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Original Article

Inhibitory effects of dimethyl α-ketoglutarate in hepatic stellate cell activation

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Abstract: Aim: The activation of Hepatic stellate cell (HSC) is a pivotal event in the initiation and progression of hepatic fibrosis and a major source of collagen deposition. A recent study found that autophagy fuels the HSC activation. α-ketoglutarate (AKG), an intermediate in the Kerbs CYCLE, has been shown to regulate the level of autophagy. In this study, we aim to investigate the potential effect of dimethyl α-ketoglutarate (DMKG), a membrane-permeable esters of AKG, on the activation of HSC. Methods: HSC and hepatocyte cell lines were treated with DMKG at gradient concentrations, MTT assay was used to assess the cell viability. Concentrations of DMKG that did not affect the cell survival were added to the culture media of HSC cells. Real-time PCR and western blot analysis was carried out to evaluate the expression of fibrogenic genes in HSC after culture for 24 hours. Results: Low dose of DMKG had little cytotoxicity to both HSCs and hepatocytes, while HSCs were more vulnerable to high dose of DMKG than hepatocytes. More importantly, DMKG inhibited the expression of α-SMA and collagen I significantly in HSCs detected by real-time PCR and western blot analysis at the concentrations that didn't decrease cell viability. Conclusions: DMKG has a significant role of inhibiting the activation of HSC and may attenuate hepatic fibrosis safely.

keywords: Hepatic fibrosis, autophagy, Dimethyl α-ketoglutarate, extracellular matrix, hepatic stellate cell

Introduction

Hepatic fibrosis represents an important cause of mortality worldwide, which is the consequence of sustained liver injury, including viruses, alcoholism, autoimmune hepatitis, and metabolic diseases. Hepatic fibrosis is characterized by excessive deposition of extracellular matrix proteins (ECM), predominantly type I collagen, and activated Hepatic stellate cells (HSCs) are considered to be a major source of ECM [1-3].

In response to hepatic injury, quiescent HSCs characterized as vitamin A storing cells transdifferentiate into myofibroblast-like cells marked by loss of retinyl ester stores, expression of α-smooth muscle actin (α-SMA), and excessive production of ECM [4]. Myofibroblastic HSCs secrete and respond to a variety of profibrogenic cytokines, such as transforming growth factor-β (TGFβ), Platelet-derived growth factor (PDGF) [5]. Whereas the signals that promote the activation of HSC during fibrogenesis are increasingly clarified, few therapeutic strategies exist to treat hepatic fibrosis, which may be attributed to the complexity of signal pathways involved in HSC activation [6].

A recent study identified that autophagy fuels the activation of HSC activation [7, 8], and some other studies carried out by Marino et al. [9-11] demonstrated that α-ketoglutarate (AKG) can decrease autophagy safely. Currently, no studies have examined the role of AKG in HSC activation and hepatic fibrosis.

We found that Dimethyl α-ketoglutarate (DMKG), membrane-permeable esters of AKG, is safe to hepatocytes, and can dramatically inhibit HSC activation and reduce the production of collagen I.

Materials and methods

Cell culture and treatment

The rat immortalized stellate cell line HSC-T6 within this study is an activated HSC phenotype

[12], which was obtained from the Institute of Basic Medical Sciences of Qilu Hospital and originally from Liver Disease Research Center of San Francisco General Hospital, CA, USA. The rat liver cell line BRL-3A was purchased from Chinese Academy of Science (Shanghai, PR China). All the culture media were Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA), and cells were grown and maintained in an atmosphere of 5% CO_2 at 37°C. For all experiments, cells were seeded on tissue culture dishes and left overnight to attach and stabilize before the addition of Dimethyl α-ketoglutarate (DMKG, TCI, Japan). DMKG at gradient concentration from 1 mM to 32 mM was added to culture media in MTT assay, and doses at 1 mM and 4 mM that did not affect cell viability were used in real-time PCR and western blot analysis.

