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LETTER TO THE EDITOR

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ABOUT COVER

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ORIGINAL ARTICLE

Basic Study

C23 ameliorates carbon tetrachloride-induced liver fibrosis in mice

Rong-Xing Tang, Xiao-Jun Xie, Yong Xiong, Su Li, Chen Luo, Yi-Gang Wang

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Abstract

BACKGROUND

C23, an oligo-peptide derived from cold-inducible RNA-binding protein (CIRP), has been reported to inhibit tissue inflammation, apoptosis and fibrosis by binding to the CIRP receptor; however, there are few reports on its role in liver fibrosis and the underlying mechanism is unknown.

AIM

To explore whether C23 plays a significant role in carbon tetrachloride (CCl4)-induced liver fibrosis.

METHODS

CCl4 was injected for 6 weeks to induce liver fibrosis and C23 was used beginning in the second week. Masson and Sirius red staining were used to examine changes in fiber levels. Inflammatory factors in the liver were detected and changes in α-smooth muscle actin (α-SMA) and collagen I expression were detected *via* immunohistochemical staining to evaluate the activation of hematopoietic stellate cells (HSCs). Western blotting was used to detect the activation status of the transforming growth factor-beta (TGF-β)/Smad3 axis after C23 treatment.

RESULTS

CCl4 successfully induced liver fibrosis in mice, while tumor necrosis factor-alpha (TNF-α), IL (interleukin)-1β, and IL-6 levels increased significantly and the IL-10 level decreased significantly. Interestingly, C23 inhibited this process. On the other hand, C23 significantly inhibited the activation of HSCs induced by CCl4, which inhibited the expression of α-SMA and the synthesis of collagen I. In terms of mechanism, C23 can block Smad3 phosphorylation significantly and inhibits

TGF-β/Smad3 pathway activation, thereby improving liver injury caused by CCl4.

CONCLUSION
C23 may block TGF-β/Smad3 axis activation, inhibit the expression of inflammatory factors, and inhibit the activation of HSCs induced by CCl4, alleviating liver fibrosis.

Key Words: C23 oligo-peptide; Carbon tetrachloride; Liver fibrosis; Transforming growth factor-beta /Smad3 axis

Core Tip: C23, an oligo-peptide derived from cold-inducible RNA-binding protein inhibits the activation of hepatic stellate cells induced with carbon tetrachloride and the expression of collagen I and α-smooth muscle actin. C23 inhibits the expression of liver inflammatory factors and downregulates transforming growth factor-beta/Smad3 pathway activation, thereby alleviating liver fibrosis.

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INTRODUCTION

At present there is no specific and effective drug for treating liver fibrosis caused by acute or chronic injury. Although preclinical research has made breakthroughs, their suitability as clinical treatments is still unknown. The activation of hepatic stellate cells (HSCs) caused by chronic inflammation is a key process in the development of liver fibrosis and activated HSCs express α-smooth muscle actin (α-SMA) and transdifferentiate into myofibroblasts with proliferation, migration and secretion abilities, synthesizing the extracellular matrix to deposit in the hepatocyte space and subsequently forming liver fibrosis[1]. Although therapeutic strategies have improved due to past few efforts there is no ideal treatment for hepatic fibrosis[2].

