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Sorafenib reduces steatosis-induced fibrogenesis in a human 3D co-culture model of non-alcoholic fatty liver disease

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) affects around 25% of the population worldwide. Non-alcoholic steatohepatitis (NASH), the more progressive variant of NAFLD, is characterized by steatosis, cellular ballooning, lobular inflammation and may culminate on hepatic stellate cell (HSC) activation, thus increasing the risk for fibrosis, cirrhosis and HCC development. The antifibrotic effects of sorafenib, an FDA-approved drug for HCC treatment, have been demonstrated in 2D cell cultures and animal models, but its mechanisms in a NAFLD-related microenvironment *in vitro* requires further investigation. Thus, a human 3D co-culture model of fatty hepatocytes and HSC was established by culturing hepatoma C3A cells, pre-treated with 1.32 mM oleic acid, with HSC LX-2 cells. The fatty C3A/LX-2 spheroids showed morphological and molecular hallmarks of altered lipid metabolism and steatosis-induced fibrogenesis similar to the human disease. Sorafenib (15 μM) for 72 h reduced fatty spheroid viability and upregulated the expression of lipid oxidation- and hydrolysis-related genes, *CPT1* and *LIPC*, respectively. Sorafenib also inhibited steatosis-induced fibrogenesis by downregulating *COL1A1*, *TGFB1*, *PDGF*, and *TIMP1* and by decreasing protein levels of IL-6, TGF-β1 and TNF-α in fatty spheroids. Demonstration of the antifibrotic properties of sorafenib on steatosis-induced fibrogenesis in a 3D *in vitro* model of NAFLD supports its clinical use as a therapeutic agent for the treatment of NAFLD/NASH patients.

Keywords:

Non-alcoholic steatohepatitis; fatty hepatocytes; hepatic stellate cells; antifibrotic therapy; tridimensional cell culture.

Abbreviations:

ACTA2, smooth muscle actin alpha 2; ANOVA, analysis of variance; APOB, apolipoprotein B; COL1A1, collagen α1 (I); CPT1, carnitine acyltransferase I; ECM, extracellular matrix; FA, fatty acids; FAS, fatty acid synthase; HSC, hepatic stellate cells; IL, interleukin; LDH, lactate dehydrogenase; LIPC, lipase C hepatic type; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium; MMP, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTTP, microsomal triglyceride transfer protein; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PDGF, platelet derived growth factor; PPARGC1A, peroxisome proliferator-activated receptor-gamma coactivator 1-alpha; PPARG, peroxisome proliferator-activated receptor gamma; SEM, standard error of the mean; SMAD, mothers against decapentaplegic homolog (transforming growth factor-beta signaling protein); SREBF1, sterol regulatory element binding protein 1; STAT3, signal transducer and activator of transcription 3; TFGB1, transforming growth factor beta 1 gene; TG, triglyceride; TIMP1, metallopeptidase inhibitor 1; TNF-α, tumor necrosis factor alpha; T2D, type 2 diabetes.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) currently affects around 25% of the population worldwide and is fast becoming a major public health problem, particularly in western countries, where NAFLD-related mortality is expected to double during the period 2016-2030.[1,2] NAFLD is a multifactorial disease associated with metabolic comorbidities such as obesity, type 2 diabetes (T2D), hyperlipidemia, hypertension and metabolic syndrome.[1,2] NAFLD can be further classified into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) subtypes. NAFL is associated to impaired lipid homeostasis leading to simple steatosis accompanied or not by lobular inflammation. On the other hand, NASH displays steatosis with lobular inflammation and cellular ballooning.[3] Comparatively, the latter is a more progressive variant of NAFLD with increased risk of fibrosis/cirrhosis development. In fact, NASH patients feature one stage progression rate of 7.1 years *versus* 14.3 years for NAFL patients.[4] Noteworthy, NASH prevalence estimate was 59.10% among NAFLD patients.[1]