MTT assay for cellular cytotoxicity

The effect of DMKG on viability of HSC-T6 and BRL-3A cells was assessed by MTT proliferation assay as described previously [13, 14]. 1×10^5 cells with 100 μL culture medium were plated in each well of 96-well culture plate, incubated in the CO_2 incubator at 37°C overnight, and treated with DMKG (0, 1 mM, 4 mM, 8 mM, 16 mM, 18 mM, 20 mM, 22 mM, 24 mM, 26 mM, 28 mM, 30 mM, 32 mM) for 24 h. At least four replicates were used for each treatment. Thereafter, 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) solution (5 mg/mL) was added per well and the cells were incubated for an additional 4 hours. At the completion of incubation period, the supernatants were discarded carefully and colored formazan crystals were dissolved by adding 150 μl Dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) to each well and vibrating for 10 min at room temperature. The optical density (OD) was read at 490nm by use of an automated plate reader, with DMSO used as the blank. The experiments were repeated thrice.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from cells using Trizol (Takara, Japan) from HSC-T6 cells. All RNA samples were treated with gDNA Eraser (Takara, Japan) to remove residual genomic DNA contamination before performing reverse transcription of total RNA. Reverse transcription of total RNA to cDNA was performed using PrimeScript RT reagent Kit (Takara, Japan) as described by manufacturer's protocol. Real-time reverse transcription PCR analysis was done with the Light Cycler 2.0 System (Roche, Mannheim, Germany) in 10 μL reactions containing 1 μL of cDNA, 1 μL of forward and reverse primers (10 μmol/L), 5μL SYBR Green 1 Master Mix (Takara, Japan), and 2 μL RNase-free H_2O. The reactions were carried out with the following cycling variables: an initial denaturation of 30 seconds at 95°C, then amplified for 40 cycles (5 seconds at 95°C, 5 seconds at 57°C, 30 seconds at 72°C) and followed by a final extension step. The relative amount of mRNA levels was calculated by $2^{-\Delta\Delta CT}$ method [15]. Relative gene expression was normalized to β-actin expression. The primers used for PCR amplification were as follows: α-SMA: 5'-AGT CGC CAT CAG GAA CCT CGA G-3' and 5'-ATC TTT TCC ATG TCG TCC CAG TTG-3', collagen I: 5'-GGA GAG AGC ATG ACC GAT GG-3' and 5'-GGG ACT TCT TGA GGT TGC CA-3', β-actin: 5'-CGT TGA CAT CCG TAA AGA CC-3' and 5'-TAG AGC CAC CAA TCC ACA CA-3'.

Western blot analysis

Whole-cell lysates were made in chilled RIPA buffer (Beyotime, China) of 50 mM Tris (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail with 1 mM phenylmethanesulfonyl fluoride (Beyotime, China), and boiled in 5× loading buffer. After separation by SDS-PAGE, proteins were electroblotted to a PVDF membrane (Millipore, USA), which was then blocked with 5% skim milk in TBST for 2 h. After that, the membrane was first incubated with indicated primary antibodies at 4°C overnight and then incubated with horseradish peroxidase-conjugated secondary antibodies specific to the appropriate species at room temperature for 2 h. Immunoreactive blots were visualized using the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The western blot signals were analyzed using an image analyzer (Alpha Innotech, San Leandro, CA, USA) and quantified with ImageJ software, version 1.47. β-actin was used as a loading control.

The following antibodies were used: α-SMA (1:500, Boster, China); collagen I (1:500, Bos-