Extracellular cold inducible RNA binding protein (CIRP) has been shown to play a role in various acute and chronic inflammatory diseases by promoting tissue inflammation and apoptosis and inducing fibrosis through its receptor Toll-like receptor 4 (TLR4)[3]. C23 is a recognized competitive inhibitor of CIRP that can competitively bind to CIRP receptors and reduce tissue damage in inflammatory diseases[4]. C23 has been shown to significantly reduce serum tumor necrosis factor-alpha (TNF-α), IL (interleukin)-6 and IL-1β levels. In addition, it can reduce tissue TLR4, TNF-α, IL-6 and IL-1β levels and inhibit the colocalization of CIRP and TLR4, which plays a significant role in systemic inflammation[5]. Research has shown that CIRP induces the inflammatory phenotype of lung fibroblasts in a TLR4-dependent manner[6]. On the other hand, CIRP is associated with markers of fibrosis and α-SMA is significantly positively correlated with CIRP. *Cirp*$^{-/-}$ mice exhibit attenuated expression of α-SMA and collagen (COL1A1 and COL3A1), decreased hydroxyproline content, decreased histological fibrosis scores, and improved pulmonary hypertension[7]. C23 inhibited the release of TNF-α, the degradation of IκB and the nuclear translocation of NF-κB in CIRP-stimulated macrophages in a dose-dependent manner and C23 treatment significantly increased the serum levels of lactic dehydrogenase, alanine aminotransferase, IL-6, TNF-α and IL-1β in septic CLP mice[8]. Based on previous research we hypothesized that C23 might alleviate liver fibrosis by inhibiting acute and chronic inflammation. As a selective hepatotoxic chemical carbon tetrachloride (CCl4). can induce inflammation and activate HSCs, promoting liver fibrosis. This study reveals the role and mechanism of C23 in CCl4-induced liver fibrosis in mice.

MATERIALS AND METHODS

Animal experiments

These animal experiments were approved by the Ethics Committee of the General Hospital of Western Theater Command (protocol code 2023EC004) and comply with the Guidelines for Animal Experiments on Laboratory Animals. Thirty-two male *C57BL/6J* mice (20 ± 2 g) were purchased from Chengdu Dashuo Experimental Animal Company and adaptively fed for 1 week at 22 °C with 50% humidity and a 12-hour day/night cycle. The animals were randomly divided into four groups: a control group, C23 group, CCl4 group, and a CCl4 + C23 group. In the CCl4 and CCl4 + C23 groups, CCl4 (0.5 μL/g) (Meryer, m81121, China) was intraperitoneally injected twice a week for 6 weeks to induce liver fibrosis. C23 (Peptide sequence: GRGFSRGGGDRGYGG) was synthesized by Tgpeptide, Inc. (China). In the CCl4 + C23 group C23 (8 mg/kg) was injected for 5 weeks. In the sham group, an equal volume of solvent was injected and in the C23 group C23 (8 mg/kg) was injected for 5 weeks. After the experiment, livers were removed for fixation and freezing.

Hematoxylin and eosin staining

The liver tissues were removed, trimmed to a size of 1 cm × 1 cm, fixed in 4% paraformaldehyde (Biosharp, BL539A, China), routinely dehydrated, paraffin embedded, sectioned at 3 μM, pasted on adhesive glass slides, and stored at room temperature after being baked in paraffin[9]. The samples were subjected to conventional dewaxing and rehydration, hematoxylin staining for 3 minutes, washing with running water for 3 minutes, differentiation with 1% hydrochloric acid (concentrated hydrochloric acid:75% alcohol = 1:99) for 5 s, washing with running water for 10 minutes, eosin staining for 5 s, washing with running water without bound eosin, and sealing with neutral gum after drying naturally. After the neutral gum was completely solidified, photos were taken under a light microscope.

Sirius red staining

Sirius red staining was performed on the paraffin sections. The procedure used before dewaxing and rehydration of the sections was the same as that used for hematoxylin and eosin (HE) staining. After dewaxing and rehydration the sections were stained with iron hematoxylin for 5 minutes, washed twice with distilled water for 3 minutes each time, stained with Sirius red dye solution (Solarbio, G1472, China) for 15 minutes, washed twice with distilled water for 3 minutes each time, dehydrated, cleared routinely, and sealed with medium gum. Finally, images were taken under a light microscope and the fibrosis ratio was determined using image-pro plus (IPP).