The presence of a pro-inflammatory microenvironment in NASH patients is a predictive factor for fibrosis progression rather than the amount of intrahepatic triglyceride (TG).[5,6] The inflammatory milieu may become the stimuli for hepatic stellate cell (HSC) activation, the main hallmark for fibrosis setting.[7] In fact, around 5–10% of NASH patients will have clinically significant fibrosis.[1,5] The inability to quell oxidative and endoplasmic reticulum stress, and subsequent lipotoxicity and necroinflammation may also contribute to liver damage and fibrosis establishment.[8] The progression to cirrhosis can occur in approximately one quarter of NASH patients[9], increasing the risk for hepatocellular carcinoma (HCC) emergence. Remarkably, NASH-related metabolic disorders are the leading cause of HCC in the US, corresponding to 32% of all attributable risk factors.[10] HCC incidence ranging from 2.4% to 12.8% in NASH patients[11–13], being the most rapidly growing cause in liver transplant candidates.[14] Currently, treatment of NASH comprises complex and tailored changes in the lifestyle of the patient since there is no FDA-approved medication for the condition. This situation highlights the urgent need for novel therapeutic strategies.[15]

In recent years, *in vitro* 3D co-culture systems have emerged as reliable tools for disease modelling, toxicity testing and drug screening, as they provide a reliable reflection of the *in vivo* situation and thus have high translational value.[16,17] Unlike homotypic 2D models, heterotypic 3D models assemble different types of cells and microenvironmental components with the possibility of recreating the cellular and molecular events involved in complex disease pathogenesis. In this manner, *in vitro* 3D models raise the clinical relevance of the otherwise limited *in vitro*-based testing methods and increase cost-efficiency in drug screening programs.[18] A number of *in vitro* 3D NAFLD/NASH models employing primary or tumoral hepatocytes, HSC, Kupffer cells and/or endothelial cells with fatty acid (FA) medium enrichment have been established and validated as useful translational tools for the investigation of predispositional and therapeutic factors for liver diseases.[19,20]

Sorafenib, a multi-kinase inhibitor approved by the FDA for the treatment of HCC, has been shown to exert antifibrotic effects during liver fibrosis in preclinical animal models.[21–24] In HCC, sorafenib blocks cell surface tyrosine kinase receptors and intracellular serine/threonine kinases in the Ras/mitogen-activated protein kinase (MAPK) cascade, thereby promoting apoptosis and decreasing cell proliferation and angiogenesis.[25] In a rat model of NASH, sorafenib restored mitochondrial function, reduced collagen deposition, increased matrix metalloproteinases (MMP) mRNA levels, and reduced the protein expression of the tissue inhibitor of metallopeptidase 1 (TIMP1) and of pro-inflammatory interleukins (IL)-6 and 10.[24] Recently, Jian et al.[26] provided further evidence *in vivo*, showing that low sorafenib dose attenuated steatosis, inflammation, and fibrosis hallmarks by AMPK activation via mitochondrial uncoupling in diet-induced mouse and monkey NASH models. The sorafenib-mediated suppression of collagen accumulation and HSC growth has also been described in human HSCs *in vitro.*[21,23] However, since the *in vitro* effects of sorafenib have been reported only in 2D homotypic culture models of liver fibrosis, further mechanistic investigation and validation in 3D multicellular NAFLD-related microenvironment models are warranted. The aim of this study was

therefore, to evaluate whether sorafenib administration attenuates steatosis and steatosis-induced fibrogenesis in a 3D model of NAFLD formed by co-culturing human fatty hepatocytes and HSCs.

2. Materials and methods

2.1. Cell lines and culture conditions

The cell lines employed in the study were C3A, a clonal derivative of human hepatoma HepG2 cells (CRL-10741™, ATCC, USA), and LX-2, immortalized human activated HSCs (kindly provided by Professor Scott L. Friedman, Mount Sinai School of Medicine, USA). Cells were grown in Eagle's Minimum Essential Medium (MEM) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific, USA) at 37ºC under a humidified atmosphere of 5% CO_2. In order to induce steatosis in hepatocytes prior to spheroid formation, C3A cells were cultivated in Corning uncoated regular culture flasks (Corning, USA) in MEM supplemented with steatogenic 1.32 mM oleic acid (OA) or 100 mM methanol (control) for 24 h as previously described.[27]

