Published in final edited form as:

Biochem Biophys Res Commun. 2016 January 15; 469(3): 463–469. doi:10.1016/j.bbrc.2015.10.101.

Deregulation of energy metabolism promotes antifibrotic effects in human hepatic stellate cells and prevents liver fibrosis in a mouse model[★]

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Abstract

Liver fibrosis and cirrhosis result from uncontrolled secretion and accumulation of extracellular matrix (ECM) proteins by hepatic stellate cells (HSCs) that are activated by liver injury and inflammation. Despite the progress in understanding the biology liver fibrogenesis and the identification of potential targets for treating fibrosis, development of an effective therapy remains elusive. Since an uninterrupted supply of intracellular energy is critical for the activated-HSCs to maintain constant synthesis and secretion of ECM, we hypothesized that interfering with energy metabolism could affect ECM secretion. Here we report that a sublethal dose of the energy blocker, 3-bromopyruvate (3-BrPA) facilitates phenotypic alteration of activated LX-2 (a human hepatic stellate cell line), into a less-active form. This treatment-dependent reversal of activated-LX2 cells was evidenced by a reduction in α -smooth muscle actin (α -SMA) and collagen secretion, and an increase in activity of matrix metalloproteases. Mechanistically, 3-BrPA-dependent antifibrotic effects involved down-regulation of the mitochondrial metabolic enzyme, ATP5E, and up-regulation of glycolysis, as evident by elevated levels of lactate dehydrogenase, lactate production and its transporter, MCT4. Finally, the antifibrotic effects of 3-BrPA were validated *in vivo* in a mouse model of carbon tetrachloride-induced liver fibrosis. Results from histopathology & histochemical staining for collagen and α -SMA substantiated that 3-BrPA

[★]This work was supported by the Abdulrahman Abdulmalik Research Fund and the Charles Wallace Pratt Research Fund.

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Conflicts of interests

B. Vogelstein has ownership interest (including patents) in PGDx and PapGene, Inc., is a consultant/advisory board member for Symex-Inostics. J.F. Geschwind is the CEO and Founder of Pre-Science Labs; Others declare no potential conflicts of interest.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2015.10.101>.

promotes antifibrotic effects *in vivo*. Taken together, our data indicate that sublethal, metronomic treatment with 3-BrPA blocks the progression of liver fibrosis suggesting its potential as a novel therapeutic for treating liver fibrosis.

Keywords

Metabolism; Liver fibrosis; 3-Bromopyruvate; Hepatic stellate cells

1. Introduction

Liver fibrosis and cirrhosis occur as a result of chronic inflammatory injury to the liver parenchyma. Irrespective of the primary cause, liver fibrosis eventually leads to cirrhosis and liver failure [1]. The pathogenesis of liver fibrosis involves progressive replacement of normal hepatic parenchyma with collagen-rich extracellular matrix (ECM) [2]. The principle cells responsible for liver fibrosis are hepatic stellate cells (HSCs) [3]. In chronic liver injury, frequent and overlapping phases of uncontrolled inflammatory and wound-healing processes result in the constant activation of HSCs leading to increased deposition and decreased degradation of collagen [4] with an estimated 4–8 fold more ECM than non-fibrotic livers [5,6]. Thus, the active-HSCs that contribute to excess accumulation of ECM in hepatic fibrogenesis remain an ideal target for anti-fibrotic therapy.

In advanced liver fibrosis or cirrhosis, there is an increased energy demand associated with increased synthesis and secretion of ECM [7,8]. Activated-HSCs are functionally dependent on a constant supply of intracellular ATP to maintain ECM synthesis and secretion. This energy demand provides an opportunity to interfere with the function of activated-HSCs. Hence we hypothesized that selective targeting of energy metabolism in activated-HSCs may be an effective antifibrotic strategy. The pyruvate analog, 3-bromopyruvate (3-BrPA) is an energy blocker that has been validated for the treatment of multiple types of malignancies (refer reviews [9,10]). Recently, we developed a microencapsulated formulation of 3-BrPA using β -cyclodextrin (β -CD) which is relevant for systemic delivery [11]. The aim of the current study is to determine the effects of sublethal dose of 3-BrPA on activated-HSCs *in vitro* and *in vivo*, and to validate that targeting energy metabolism is a rational and viable strategy to treat liver fibrosis.