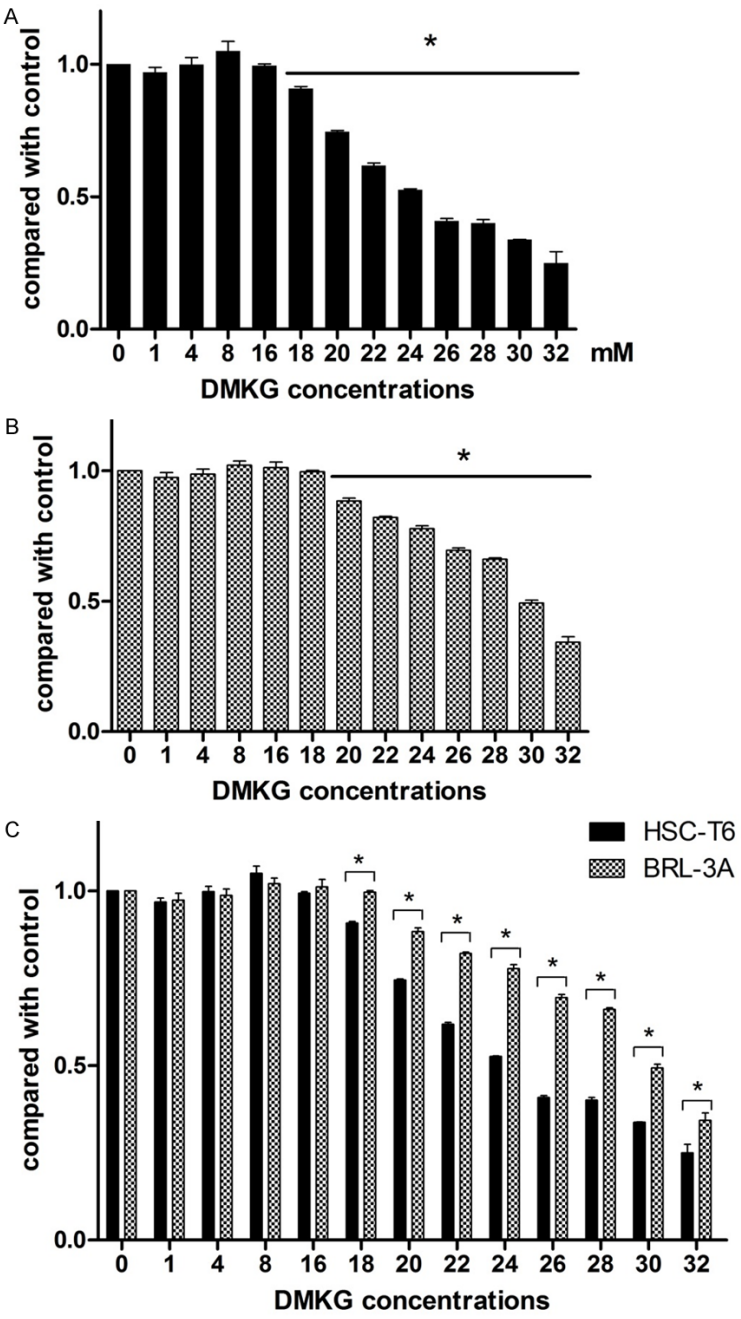

Figure 1. Evaluation of the viability of HSC-T6 and BRL-3A cells treated with DMKG in 24 hours. A. The HSC-T6 cell viability was suppressed in a dose-dependent manner upon treatment with DMKG. B. Dose-dependent inhibition of BRL-3A cell survival upon treatment with DMKG. C. Comparison of cell viability between HSC-T6 and BRL-3A exposed to the same doses of DMKG. The data shown are representative of three independent experimental results (*$P < 0.05$).

ter, China); β-actin (1:5000, CST, USA); horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:5000, CST, USA).

Statistical analysis

All the results are expressed as the mean with the SD. All figures were processed using the Graph Pad Prism 5 software, version 5.01. Data were analyzed using the SPSS software, version 22.0 or the Graph Pad Prism 5 software, version 5.01. Independent sample T-test, paired T-test or One way ANOVA followed by Dunnett's post hoc test was used for the statistical analysis. $P < 0.05$ was taken as the minimum level of significance.

Results

Lower dose concentrations of DMKG didn't effect cell viability

To further highlight the role of DMKG in the viability of HSC-T6 and BRL-3A cells, MTT assays were performed. Both cells responded significantly to DMKG tested in a dose dependent manner. Both cells exposed to lower concentration of DMKG (1–18 mM for BRL-3A, 1-16 mM for HSC-T6) have shown no significant reduction in percent cell in 24 hours. Whereas, higher concentrations of DMKG treatment (20-32 mM for BRL-3A, 18-32 mM for HSC-T6) gradually reduced the cell viability of both cells significantly. Together, though high concentrations of DMKG were cytotoxic to cells, treatment of lower dose of DMKG from 1-16 mM with both HSC-T6 and BRL-3A cell lines didn't showed significant level of inhibition to viability. Moreover, the MTT data showed that HSC-T6 cells were more sensitive to the citotoxic effect of DMKG at high concentrations. (**Figure 1**; **Table 1**) Viabilities of Both cells