Masson staining

Masson (Solarbio, G1340, China) staining was performed on the paraffin sections. The procedure used before dewaxing and rehydration of the sections was the same as that used for HE staining. After dewaxing and rehydration, mordant staining was performed in a 60 °C incubator for 1 h and the sections were rinsed with running water for 10 min, stained with azurol blue for 3 minutes, washed with distilled water twice for 10 s each time, subjected to Mayer hematoxylin stain -ing for 3 minutes and washed twice with distilled water for 10 s each time. After differentiation in acidic differentiation solution for 5 s the differentiation was terminated by washing with water and then rinsing with distilled water for 10 minutes. Ponceau fuchsin was added for 10 minutes and the sections were subsequently washed twice with distilled water for 10 s each time. Phosphomolybdic acid solution was added for 10 minutes and aniline blue staining solution was added dropwise for 5 minutes. The sections were subsequently covered with weak acid working solution for 2 minutes. The sections were normally dehydrated, made transparent, and sealed with neutral gum. Finally, images were taken under a light microscope and the fibrosis ratio was determined using IPP.

Immunohistochemistry

Immunohistochemical staining was performed on paraffin-embedded sections. The procedure used before dewaxing and rehydration of the sections was the same as that used for HE staining. The sections were boiled in acid repair solution for 10 minutes to expose the antigen, blocked with 5% goat serum (ZSGB-BIO, ZLI-9056, China), incubated with primary antibodies against α-SMA (Abcam, ab7817, United Kingdom) and collagen I (Abcam, ab270993, United Kingdom) at a dilution ratio of 1:100, and incubated overnight at 4 °C. According to the manufacturer's instructions for the secondary antibody (ZSGB-BIO, PV9000, China), the secondary antibody was added dropwise and incubated at room temperature for 30 minutes. Diaminobenzidine (ZSGB-BIO, ZLI-9018, China) was used for color development followed by hematoxylin staining for 3 minutes, rinsing with running water for 3 minutes, differentiation with 1% hydrochloric acid alcohol (concentrated hydrochloric acid: 75% alcohol = 1:99) for 5 s, rinsing with running water for 10 minutes, and sealing with neutral gum (Biosharp, BL704A, China) after natural drying. After the neutral gum was completely solidified, photos were taken under a light microscope and the proportion of positive areas was determined using IPP.

Transcriptome array

At 5 weeks after the procedure the mice were euthanized and their livers were isolated and stored in RNA later at 80 °C. Total RNA was transcribed into cDNA, synthesized into cRNA and labeled with cyanine-3-CTP. A mouse microarray was generated and a heatmap was plotted at https://www.bioinformatics.com.cn (last accessed on 20 Feb 2024), an online platform for data analysis and visualization. The total dataset included 3 biological replicates for the two groups. Each sample consisted of 3 mouse livers.

Enzyme-linked immunosorbent assays

The frozen liver was added to phosphate buffered solution, homogenized, and centrifuged at 10000 rpm for 20 minutes, after which the supernatant was removed. Liver TNF-α (Nanjing Jiancheng BIO, H052-1-2, China), IL-1β (Nanjing Jiancheng BIO, H002-1-2, China), IL-6 (Nanjing Jiancheng BIO, H007-1-2, China) and IL-10 (Nanjing Jiancheng BIO, H009-1-2, China) levels were detected according to the kit instructions.

Western blot analysis

Total protein was extracted from 100 mg of frozen liver tissue according to the instructions of the protein extraction kit (KeyGEN, KGB5303, China) and the protein content was calculated with a BCA kit (KeyGEN, KGB2101, China). Proteins were separated with SDS-PAGE electrophoresis, transferred to PVDF membranes (Millipore, IPFL00010, United States), blocked with 5% nonfat dry milk for 30 minutes, and incubated with primary antibodies against Smad3 (CST, 9523, United States) (dilution ratio, 1:1000), phosphorylated Smad3 (CST, 9520, United States) (dilution ratio, 1:500), TGF-β (Abcam, ab179695, United Kingdom) (dilution ratio, 1:1000) and β-actin (CST, 4967, United States) (dilution ratio, 1:4000) overnight at 4 °C. Goat anti-rabbit antibody (dilution ratio, 1:2000) was added dropwise and the samples were incubated

at room temperature for 30 minutes. The gray value of each group was calculated after chemiluminescence.

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics software V19.0 (IBM, United States). The data are expressed as means ± SDs. The normality of the data distribution was tested using the Shapiro-Wilk test. Unpaired Student's *t* tests (two-tailed) were used to compare two groups and one-way ANOVA and Tukey's post hoc test were used to compare the mean values of each group with those of the other group. A P value < 0.05 was considered statistically significant. The statistical methods were reviewed by Yao-Lei Zhang from the General Hospital of Western Theater Command.