2. Materials and methods

2.1. Chemicals, reagents and media

Unless otherwise mentioned, all chemicals including 3-BrPA were purchased from Sigma-Aldrich Co., (St. Louis, MO, USA). Cell culture media, antibiotics and geltrex were procured from Invitrogen/Life Technologies Inc., (Carlsbad, CA, USA). Chamber slides used for confocal microscopy were purchased from Nalgene/Nunc Inc., (Waltham, MA, USA). Primary antibodies used for immunoblotting and immunofluorescence were MMP-2, MMP-9 and cytokeratin-18 (CK-18) (Cell Signaling Inc., Danvers, MA), Monocarboxylate Transporter (MCT)-4, MCT-1, Adenine tri phosphatase 5E (ATP5E) and lactate dehydrogenase A (LDH-A) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and α -smooth

muscle actin (α -SMA) and anti-leptin antibodies (Sigma Aldrich Co.). Secondary antibodies were purchased from Santa Cruz Biotechnology Inc.

2.2. Cell culture

LX-2, a human stellate cell line, was originally gifted by Dr. Scott L. Friedman from the Mount Sinai School of Medicine (New York, NY, USA). LX-2 cells when cultured on regular tissue culture plasticwares exhibit fibrogenic phenotype hence referred as activated-LX-2 [12]. De-activated (quiescent) LX-2 cells were generated by culturing them on Matrigel [12–14] and were used as positive controls. The activated and de-activated LX-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 0.1% antibiotic (Penicillin-Streptomycin) and 0.1% Fungizone as described previously [15]. Human primary hepatocytes were obtained from Invitrogen and cultured in William's medium (without phenol red) along with plating and maintenance supplements.

2.3. Metronomic therapy for activated-LX-2 cells

To analyze the effect of 3-BrPA on activated-LX-2 cells, the principle of metronomic therapy, a repeated but low, nonlethal dose of 3-BrPA (25 μ M) was adopted. The maximum tolerated, nonlethal dose was determined by cell viability/proliferation assays. For treatment, a known number of cells were plated in 6-well plates, 10 cm petri dishes or chamber slides as required and the culture media was replaced with fresh media containing the drug(s) on alternative days. The cells were treated for a period of 13 days for immunofluorescence studies and 6 days for all other studies including biochemical analysis. Monotherapy involved treating the cells with 3-BrPA alone or AICAR (0.5 mM) in DMEM. The concentration of AICAR was chosen based on the literature [16]. Combination therapy involved using half of the dose used for the monotherapy of AICAR and 3-BrPA.

2.4. Immunofluorescence (confocal microscopy)

LX-2 cells grown in chamber slides were fixed with 4% formaldehyde for 10 min, rapidly rehydrated with Phosphate-buffered saline (PBS) and permeabilized with a 0.2% Triton X-100 solution in PBS for imaging of MCT-1, MCT-4 and ATP5E. The cells were incubated with primary antibodies in PBS for 1 h at room temperature in a humidified chamber. They were then washed and incubated at room temperature with secondary antibody labeled with Texas Red or FITC (CK-18) for 30 min, followed by nuclear staining with DAPI (300 nM) for 1–5 min. The slides were mounted with ProLong Gold anti-fade (Invitrogen, USA) mounting media. Images were acquired with the Zeiss 510 Meta LSM Confocal Microscope.

2.5. Immunoblotting

For immunoblotting, control and treated cells were washed in PBS, lysed in RIPA lysis buffer (Sigma–Aldrich) containing protease and a phosphatase inhibitor cocktails (both from Sigma–Aldrich) at 4°C using a dounce homogenizer. The lysates were centrifuged at 12,000 $\times g$ for 15 min at 4°C to remove any cell debris. The clear supernatants were collected and the protein concentrations were determined using a 2-D Quant protein assay Kit (GE Healthcare) as described [17]. Then, protein samples were resolved on a 4–12% Bis-Tris gel by electrophoresis with MOPS buffer or 12% Bis-Tris gel electrophoresis with MES running

buffer (for low molecular weight targets) and blotted onto PVDF membranes (BioRad, Hercules, CA, USA) followed by immunoblotting with specific antibodies. Immune complexes were visualized by ECL-detection kit (GE Healthcare).