2.2. Spheroid formation and treatment with sorafenib

In order to establish the C3A/LX-2 spheroid model, hepatoma C3A (non-fatty and fatty) cells were seeded at concentrations of 1×10^4, 2.5×10^4, 5×10^4, 1×10^5 and 2.5×10^5 cells/mL together with LX-2 cells at 10-fold lower concentrations[28], and co-cultured in Corning Ultra-Low Attachment flasks or plates (6- or 96-well) for 24, 48 or 72 h. Details of the formation, morphological, biochemical and ultrastructural characterization of C3A/LX-2 spheroids can be found in the Supplementary Assays A1 and A2. The optimized fatty C3A/LX-2 (NAFLD model) spheroids were treated with sorafenib (Bayer HealthCare Pharmaceuticals, Germany) at concentrations of 2.5, 7.5 or 15 µM or dimethyl sulfoxide (Sigma-Aldrich) as control for 24, 48 and 72 h. The concentrations of sorafenib employed were based on the reported antifibrotic efficacy of the agent on LX-2 cells[23] and on the steady-state serum concentration in HCC patients treated with 200 to 800 mg sorafenib/day.[29]

2.3. Cell viability and cytotoxicity analyses

Cell viability was assayed by incubating spheroids, cultured in Ultra-Low 96-well plates (Corning, USA), with 0.5 mg/mL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) for 3 h followed by addition of 100 µL dimethyl sulfoxide/well to dissolve crystals of formazan crystals and subsequent measurement of absorbance at 620 nm. Cytotoxicity was assessed by determining the amount of lactate dehydrogenase (LDH) released from dead or severely damaged cells using a commercial LDH Cytotoxicity Detection kit (Roche Diagnostics, USA) according to the instructions of the manufacturer with absorbance recorded at 620 nm, using a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, USA).

2.4. Extraction of mRNA, cDNA synthesis and RT-qPCR analysis

Total RNA was extracted from spheroids cultured in Ultra-Low 6-well plates (Corning, USA), using a column-based RNAspin Mini RNA Isolation kit (GE Healthcare, USA) following the instructions of the manufacturer. The quantity and integrity of extracted RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and electrophoresis on 1.5% agarose gel, respectively. Total RNA was reverse transcribed to cDNA in an Applied Biosystems ABI Prism 7500 RT-qPCR system (Thermo Fisher Scientific, USA) using random primers and a Life Technologies (Thermo Fisher Scientific, USA) SVILO Master Mix kit. The primers and probes employed in the RT-qPCR assays are described in the Supplementary Table 1. The qPCR protocol involved activation at 50°C for 2 min and denaturation at 95°C for 10 min, followed by 40 cycles at 90°C for 15 s and extension at 60°C for 1 min. Relative quantification was estimated according to the $2^{(-\Delta\Delta Ct)}$ method[30] with normalization against the 18S rRNA reference gene. Data were visualized in the form of expression-based heat maps constructed using the Heatmapper platform.[31]

2.5. Cytokine analysis

Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA) were used to measure levels of interleukin (IL)-6 (# D6050), tumor necrosis factor alpha (TNF-α, # DTA00D) and transforming growth factor beta 1 (TGF-β1, # DB100B) in the supernatants of spheroids cultured in Ultra-Low 96-well plates (Corning, USA). Absorbance was measured at 450 nm with wavelength correction at 570 nm using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, USA). Two independent assays were performed for each parameter.

2.6. Statistical analysis

Results are presented as mean values ± standard error of the mean (SEM). Six subcultures were used per treatment. Data relating to the 3D model establishment, namely non-fatty spheroids *vs.* fatty spheroids, and timepoint *vs.* timepoint, were analyzed using the Student *t* test. Data concerning treatments with sorafenib were analyzed by one-way analysis of variance (ANOVA) and *post-hoc* Tukey tests. The level of significance for statistical tests was set at an alpha value of 0.05.

3. Results

3.1. Establishment of a NAFLD spheroid model

Cell viability (MTT method) and cytotoxicity (LDH method) of non-fatty and fatty spheroids were evaluated at 24, 48 and 72 h after establishment. The growth rates of fatty spheroids were generally higher than those of their non-fatty counterparts at 24 h ($p = 0.0079$ considering all concentrations), while growth thresholds for both non-fatty and fatty spheroids were observed at 72 h at all studied concentrations. Non-fatty and fatty spheroids established with the two highest concentrations of cells showed reductions in viability at 48 and 72 h (Supplementary Fig. S1). Fatty spheroids exhibited increased levels of cytotoxicity in comparison with their non-fatty counterparts irrespective of the concentration of seeded cells (Supplementary Fig. S2). The most regular time-dependent increase in cytotoxicity was observed in fatty spheroids formed with an intermediate