RESULTS

C23 inhibits CCl4-induced liver fibrosis

HE staining was used to evaluate the liver pathological changes in each group. Compared with those in the control group CCl4-induced liver diffuse inflammatory cell infiltration, vacuolar fat changes and fibrosis were not caused by C23 injection alone. Compared with those in the CCl4 group diffuse inflammatory cell infiltration and the areas of fibrosis and vacuolar fat were significantly lower in the CCl4+C23 group (Figure 1). These results suggest that C23 can significantly ameliorate CCl4-induced liver fibrosis and hepatocyte inflammation.

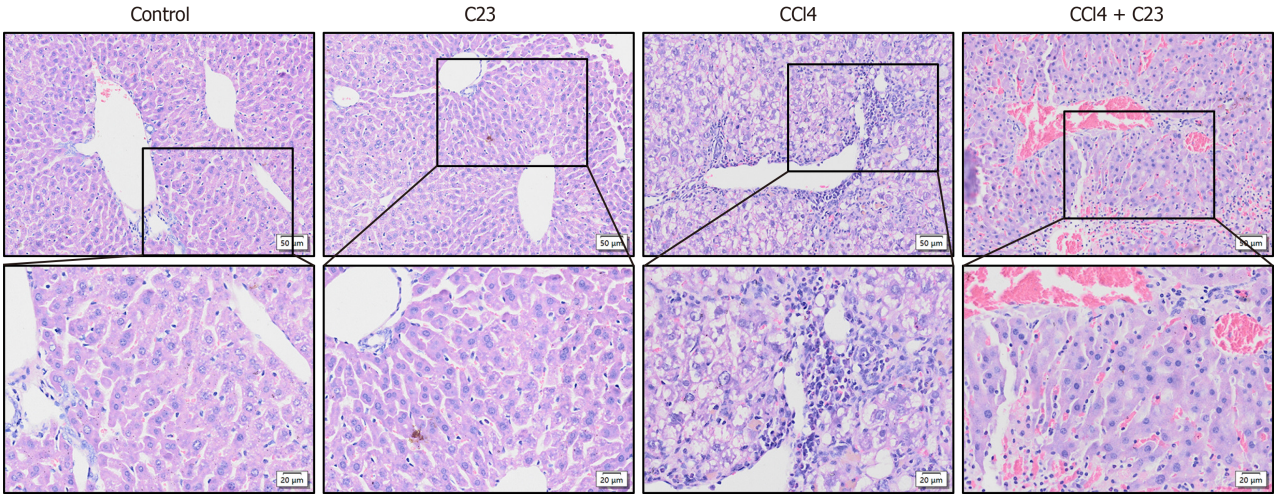

Figure 1 C23 inhibits carbon tetrachloride-induced liver fibrosis and hepatocyte inflammation. Hematoxylin and eosin staining was used to examine the effects of C23 on carbon tetrachloride-induced liver fibrosis and liver inflammation. CCl4: Carbon tetrachloride.

C23 inhibits CCl4-induced hepatic collagen synthesis

To further investigate the effect of C23 on collagen synthesis in the liver, changes in collagen expression in each group were detected with Sirius red and Masson staining (Figure 2A and B). Compared with the control C23 injection alone did not significantly change the collagen content (P > 0.05), and CCl4 significantly promoted the expression of type I and type III collagen (P < 0.05) (Figure 2C). Compared with CCl4, C23 significantly inhibited the production of type I and type III collagen (Figure 2C). These results were consistent with the above results (Figure 2B and D). These data further suggest that C23 could significantly improve CCl4-induced liver fibrosis.