2.6. Gelatin zymography for matrix metalloproteinase (MMP) activity

The level of MMP activity in the conditioned medium of control and treated LX-2 cells was quantified by gelatin zymography. In brief, upon the completion of drug treatment protocol, media from both control and treated cells were removed, cells were rinsed with PBS and replaced with serum-free DMEM media and cultured overnight at 37°C prior to the collection of media containing secreted proteins for further analysis. The media thus collected were concentrated using a 3000Da centrifugal filter (Amicon Inc., Pineville, NC, USA) and stored at -80°C until further analysis. Protein (~10 µg) samples from the conditioned media of control and treated cells were subjected to zymography as described [18]. MMP activity was visualized as clear bands. The identity of the MMP associated with zymogram signal (activity) was verified by immunoblot analysis.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Gene expression analysis was performed by using qRT-PCR with a sequence detection system (ABI 7900HT; Applied Biosystems, Bedford, MA, USA). The total RNA was extracted and the cDNA thus synthesized were subjected to qRT-PCR analysis using TaqMan PCR Kit for Coll 1α (Mm 00801666_g1). 18S was used as internal control (Applied Biosystems).

2.8. Two dimensional (2D) gel electrophoresis

For 2D gel electrophoresis the cells were washed in ice-cold PBS and lysed using 2% SDS in PBS with protease and phosphatase inhibitors. The lysate was then sonicated at 15% amplitude for 9 s and centrifuged to remove any precipitate. Protein samples were subjected to 2D clean up (GE Healthcare Inc., Piscataway NJ, USA) and re-suspended with DeStreak Rehydration solution. For the first dimension, samples were loaded on to immobilized pH 3–10 IEF strips (GE Healthcare) and focused for 16 h from 50 V–3000 V. The second dimension, SDS- PAGE was carried out using 4–12% Bis-Tris ZOOM gels for 2 h at room temperature. The proteins were then transferred on to PVDF membranes for detection by immunoblotting as described elsewhere.

2.9. Animal studies

Male mice (*C57BL/6*) 3–4 weeks of age were purchased from Charles River laboratories (Wilmington, MA, USA) and maintained in a temperature-controlled room with an alternating 12 h dark and light cycle [19]. Animal studies were performed as approved by the Johns Hopkins University Animal Care and Use Committee. All animal experiments were conducted in accordance with the approved protocol as per the guidelines and regulations of the institute. Microencapsulated 3-BrPA (β-CD-3-BrPA) used for animal experiments was prepared as described [11]. Mice were randomly divided into control (vehicle-olive oil) group, CCl₄ (fibrosis) group, and CCl₄ + β-CD-3-BrPA (treatment of fibrosis) group. Ten mice received intra-peritoneal injection of CCl₄ biweekly as 100 µL of a

2.5% solution of CCl₄ in olive oil per gram body weight. Five of these mice were treated with β -CD-3-BrPA at a dose of 1 mg/kg body weight the day after CCl₄ injection. Five control mice received an isovolumetric dose of olive oil as that of the CCl₄. The biweekly treatment regimen was carried out for 4 weeks at the end of which the animals were sacrificed. At the time of sacrifice liver was removed and either (a) fixed in 10% buffered formaldehyde and embedded in paraffin, or (b) homogenized in RIPA buffer and stored at -80°C for biochemical assays.

2.10. Histology and immunofluorescence

Liver tissues from different animal groups were fixed in 10% phosphate-buffered formalin (Polysciences, Warrington, PA), dehydrated with graded ethanol, embedded in wax (Paraplast Plus; McCormick Scientific, Richmond, IL), sliced at 5 mm, mounted on slides, and oven dried and deparaffinized and subjected to hematoxylin-eosin staining as described [20]. Immunofluorescence staining for the profibrotic marker, α -SMA was performed as described with few modifications. In brief, prior to primary antibody incubation, tissue sections were subjected to deparaffinization, antigen retrieval and blocking with 10% goat serum. Rest of the protocol for immune staining was performed as described before. The slides were viewed with fluorescent microscopy, and the images were acquired with Zeiss 510 Meta LSM Confocal Microscope.