Table 1. Evaluation of the viability of HSC-T6 and BRL-3A cells treated with DMKG at 24 h

DMKG concentrations (mM)	HSC-T6 cells (%)	BRL-3A cells (%)
1	96.81±1.96	97.34±3.37
4	99.77±2.67	98.68±3.17
8	104.99±3.59	102.06±2.72
16	99.31±0.77	101.11±3.67
18	90.79±0.80[a]	99.59±0.77[c]
20	74.43±0.57[a]	88.33±1.87[b,c]
22	61.79±0.87[a]	82.07±0.64[b,c]
24	52.53±0.37[a]	77.72±1.98[b,c]
26	40.83±0.97[a]	69.45±1.53[b,c]
28	40.08±1.32[a]	66.08±0.74[b,c]
30	33.73±0.16[a]	49.27±1.81[b,c]
32	24.98±4.25[a]	34.30±3.612[b,c]

Data are mean ± SD, n = 3. [a]$P < 0.05$ compared with 24 h untreated HSC-T6 cells. [b]$P < 0.05$ compared with 24 h untreated BRL-3A cells. [c]$P < 0.05$ compared with HSC-T6 cells treated with the same concentrations of DMKG at 24 h.

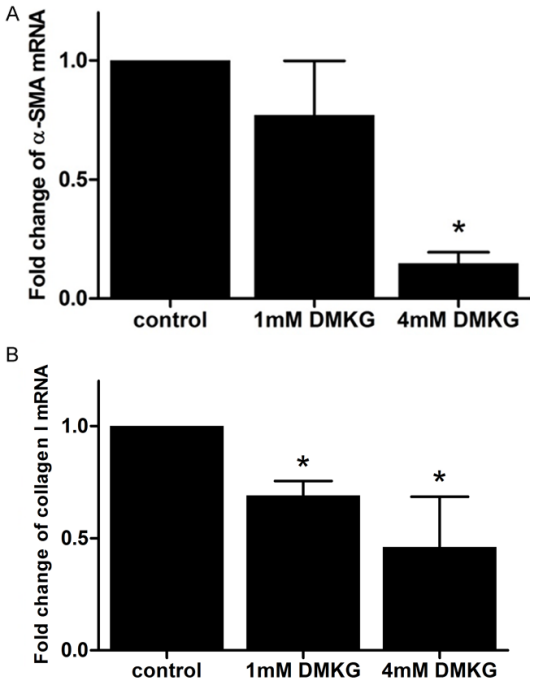

Figure 2. HSC-T6 cells were treated as described above and relative mRNA abundance was assessed by quantitative RT-PCR. A. Decrease of α-SMA mRNA with DMKG treatment. B. DMKG inhibited the expression of collagen I mRNA. Each column represents the mean ± SD (n = 4). *$P < 0.05$.

were determined from three independent experiments ($P < 0.05$ as a statistically significant difference compared with the control).

According to result of MTT assay, we choose 1 mM and 4 mM as the optimum concentrations for HSC-T6 cells in the following experiments, at which concentrations DMKG has no effect on survival of both HSC-T6 and BRL-3A cells.

Effects of DMKG on HSC-T6 activation detected by real-time PCR

HSC-T6 cells were maintained in the culture medium for 24 h with the addition of DMKG at the concentration of 1 mM and 4 mM, and then, total RNA were isolated. Inhibition of HSC-T6 activation was confirmed by quantitative RT-PCR (**Figure 2**). DMKG at 4 mM but not 1 mM decrease the expression of α-SMA mRNA. Treatment of HSC-T6 with 1 mM- and 4mM-DMKG led to an down-regulation of collagen I mRNA.

Effects of DMKG on HSC-T6 activation detected by western blot

Additional evidence that DMKG can inhibit the activation of HSC-T6 cells came from western blotting analysis. DMKG treatment at the concentrations of 1 mM and 4 mM significantly decreased the protein levels of α-SMA and collagen I compared with the control group for 24 h. Both protein levels did not differ significantly between the 1 mM-DMKG treated group and the 4 mM-DMKG treated one (**Figure 3**).