C23 inhibits the activation of HSCs induced by CCl4

α-SMA is an important marker of HSC proliferation and activation. Activated HSCs express a large amount of collagen I, which leads to liver fibrosis. According to the immunohistochemical results (Figure 3A and B), compared with the control C23 injection alone did not affect α-SMA or collagen I expression (P > 0.05). Compared with the control CCl4 significantly promoted the expression of α-SMA and collagen I (P < 0.05), and C23 inhibited the expression of α-SMA and collagen I (P < 0.05) (Figure 3C and D). These results suggest that C23 could inhibit the activation of HSCs induced by CCl4 and alleviate liver fibrosis.

C23 inhibits CCl4-induced liver inflammation

Inflammation is an important part of CCl4-induced fibrosis. Changes in the expression of inflammatory factors in the liver were detected using enzyme-linked immunosorbent assays. Compared with the control, CCl4 promoted the expression of inflammatory factors (TNF-α, IL-1β, and IL-6) (P < 0.05) (Figure 4A-C) and the expression of the anti-inflammatory factor IL-10 was downregulated (P < 0.05) (Figure 4D). Compared with CCl4, C23 significantly inhibited TNF-α, IL-1β, and IL-6 expression (P < 0.05) (Figure 4A-C) and promoted IL-10 expression (P < 0.05) (Figure 4D). These results