2.11. Collagen staining

Tissue sections were deparaffinized in xylene followed by rehydration through graded series of alcohol followed by staining for collagen using Masson's Trichrome stain (Sigma Aldrich, St. Louis, MO) or Sirius Red stain (PolySciences Inc. Warrington, PA). For *in vitro* studies, the LX-2 cells were grown in chamber slides containing appropriate growth medium. They were fixed with 4% formaldehyde for 10 min, rapidly rehydrated with Phosphate-buffered saline (PBS), following which, the staining was performed.

2.12. Statistical analysis

All experiments were repeated at least thrice with triplicates each time. The mean and the standard error were calculated. Data were analyzed using Student's *t* test or by two way analysis of variance (ANOVA) when comparing means of more than two groups.

3. Results

3.1. 3-BrPA deactivates the phenotype of activated-LX-2 cells

De-activated LX-2 cells were prepared as described in the methods and used as positive control. We determined the MTD of activated-LX-2 cells to be 25 μ M 3-BrPA. 3-BrPA treatment of activated-LX-2 cells induced changes in phenotype including an elongated, spindle-shaped morphology which resembled the normal, de-activated LX-2 cells (Fig. 1A). To determine if the phenotypic alterations of the activated-LX-2 cells are dependent on cellular stress, we treated the cells with an energy stress inducer, AICAR which is known to increase cellular ROS levels. Surprisingly, AICAR did not induce any change in morphology (Fig. 1A), whereas 3-BrPA promoted the changes even in the presence of AICAR. (Fig. 1A). The phenotypic changes in activated-LX-2 cells treated with 3-BrPA were stable, even after

withdrawal of the drug (Fig. 1B). We then investigated whether the effect of 3-BrPA is specific to activated-LX-2 cells, as nonspecific targeting may cause unwanted toxicity to hepatocytes. Results showed that at sublethal dose 3-BrPA did not affect the viability of human hepatocytes (Fig. 1C,D). Thus, a metronomic treatment with low dose 3-BrPA selectively promotes alterations in activated-LX-2 cells that alters the phenotype to resemble a de-activated LX-2 cell.

3.2. 3-BrPA-treatment affects the profibrogenic capacity of activated-LX-2 cells

Activated-LX-2 cells exhibit a high level of α -SMA and CK-18 expression, collagen secretion, and reduced level of extracellular MMPs, representing the typical profibrogenic phenotype. 3-BrPA treatment reduced the level of α -SMA in activated-LX-2 cells (Fig. 2A,B) and elevated the secretion of MMPs (Fig. 2C). 3-BrPA decreased liver collagen and α_1 (I) collagen mRNA (Fig. 2D,E). A prominent decrease in the level of CK-18 was also observed in 3-BrPA treated activated-LX-2 cells (Fig. 2F). Thus, 3-BrPA treatment reversed the biochemical (α -SMA, extracellular MMP activity, CK-18) and functional markers (secretion of MMPs and collagen) of profibrogenic, activated-LX-2 cells to resemble de-activated HSCs.

3.3. 3-BrPA treatment deregulates energy metabolism in activated-LX-2 cells

Cellular uptake of 3-BrPA is dependent on the expression of MCT-1. Our data show that activated LX-2 cells express MCT-1 and this is unaffected by 3-BrPA treatment (Fig. 3A). 3-BrPA treatment significantly downregulated the expression of mitochondrial-membrane bound enzyme, ATP5E (also known as F_1F_0 ATPase) (Fig. 3B,D) indicating a decrease in mitochondrial function. Notably, the expression levels of the lactate transporter, MCT-4 (Fig. 3C,D) and the enzyme lactate dehydrogenase (LDH) were increased (Fig. 3D). In corroboration, 3-BrPA treatment also increased the level of lactate secretion (Fig. 3E) indicating a metabolic reprogramming towards increased rate of glycolysis.