concentration of seeded cells (5×10^4 C3A cells/mL and 5×10^3 LX-2 cells/mL). In addition, these fatty spheroids showed well-defined morphology, mostly at 72 h (Fig. 1A), and ultrastructural features characteristic of NAFLD/NASH (Fig. 1B). Fatty spheroids displayed intracytoplasmic lipid droplets, as shown in representative micrographs of TEM (Fig. 1B) and toluidine blue-stained sections (Fig. 1C), in keeping with increased TG content at 72 h ($p < 0.0001$) (Fig. 1D). Moreover, fatty spheroids showed clear collagen deposition compared to non-fatty ones ($p < 0.0001$), as demonstrated in representative photomicrographs (Fig. 1E) and semiquantitative analysis of Sirius red-stained sections (Fig. 1D). According to MTT, LDH and morphological analysis, the intermediate concentration of cells and the timepoint of 72h were, therefore, employed in further assays.

3.2. Effects of sorafenib on the viability and cytotoxicity of NAFLD spheroids

Sorafenib did not modify the OA-induced increase in cytotoxicity at any timepoint or in cell viability at 24 h (Fig. 2B). However, treatment with 15 µM sorafenib reduced significantly the viability of fatty spheroids at 48 h ($p < 0.001$) and 72 h ($p < 0.001$) after seeding, an effect that was not observed following treatment with 2.5 or 7.5 µM sorafenib (Fig. 2A). Interestingly, the viability of fatty spheroids treated with 15 µM sorafenib was similar to those of non-fatty spheroids (Fig. 2A). Based on these findings, further analyses were performed on 15 µM sorafenib-treated spheroids at 72 h.

3.3. Effects of sorafenib on lipid oxidation- and hydrolysis-related genes

Transcriptional modulation of genes related to lipid metabolism in fatty spheroids was substantially different from that observed in their non-fatty counterparts (Fig. 3A). Treatment with 15 µM sorafenib did not suppress the OA-mediated upregulation of the lipogenesis-related gene *FAS*, or the downregulation of lipid transport genes *APOB* and *MTTP* in fatty C3A/LX-2 spheroids. In contrast, sorafenib inhibited significantly the OA-induced reduction of *CPT1* ($p = 0.021$) and *LIPC* ($p = 0.0045$) expression in fatty spheroids by increasing the mRNA levels of these lipid oxidation-

and hydrolysis-related genes by 25% and ~50%, respectively. The sorafenib-mediated downregulation of *CPT1* and *LIPC* is clearly featured on the heatmap (Fig. 3B).

3.4. Effects of sorafenib on fibrogenesis-associated genes

Steatosis-induced fibrogenesis was confirmed unambiguously by the observed upregulation of *COL1A1*, *ACTA2*, *PDGF* and *TIMP* genes in fatty spheroids compared with their non-fatty counterparts (Fig. 3A). Except for *ACTA2*, all of these genes were downregulated significantly in sorafenib-treated fatty spheroids with mRNA levels for pro-fibrotic *COL1A1* ($p = 0.029$), *TGFB1* ($p = 0.0055$) and *PDGF* ($p < 0.0001$) reduced by 42.56, 23.52 and 59.52%, respectively, and the mRNA level for the matrix remodeling-related *TIMP1* ($p < 0.0001$) reduced by 61%. This clear downregulation of fibrogenesis-related genes is also illustrated on the heatmap (Fig. 3B).

3.5. Effects of sorafenib on pro-inflammatory cytokines

According to ELISA, sorafenib treatment inhibited significantly the OA-induced increase in pro-inflammatory cytokines with reductions in protein levels of IL-6 ($p < 0.0001$), TGF-β1 ($p = 0.023$) and TNF-α ($p = 0.0075$) in fatty spheroid supernatants of 66, 54 and 85%, respectively (Fig. 4).