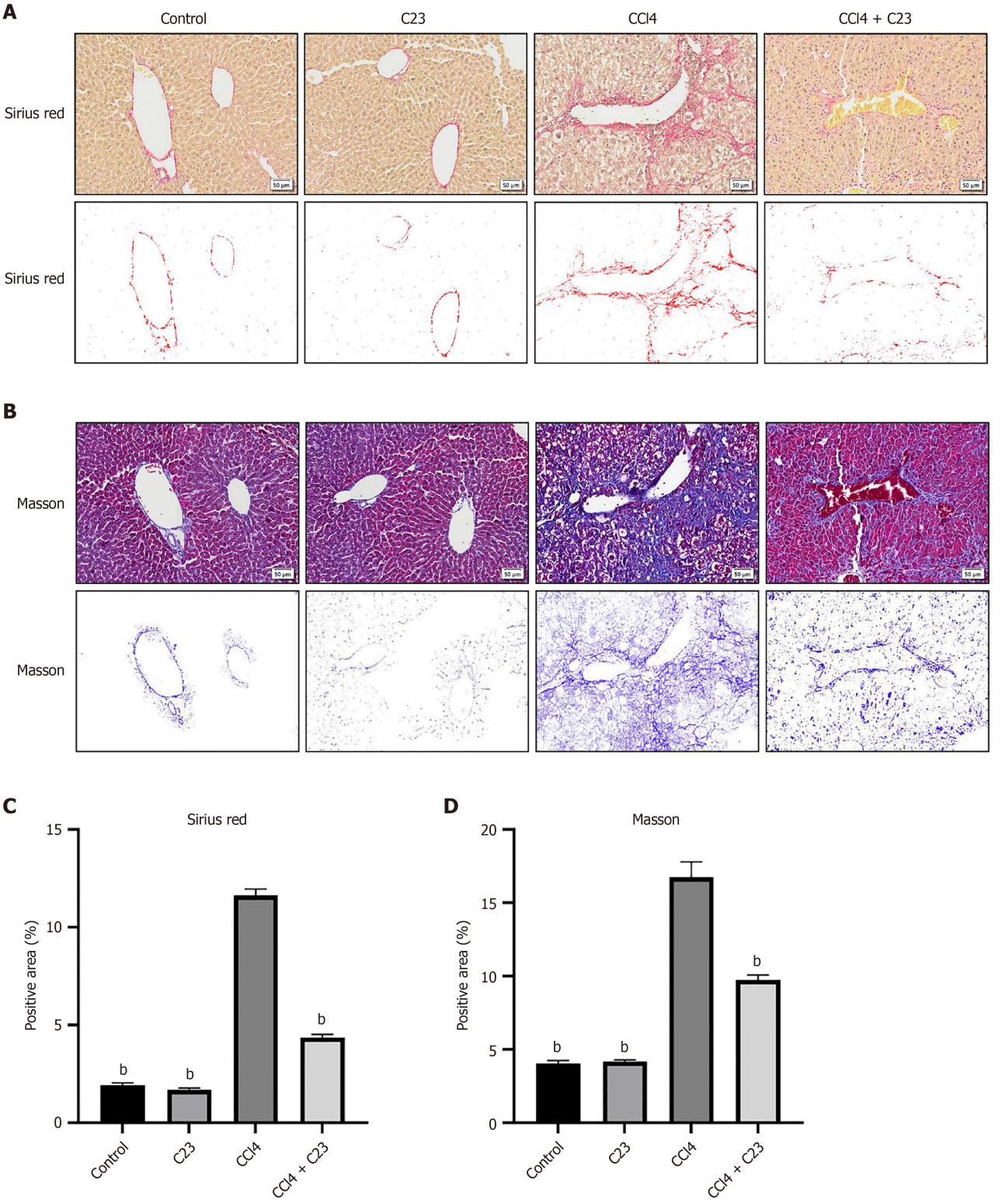

Figure 2 C23 inhibits carbon tetrachloride-induced hepatic collagen synthesis. A: Sirius red staining was used to examine changes in collagen fiber expression in each group; B: Masson staining was used to examine changes in collagen fiber expression in each group. The location of the collagen fibers was visualized with image-pro plus (IPP); C: The IPP concentration was used to calculate the Sirius red-positive area of each group; D: The IPP concentration was used to calculate the Masson positive area of each group. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* carbon tetrachloride. CCl4: Carbon tetrachloride.

suggest that C23 could significantly alleviate CCl4-induced liver inflammation.

C23 inhibits TGF-β/Smad3 activation in the fibrotic pathway

Five weeks after the procedure the livers were harvested for transcriptome array analysis. Among the 30 genes involved in the regulation of fibrosis function, 5 were significantly upregulated while 8 genes were downregulated in the CCl4+C23 group compared with the CCl4 group (Figure 5A). Notably, *TGF-β* was the gene whose expression was most