3.4. Microencapsulated 3-BrPA blocked the progression of liver fibrosis in vivo

In vivo validation of the antifibrotic effects of 3-BrPA was performed in CCl_4 -induced liver fibrosis in *C57BL/6* black mice. As mentioned elsewhere, due to the short half-life of 3-BrPA, for the *in vivo* experiments we used our recently developed micro-encapsulated 3-BrPA using β -cyclodextrin (β -CD) [11]. β -CD-3-BrPA treatment decreased hepatocyte ballooning degeneration, necrosis (Fig. 4A) and fibrosis induced by CCl_4 (Fig. 4B,C). The fibrotic markers α -SMA and leptin decreased in β -CD-3-BrPA treated animals (Fig. 4D,E). Immunoblot analysis of liver tissue from the control and treated animals showed a β -CD-3-BrPA-dependent elevation in the level of MCT-4 with a concomitant reduction in mitochondrial-membrane bound enzyme, ATP5E (Fig. 4E). The results indicate that the antifibrotic effects of 3-BrPA involves deregulation of energy metabolism in this mouse model of liver fibrosis. (Fig. 4E).

4. Discussion

The present study demonstrates that sublethal dose of 3-BrPA effectively reverses the profibrogenic phenotype of HSCs *in vitro*, and blocks the progression of liver fibrosis *in*

vivo. Notably, we also demonstrate that the principle mechanism underlying 3-BrPA-mediated effect involves deregulation of energy metabolism as evident by a decrease in the level of mitochondrial ATP5E with a corresponding increase in the level of the glycolytic enzyme LDH-A, the lactate transporter MCT-4 and increased cellular lactate secretion. The indicated dose of 3-BrPA was not toxic to human primary hepatocytes (*in vitro*) and mouse hepatocytes (*in vitro* and *in vivo*) (data not shown). Thus, the efficacious antifibrotic dose of 3-BrPA is also safe for normal hepatocytes. In liver fibrosis or cirrhosis, preservation or conservation of healthy hepatocytes is critical for the maintenance of functional liver [21]. Collectively, the findings of the current study are in congruent with a potentially effective antifibrotic agent: selective targeting of activated-HSCs, reversal of functional morphology, and lack of toxic effects on primary hepatocytes [22].

The validation of our findings relies on several lines of experimental evidence. 3-BrPA dependent phenotypic alterations were compared with a (positive) control represented by de-activated LX-2 that mimics normal HSCs. 3-BrPA dependent phenotypic alteration of activated-LX-2 cells was substantiated by a decrease in the fibrotic markers α -SMA and CK-18, a decrease in collagen mRNA, and a corresponding increase in MMPs. Furthermore, the effect of 3-BrPA on fibrotic markers were also validated in rat HSCs (data not shown). 3-BrPA-mediated reversal of activated-LX-2 (fibrogenic) into de-activated (non-fibrogenic) phenotype was stable indicating the prevention of recurrence, a potential concern in treating fibrosis [23,24]. Finally, the mechanism, i.e. metabolic reprogramming towards glycolysis, was validated both *in vitro* and *in vivo*. Importantly, 3-BrPA could promote its antifibrotic effects even in the presence of a stress inducer like AICAR. Intracellular stress and its enhancers (e.g. reactive oxygen species (ROS)) play a pivotal role in the propagation of fibrosis, and its progression towards cirrhosis [25,26] and carcinogenesis [27,28]. Thus an effective antifibrotic agent potentially should have the ability to promote its effects despite the presence of stress inducers.

Interestingly, unlike 3-BrPA's anticancer effects which rely on the inhibition of glycolysis, its antifibrotic effects in activated-HSCs primarily involve down-regulation of mitochondrial ATP5E while facilitating glycolysis. This biochemical paradox is comprehensible due to the fact that in order to achieve antiglycolytic, anticancer effects 3-BrPA is required at a significantly higher dose [8–10 fold *in vitro* [29] or 4–5 fold *in vivo* [30]]. It has been known that fibrotic or cirrhotic cells require enormous amount of intracellular energy to meet their energy demands owing to increased synthetic and secretory activities. This in turn necessitates the prevalence of functionally efficient glycolytic and mitochondrial metabolic pathways. Chen and co [30] have demonstrated that blocking hedgehog signaling pathway that affects glycolysis resulted in the mitigation of fibrotic phenotype. Here we demonstrate that selective targeting of mitochondrial-ATP5E is sufficient to block the progression of activated-HSCs. In conclusion, this study shows that targeting energy metabolism of activated-HSCs impairs their functional capacity resulting in the inhibition of profibrotic phenotype. Further studies to validate the efficacy and relevance of targeting energy metabolism as a therapeutic strategy are needed to determine its translational potential for the treatment of liver fibrosis and cirrhosis.