Discussion

Hepatic fibrosis is a consequence of excess collagen production and deposition in response to various liver injuries. Persistent HSC activation is usually considered to be critical in hepatic fibrosis. Upon liver injury, HSC changes in phenotypical transformation to α-SMA positive myofibroblast and produces excessive ECM, mainly the collagen I. Therefore, it is well established that inhibition of HSC activation has become an attractive target for fibrosis treatment [1-6].

A recent study identifying that autophagy provides ATP that is essential to support HSC activation through lipid droplets (LD) mobilization, liberation of free fatty acids (FFAs), and mitochondrial β-oxidation [7, 8]. That is to say, autophagy fuels HSC activation. In the same study, pharmacologic or genetic inhibition of

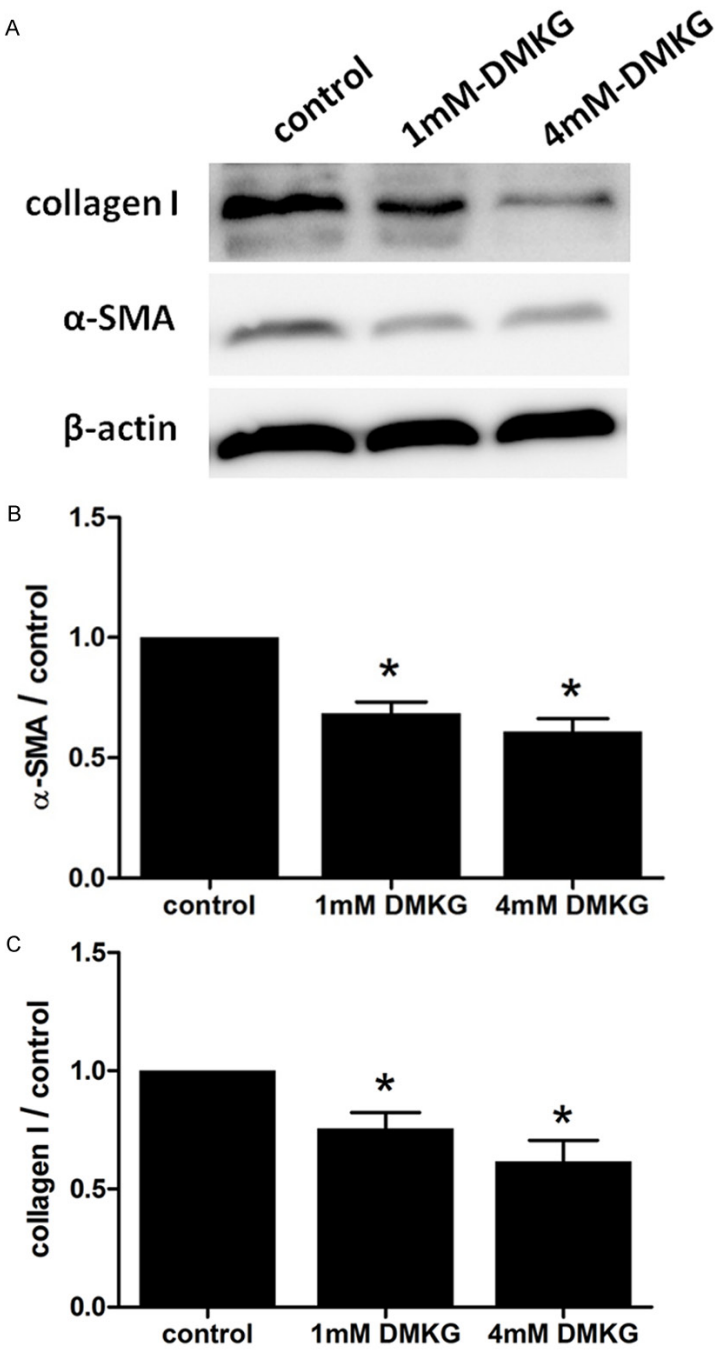

Figure 3. (A) Western blot analysis of protein levels of α-SMA and collagen I in HSC-T6 cells with DMKG treatment at the indicated concentrations for 24 hours. control, cells without DMKG treatment. Data in (B, C) were normalized to β-actin (loading control); Western-blot were independently performed five times and mean ± SD are indicated (*$P < 0.05$).

autophagy reduce activation and fibrosis not only in HSC and liver but also other fibrogenic cells and relevant tissues, such as lung and kidney. Nevertheless, the most commonly used agents to efficiently suppress autophagy in vivo, such as chloroquine, 3-methyladenine and taxanes, confer major toxic side effects [9]. For this reason, our study focused on substances made naturally by the body.