4. Discussion

There is no FDA-approved therapy for NAFLD even though the disease is not only an increasing epidemiological burden but is also associated with high economic costs that are predicted to reach US$ 2.60 billion each year in the USA alone.[32] In this context, we have evaluated the effects of sorafenib on steatosis and steatosis-induced fibrogenesis in 3D *in vitro* co-culture model of human fatty hepatocytes and HSCs. This investigation is of particular importance since it substantiates the antifibrotic potential of the widely applied multi-kinase inhibitor sorafenib. Of note, C3A spheroids maintain important organ-like characteristics such as albumin and urea production and secretion, functional canalicular transporters, zonation, direct cell-cell contacts, CYP2E1 expression, as well as

a high predictive value of hepatotoxicity during drug screening (Gaskell et al., 2016).[33] The results revealed that the fatty C3A/LX-2 model displayed increased TG content compared to non-fatty spheroids, a featured attributed to the strong steatogenic property of OA pre-treatment in C3A hepatocytes.[27] In keeping with these findings, fatty spheroids showed upregulation of the lipogenesis-related gene *FAS* as well as downregulation of genes associated with lipid transport (*APOB* and *MTTP*), oxidation (*CPT1*) and lipolysis (*LIPC1*). It is important to note that these steatosis-related molecular hallmarks of the 3D *in vitro* model of NAFLD were similar with those observed in human liver disease and/or rodent models.[7,8]

The effects of sorafenib on lipid homeostasis have not been investigated in depth and, to the best of our knowledge, the present report is the first to describe sorafenib-mediated upregulation of *LIPC* and *CPT1* genes. Carnitine palmitoyltransferase 1 (CPT1) mediates the translocation of FAs from the cytosol to the mitochondrial matrix, thus promoting FA β-oxidation.[34] Experimentally, a moderate increase in CPT1 activity in diet-induced NASH in rats and in FA-treated isolated rat hepatocytes increases FA oxidation, resulting in the reduction of hepatocyte TG content.[34]. On the other hand, hepatic lipase (LIPC) catalyzes the hydrolysis of phospholipids and TG in lipoproteins, which increases FA availability. However, while LIPC-mediated lipoprotein remodeling seems to favor TG reesterification and accumulation in the liver *in vivo*[35], sorafenib-induced upregulation of *LIPC* may promote the release of FAs, thereby fueling further CPT1-mediated β-oxidation and contributing to lipid clearance in fatty spheroids. The finding that sorafenib stimulated the lipid oxidation-related *CPT1* gene is in accordance with the report by Stefano et al.[24] , demonstrating that sorafenib influences the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*)-coding gene *in vivo* leading to improvement of mitochondrial respiration. However, further clarification of the specific role of sorafenib in lipid clearance is needed.

The pre-treatment of C3A cells with OA clearly contributed to the enhancement of a fibrogenic phenotype in LX-2 cells after spheroid formation since the levels of *COL1A1*, *ACTA2*, *TGFB1*, *PDGF*, *TIMP1* mRNA and IL-6, TGF-β1 and TNF-α protein were increased. The cellular

and molecular landscape of HSC activation during NASH-related fibrogenesis is well characterized and involves the transition from a quiescent to a proliferative, contractile, migratory and fibrogenic phenotype.[36] When activated, HSCs are responsible for sustaining and promoting the deposition and remodeling of extracellular matrix (ECM), and for exacerbating the inflammatory response through the production of cytokines.[36] *In vitro* studies employing 2D and 3D transwell models have indicated that both cell-to-cell proximity and paracrine signaling between fatty hepatocytes and HSCs participate in HSC transactivation.[37,38] The initial triggers of HSC activation in NAFLD are thought to include lipotoxicity-induced oxidative stress, paracrine signals from fatty hepatocytes (as adipokines), and necrosis-induced cell death[18,36], the latter being a feature that is often observed in the inner core of spheroid models. Enhanced FA influx in the hepatocytes promotes the production of pro-inflammatory cytokines (e.g. IL-6, IL-8 and TNF-α) that are strongly correlated with HSC activation and survival[39], and the generation of lipotoxic metabolites that may ultimately culminate in oxidative stress and necrosis-induced cell death.[36] In addition, damage-associated molecular patterns and reactive oxygen species that are released following hepatocyte death or damage, as confirmed by the increased LDH levels in fatty spheroids observed in the present study, can stimulate receptors and downstream effectors in the HSCs leading to the epithelial-to-mesenchymal transition.[36,40] Furthermore, activated HSCs produce cytokines and growth factors such as IL-6 and TGF-β, which perpetuate their activation in an autocrine loop, followed by the production of ECM and the modulation of ECM-turnover mediators including TIMPs and MMPs.[41,42]