Acknowledgments

The authors gratefully acknowledge the assistance of Dr. Esther Kieserman and Dr. Barbara Smith from the Microscope facility, Johns Hopkins University School of Medicine.

List of Abbreviations

3-BrPA	3-bromopyruvate
α-SMA	α -smooth muscle actin
CK-18	Cytokeratin-18
MCT	Monocarboxylate Transporter
ECM	Extracellular matrix

References

1. Pinzani M, Rosselli M, Zuckermann M. Liver cirrhosis. *Best Pract Res Clin Gastroenterol.* 2011; 25:281–290. [PubMed: 21497745]
2. Gines P, Fernandez J, Durand F, Saliba F. Management of critically-ill cirrhotic patients. *J Hepatol.* 2012; 56(Suppl. 1):S13–S24. [PubMed: 22300462]
3. Wu J, Zern MA. Hepatic stellate cells: a target for the treatment of liver fibrosis. *J Gastroenterol.* 2000; 35:665–672. [PubMed: 11023037]
4. Ahmad A, Ahmad R. Understanding the mechanism of hepatic fibrosis and potential therapeutic approaches. *Saudi J Gastroenterol.* 2012; 18:155–167. [PubMed: 22626794]
5. Iredale JP, Thompson A, Henderson NC. Extracellular matrix degradation in liver fibrosis: biochemistry and regulation. *Biochim Biophys Acta.* 2013; 1832:876–883. [PubMed: 23149387]
6. Wells RG. Cellular sources of extracellular matrix in hepatic fibrosis. *Clin Liver Dis.* 2008; 12:759–768. viii. [PubMed: 18984465]
7. Ganapathy-Kanniappan S, Karthikeyan S, Geschwind JF, Mezey E. Is the pathway of energy metabolism modified in advanced cirrhosis? *J Hepatol.* 2014; 61:452. [PubMed: 24810232]
8. Nishikawa T, Bellance N, Damm A, Bing H, Zhu Z, Handa K, Yovchev MI, Sehgal V, Moss TJ, Oertel M, Ram PT, Pipinos II, Soto-Gutierrez A, Fox IJ, Nagraath D. A switch in the source of ATP production and a loss in capacity to perform glycolysis are hallmarks of hepatocyte failure in advance liver disease. *J Hepatol.* 2014; 60:1203–1211. [PubMed: 24583248]
9. Ganapathy-Kanniappan S, Vali M, Kunjithapatham R, Buijs M, Syed LH, Rao PP, Ota S, Kwak BK, Loffroy R, Geschwind JF. 3-bromopyruvate: a new targeted antiglycolytic agent and a promise for cancer therapy. *Curr Pharm Biotechnol.* 2010; 11:510–517. [PubMed: 20420565]
10. Ganapathy-Kanniappan S, Kunjithapatham R, Geschwind JF. Anticancer efficacy of the metabolic blocker 3-bromopyruvate: specific molecular targeting. *Anticancer Res.* 2013; 33:13–20. [PubMed: 23267123]
11. Chapiro J, Sur S, Savic LJ, Ganapathy-Kanniappan S, Reyes J, Duran R, Thiruganasambandam SC, Moats CR, Lin M, Luo W, Tran PT, Herman JM, Semenza GL, Ewald AJ, Vogelstein B, Geschwind JF. Systemic delivery of microencapsulated 3-bromopyruvate for the therapy of pancreatic cancer. *Clin. Cancer Res.* 2014; 20:6406–6417.
12. Gaca MD, Zhou X, Issa R, Kiriella K, Iredale JP, Benyon RC. Basement membrane-like matrix inhibits proliferation and collagen synthesis by activated rat hepatic stellate cells: evidence for matrix-dependent deactivation of stellate cells. *Matrix Biol.* 2003; 22:229–239. [PubMed: 12853033]
13. Sohara N, Znorko I, Levy MT, Trojanowska M, Reuben A. Reversal of activation of human myofibroblast-like cells by culture on a basement membrane-like substrate. *J Hepatol.* 2002; 37:214–221. [PubMed: 12127426]