Besides its role as a key intermediate for the Krebs citric acid cycle and an important nitrogen transporter in the metabolic pathways [16], α-Ketoglutarate (AKG) has been proved to inhibit autophagy [9-11].

The study of Marino et al. [9, 10] showing that AKG can converted into acetyl-coenzyme A (AcCoA) depending on either of IDH1 or IDH2 (two isocitrate dehydrogenase isoforms), and also relying on ACLY (ATP citrate lyase). Upregulation of the level of AcCoA in the cytoplasm could inhibit autophagic flux through the acetyltransferase EP300 and the hyperacetylation of autophagy related proteins in mice model and human cells.

In another study [11], Duran et al. demonstrated that glutamine in combination with leucine can enhance glutaminolysis and the production of AKG, which was then proved to have a great contribution to GTP loading of RagB and lysosomal translocation and subsequent activation of mTORC1. Finally, autophagy, the process that can be induced by mTORC1 inhibition, could be suppressed by the increase of cytoplasmic AKG.

Dimethyl α-ketoglutarate (DMKG), also known as dimethyl 2-oxoglutarate (MOG), is the membrane-permeable ester of AKG and can be cleaved to AKG in cytoplasm [16]. Cell-permeable α-ketoglutarate analogs, such as DMKG, were used in the studies as mentioned above [10, 11], and proved to

be able to increase cytoplasmic AKG and reduce the level of autophagy.

Although there is some supportive theoretical rationale, thus far there is no evidence that DMKG reduce the activation of HSC safely. Therefore, this study evaluated the effect of DMKG on cell viability and expression of fibrogenic genes in HSC cells.

The present study demonstrates that DMKG at relatively low concentrations (less than 16 mM) didn't decrease the survival rate of both HSC and hepatocyte cell lines by observing MTT assay. In addition, hepatocytes showed better tolerance of chemical toxicity associated with higher concentrations of DMKG (more than 18 mM) than HSC. These results suggest that DMKG at low concentrations (such as 1 mM and 4 mM that we used in following experiments) is low cytotoxicity, this idea is also supported by the fact that daily intra-peritoneal injections of DMKG didn't kill the mouse in Marino et al.'s study [9, 10].

More importantly, HSC treated with DMKG displayed reduced cell activation and collagen I production. In the mouse immortalized stellate cell line HSC-T6, supplementation with 1 mM or 4 mM DMKG leads to reduced expression of critical fibrogenic genes (α-SMA and collagen I) and their corresponding proteins by real-time PCR and western-blot respectively. Furthermore, the inhibition by DMKG seemed more obvious at 4 mM than 1 mM.

Although the idea that AKG can suppress autophagy was demonstrated by Marino et al. and Duran et al. [9-11], Chin et al. [17] found that autophagy, which is activated by mTOR inhibition, is markedly increased in worms feeded on AKG or in human cells treated with octyl-AKG (another membrane-permeable ester of AKG). In the same research carried out by Chin, AKG was proved to inhibit mTOR and induce autophagy through the direct and uncompetitive suppression of ATP synthase and the reduction of ATP levels. But even the idea is true that autophagy could be upregulated by AKG, the decrease of ATP levels can also inhibit the activation and collagen production in HSC according to Hernandez-Gea et al.'s research [7, 8].

Notwithstanding the disagreement with the effect of AKG on autophagy, the present study demonstrated that DMKG has a significant role of inhibiting the activation of HSC and may attenuate hepatic fibrosis safely. The exact mechanism warrants further research.

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Disclosure of conflict of interest

None.

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