Exposure to sorafenib lowered the levels of PDGF mRNA and of both TGFB1 mRNA and TGF-β1 protein in fatty spheroids, similar to results obtained previously in 2D HSC monoculture models.[21,23] Both PDGF and TGF-β1 are potent mitogens for HSCs. The TGF-β1 ligand binds to type I receptors leading to downstream phosphorylation of the SMAD protein family and increasing the levels of type I and III collagen.[43] The PDGF and TGF-β1 ligands also activate MAPK signaling pathways, thus promoting HSC proliferation.[21,44] Moreover, it has been suggested that there is an interaction between PDGF and TGF-β1 in HSCs, since the latter upregulates PDGF-β receptor mRNA

via the PI3K/AKT/p70(S6K) signaling pathway, which is also a sorafenib target.[45] Considering that sorafenib is a universal tyrosine kinase inhibitor, the observed reductions in *PDGF* and *TGFB1* mRNAs and TGF-β1 protein clearly show that the drug inhibits HSC activation, as verified by the decrease in levels of *COL1A1* mRNA. Recently, increased *PDGFA*-related signaling was found to be involved in hepatic insulin resistance during NAFLD associated to T2D [46], which is featured in 23% of NAFLD patients[1]. Therefore, as observed in Sorafenib-treated fatty spheroids, PDGF negative modulation may be considered a potential avenue for halting the close interplay between T2D and NAFLD development.[47]

Sorafenib treatment led to a marked reduction in the level of IL-6 protein present in the culture medium of fatty spheroids. According to Su *et al.*[48], phosphorylation of signal transducer and activator of transcription 3 (STAT3), a downstream member of the IL-6 signaling pathway, is suppressed in HSC following treatment with sorafenib. It is likely that sorafenib interferes negatively with the IL-6 signaling cascade in HSC, which is potentially mediated by paracrine signaling between fatty hepatocytes and HSC[39], thereby contributing to the decrease in steatosis-induced upregulation of *COL1A1* in spheroids. In addition to hindering collagen synthesis, sorafenib may also promote collagen degradation since exposure to the drug led to a substantial downregulation of *TIMP1* mRNA indicating increased ECM degradation, an effect also observed previously in a LX-2 monoculture model and *in vivo.*[21,23]

Considering that fibrotic/cirrhotic backgrounds present an increased risk for HCC emergence[11–13], Sorafenib intervention – as proposed herein and in accumulating *in vivo* and *in vitro* data[21–24] – may exert direct and indirect preventive effects on the setting of HCC. While further investigation is warranted, Sorafenib treatment during NASH context could indirectly hinder the early stages of hepatocarcinogenesis by attenuating fibrosis or by directly acting on pre-malignant liver lesions, as demonstrated in a rodent model.[49] Preventive Sorafenib intervention could also be considered for individuals with increased risk for HCC recurrence, as those who had undergone loco-regional treatment of NAFLD-HCC. Further investigations considering the potential combinatorial effects of

Sorafenib with Statins (as Fluvastatin), angiotensin-II receptor blockers or fasting on attenuating liver fibrosis and/or preventing HCC are warranted.[50–52]

The multicellular spheroid model employed in this study was able to mimic in part the complexity of the NAFLD-related hepatic microenvironment, enabling the screening of Sorafenib intervention effects. However, some limitations can be pointed, as the unfeasibility on unveiling the adverse effects of Sorafenib in *in vitro* models, as skin reactions, diarrhea, and fatigue commonly observed in response to Sorafenib use for human HCC treatment.[53] Conversely, proactive management strategies for these side effects are currently encouraged.[54] Nonetheless, this is a matter of high importance for patient care and should addressed in further clinical studies. The high cost burden of this drug is also of concern during HCC treatment, but dose adjustment could be suggested in order to increase the cost-effectiveness in the context of both chronic antifibrotic and/or HCC treatments.[55]

In conclusion, the results obtained indicate that sorafenib applied at levels reflecting therapy-based serum concentrations exerts clear antifibrotic effects against steatosis-induced fibrogenesis in the 3D *in vitro* model of NAFLD. Although the effects of sorafenib on lipid metabolism and homeostasis require further investigation, the drug could be considered as a potential antifibrotic therapeutic agent in the clinical treatment of NAFLD/NASH patients.