14. Troeger JS, Mederacke I, Gwak GY, Dapito DH, Mu X, Hsu CC, Pradere JP, Friedman RA, Schwabe RF. Deactivation of hepatic stellate cells during liver fibrosis resolution in mice. *Gastroenterology*. 2012; 143:1073–83.e22. [PubMed: 22750464]
15. Tang M, Potter JJ, Mezey E. Activation of the human alpha1(I) collagen promoter by leptin is not mediated by transforming growth factor beta responsive elements. *Biochem Biophys Res Commun*. 2003; 312:629–633. [PubMed: 14680811]
16. Lim JY, Oh MA, Kim WH, Sohn HY, Park SI. AMP-activated protein kinase inhibits TGF-beta-induced fibrogenic responses of hepatic stellate cells by targeting transcriptional coactivator p300. *J Cell Physiol*. 2012; 227:1081–1089. [PubMed: 21567395]
17. Kunjithapatham R, Karthikeyan S, Geschwind JF, Kieserman E, Lin M, Fu DX, Ganapathy-Kanniappan S. Reversal of anchorage-independent multicellular spheroid into a monolayer mimics a metastatic model. *Sci Rep*. 2014; 4:6816. [PubMed: 25351825]
18. Wang L, Potter JJ, Rennie-Tankersley L, Novitskiy G, Sipes J, Mezey E. Effects of retinoic acid on the development of liver fibrosis produced by carbon tetrachloride in mice. *Biochim Biophys Acta*. 2007; 1772:66–71. [PubMed: 17011172]
19. Ganapathy-Kanniappan S, Kunjithapatham R, Torbenson MS, Rao PP, Carson KA, Buijs M, Vali M, Geschwind JF. Human hepatocellular carcinoma in a mouse model: assessment of tumor response to percutaneous ablation by using glyceraldehyde-3-phosphate dehydrogenase antagonists. *Radiology*. 2012; 262:834–845. [PubMed: 22357885]
20. Fausto N, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mech Dev*. 2003; 120:117–130. [PubMed: 12490302]
21. Anders HJ, Vielhauer V. Identifying and validating novel targets with in vivo disease models: guidelines for study design. *Drug Discov Today*. 2007; 12:446–451. [PubMed: 17532528]
22. Friedman SL. Evolving challenges in hepatic fibrosis. *Nat Rev Gastroenterol Hepatol*. 2010; 7:425–436. [PubMed: 20585339]
23. Schuppan D, Kim YO. Evolving therapies for liver fibrosis. *J Clin Invest*. 2013; 123:1887–1901. [PubMed: 23635787]
24. Hernandez-Gea V, Hilscher M, Rozenfeld R, Lim MP, Nieto N, Werner S, Devi LA, Friedman SL. Endoplasmic reticulum stress induces fibrogenic activity in hepatic stellate cells through autophagy. *J Hepatol*. 2013; 59:98–104. [PubMed: 23485523]
25. Paik YH, Kim J, Aoyama T, De Minicis S, Bataller R, Brenner DA. Role of NADPH oxidases in liver fibrosis. *Antioxid Redox Signal*. 2014; 20:2854–2872. [PubMed: 24040957]
26. Han Y, Chen JZ. Oxidative stress induces mitochondrial DNA damage and cytotoxicity through independent mechanisms in human cancer cells. *Bio-med Res Int*. 2013; 2013:825065.
27. Sosa V, Moline T, Somoza R, Paciucci R, Kondoh H, LLeonart ME. Oxidative stress and cancer: an overview. *Ageing Res Rev*. 2013; 12:376–390. [PubMed: 23123177]
28. Ganapathy-Kanniappan S, Geschwind JF, Kunjithapatham R, Buijs M, Syed LH, Rao PP, Ota S, Kwak BK, Loffroy R, Vali M. 3-bromopyruvate induces endoplasmic reticulum stress, overcomes autophagy and causes apoptosis in human HCC cell lines. *Anticancer Res*. 2010; 30:923–935. [PubMed: 20393016]
29. Schaefer NG, Geschwind JF, Engles J, Buchanan JW, Wahl RL. Systemic administration of 3-bromopyruvate in treating disseminated aggressive lymphoma. *Transl Res*. 2012; 159:51–57. [PubMed: 22153810]
30. Chen Y, Choi SS, Michelotti GA, Chan IS, Swiderska-Syn M, Karaca GF, Xie G, Moylan CA, Garibaldi F, Premont R, Suliman HB, Piantadosi CA, Diehl AM. Hedgehog controls hepatic stellate cell fate by regulating metabolism. *Gastroenterology*. 2012; 143:1319–29.e1–11. [PubMed: 22885334]

