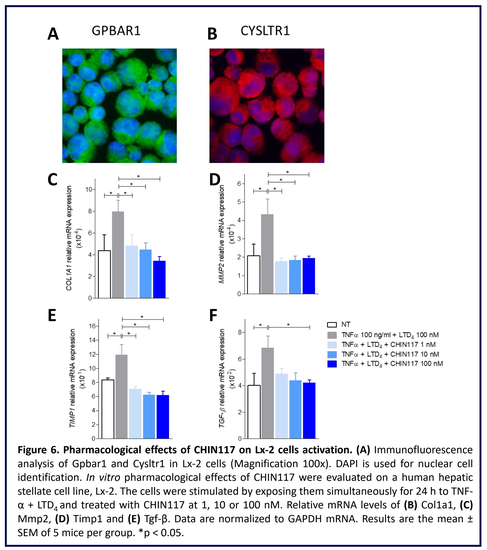

Figure 6. Pharmacological effects of CHIN117 on Lx-2 cells activation. (A) Immunofluorescence analysis of Gpbar1 and Cysltr1 in Lx-2 cells (Magnification 100x). DAPI is used for nuclear cell identification. *In vitro* pharmacological effects of CHIN117 were evaluated on a human hepatic stellate cell line, Lx-2. The cells were stimulated by exposing them simultaneously for 24 h to TNF-α + LTD_4 and treated with CHIN117 at 1, 10 or 100 nM. Relative mRNA levels of **(B)** Col1a1, **(C)** Mmp2, **(D)** Timp1 and **(E)** Tgf-β. Data are normalized to GAPDH mRNA. Results are the mean ± SEM of 5 mice per group. *p < 0.05.

CONCLUSIONS

CHIN117 is a dual GPBAR1 agonist and CYSLTR1 antagonist that effectively protects against acute liver failure induced by APAP and attenuates inflammation-driven fibrosis in mice challenged with CCL4 by acting at the interface between LSEC, Kupffer cells and hepatic stellate cells.

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