Declaration of competing interests

The authors declare no competing interests that could have influenced the work reported in this paper.

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Figure captions

Fig. 1. Morphological, biochemical and ultrastructural features of non-fatty and fatty C3A/LX-2 spheroids. **(A)** Representative bright-field photomicrographs of time-dependent spheroid formation in Ultra-Low attachment plates after 24, 48 and 72h. The C3A/LX-2 spheroids display a well-defined and round morphology at both 48 and 72 h after seeding. **(B)** TEM ultrastructural features of fatty C3A/LX-2 spheroids at 72 h seeding, showing a C3A cell with clear lipid accumulation (white arrows, low electron-dense areas) and a LX-2 cell without lipid droplets accompanied by intra-spheroid collagen deposition (black arrow). **(C)** Representative photomicrograph of toluidine blue-stained semi-thin sections of non-fatty and fatty C3A/LX-2 spheroids after 72 h after seeding (200 × magnification), showing clear intracytoplasmic lipid accumulation (arrowheads) in fatty spheroids. **(D)** Biochemical evaluation of triglyceride content of non-fatty and fatty C3A/LX-2 spheroids at 72 h after seeding. **(E)** Representative photomicrographs of Sirius red-stained sections of non-fatty and fatty C3A/LX-2 spheroids after 72 h after seeding (200 × magnification). Steatosis-induced intra-spheroid collagen accumulation (arrowheads) is clear in fatty-spheroids while absent in non-fatty ones. **(F)** Evaluation of collagen morphometry (%) in Sirius red-stained sections in non-fatty and fatty C3A/LX-2 spheroids at 72 h after seeding. Data are expressed as mean values ± SEM. *Triglyceride and collagen contents of non-fatty and fatty spheroids are significantly different (Student´s *t* test; $p < 0.05$).

Fig. 2. Effects of sorafenib treatment on: (**A**) cell viability and (**B**) cytotoxicity of C3A/LX-2 spheroids at 24, 48 and 72 h after seeding. Non-fatty and fatty spheroids were formed by mixing C3A (5×10^4 cells/mL) and LX-2 (5×10^3cells /mL) in a 96-well plate. Treatments consisted of non-fatty spheroids (□), non-treated fatty spheroids (■), fatty spheroids + 2.5 µM sorafenib (■), fatty spheroids + 7.5 µM sorafenib (■) and fatty spheroids + 15 µM sorafenib (■). Data are expressed as mean values ± SEM of six subcultures per treatment. Within the same timepoint, bars bearing

different lowercase letters indicate statistically significant differences between groups (ANOVA and Tukey's test; $p < 0.05$).

Fig. 3. Effects of sorafenib treatment on the expression of genes related to lipid metabolism and fibrogenesis in C3A/LX-2 spheroids at 72 h after seeding: (**A**) relative gene expression and (**B**) heatmap analysis. Non-fatty and fatty spheroids were formed by mixing C3A (1.5×10^6 cells/mL) and LX-2 (1.5×10^5cells/mL) in a 6-well plate. Treatments consisted of non-fatty spheroids (□), non-treated fatty spheroids (▒), and fatty spheroids + 15 μM sorafenib (■). Data are expressed as mean values ± SEM of six subcultures per treatment. Bars bearing different lowercase letters indicate statistically significant differences between groups (ANOVA and Tukey's test; $p < 0.05$).

Fig. 4. Effects of sorafenib treatment on the levels of cytokines in C3A/LX-2 spheroid supernatants at 72 h after seeding. Non-fatty and fatty spheroids were formed by mixing C3A (5×10^4 cells/mL) and LX-2 (5×10^3cells/mL) in a 96-well plate. Treatments consisted of non-fatty spheroids (□), non-treated fatty spheroids (▒), and fatty spheroids + 15 μM sorafenib (■). Data are expressed as mean values ± SEM of six subcultures per treatment. Bars bearing different lowercase letters indicate statistically significant differences between groups (ANOVA and Tukey's test; $p < 0.05$).