Fig. 1. 3-BrPA treatment alters the phenotype of activated-LX-2 cells. (A) Morphology of activated-LX-2 cells upon treatment with AICAR, 3-BrPA (25 μ M), a combination of both and de-activated LX-2 cells. (B) LX-2 cells treated with 3-BrPA, and 6 days post-treatment. Scale = 0.5 mm. (C) Human hepatocytes treated with 3-BrPA. Scale = 100 μ m. (D) Cell viability assay showing that human hepatocytes remain unaffected.

Fig. 2.

3-BrPA treatment affects the fibrogenic capacity of activated-LX-2 cells. (A) Immunofluorescent images of α -SMA. Bar graph represents the quantification of mean fluorescent intensity from at least triplicate experiments. Scale = 20 μ m. (B) 2D-gel immunoblots of α -SMA protein. (C) Zymogram and corresponding immunoblots of MMP-2 and MMP-9. (D) Trichrome staining of collagen. Insert shows a higher magnification of a region of interest. Scale = 0.5 mm, insert = 2.5 mm. (E) α_1 (I) collagen mRNA. (F) Immunofluorescent images of cytokeratin (CK)-18 Scale = 20 μ m. Bar graph represents the quantification of mean fluorescent intensity from at least triplicate experiments.

Fig. 3.

3-BrPA treatment deregulates energy metabolism and facilitates glycolysis in activated-LX-2 cells. Immunofluorescent images showing (A) MCT-1 to facilitate cellular uptake of 3-BrPA in LX-2 cells, (B) A decrease in the expression level of F_1F_0 ATPase (ATP5E) and (C) an increase in the expression of MCT-4 (lactate transporter) in 3-BrPA treated LX-2 cells. Scale: left = 20 μm; right = 40 μm. (D) Immunoblot showing 3-BrPA-dependent changes in the level of protein expression and numerical data represent the densitometric quantification of corresponding signals. (E) Lactate secretion in LX-2 cells. MCT-1; monocarboxylate transporter (MCT) 1, ATP5E; Adenine tri phosphatase 5E, LDH-A; lactate dehydrogenase A.

Fig. 4.

β-CD-3-BrPA treatment blocks the progression of fibrosis *in vivo*. (A) H & E staining showing changes in liver pathology in CCl₄ and β-CD-3-BrPA-treated mice. Scale: top panel = 20 μm, bottom panel = 100 μm. Liver sections stained with (B) Trichrome and (C) Sirius red. Scale: For (B) top panel = 200 μm, bottom panel = 100 μm and for (C) top panel = 20 μm, bottom panel = 100 μm. The level of fibrosis was measured by Sirius red staining and densitometry of various groups. The density of fibrosis was determined as intensity of Sirius red staining, divided by the area of the captured field. Total of 30 fields were captured from livers of each group of mice. The values are expressed as means ± S.E. (D) Representative confocal microscopic images showing the level of α-SMA expression with quantitative analysis of the fluorescent intensities from triplicates. Scale = 20 μm. (E) Immunoblot showing levels of leptin, ATP5E and MCT-4.