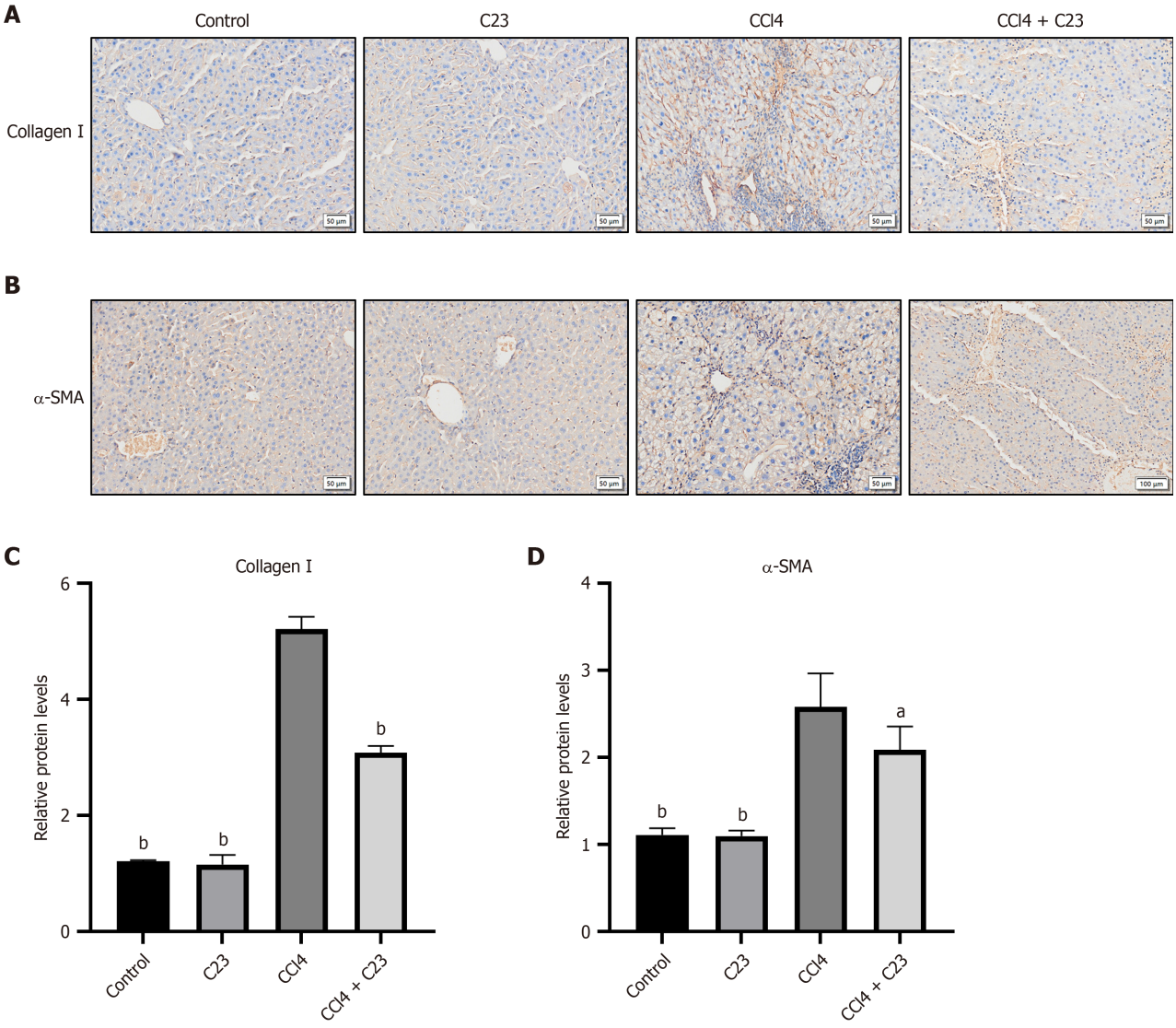

Figure 3 C23 inhibited the activation of hepatic stellate cells induced by carbon tetrachloride. A: The generation and localization of collagen I in the liver were analyzed using immunohistochemistry (IHC); B: The generation and localization of α-smooth muscle actin (α-SMA) in the liver were analyzed using IHC; C: The expression ratio of collagen I in each group was determined with image-pro plus (IPP); D: The expression ratio of α-SMA in each group was determined with IPP. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* carbon tetrachloride. CCl4: Carbon tetrachloride; α-SMA: α-smooth muscle actin.

significantly decreased on Day 30 (Figure 5A). To confirm the change in TGF-β expression in the liver, we measured the protein level using western blotting (Figure 5B). The TGF-β/Smad3 pathway is an important regulatory pathway involved in the progression of fibrosis. The specific pathway by which C23 alleviates liver fibrosis was revealed by detecting changes in the expression of genes involved in the TGF-β/Smad3 pathway. Compared with the control, CCl4 did not affect the expression level of total Smad3 ($P > 0.05$) (Figure 5B and C) but significantly activated Smad3, and p-smad3 expression was significantly upregulated ($P < 0.05$) (Figure 5B and C). Injection of C23 alone had no significant effect on total Smad3, p-smad3 or TGF-β expression ($P > 0.05$) (Figure 5C and D). Compared with CCl4, C23 significantly inhibited Smad3 activation ($P < 0.05$) and TGF-β expression ($P < 0.05$) (Figure 5B and D). These data indicate that C23 inhibits CCl4-induced liver fibrosis through the TGF-β/Smad3 signaling pathway.

DISCUSSION

This study revealed for the first time the inhibitory effect of C23 on liver fibrosis and the underlying mechanism involved. C23 was first shown to inhibit the activation of HSCs induced by CCl4 and to inhibit the expression of collagen I and α-SMA. C23 inhibits the expression of liver inflammatory factors and downregulates TGF-β/Smad3 pathway activation, thereby alleviating liver fibrosis (Figure 6).

Liver fibrosis is a progressive pathological process involving the failure of the regenerative capacity of hepatocytes eventually leading to the development of liver cirrhosis and even hepatocellular carcinoma[10]. Liver fibrosis is a multilayered process that involves inflammation, injury, and other responses. At present, the treatment of liver fibrosis is limited by many factors. Previous studies have shown that interstitial fibrosis caused by chronic inflammation is an

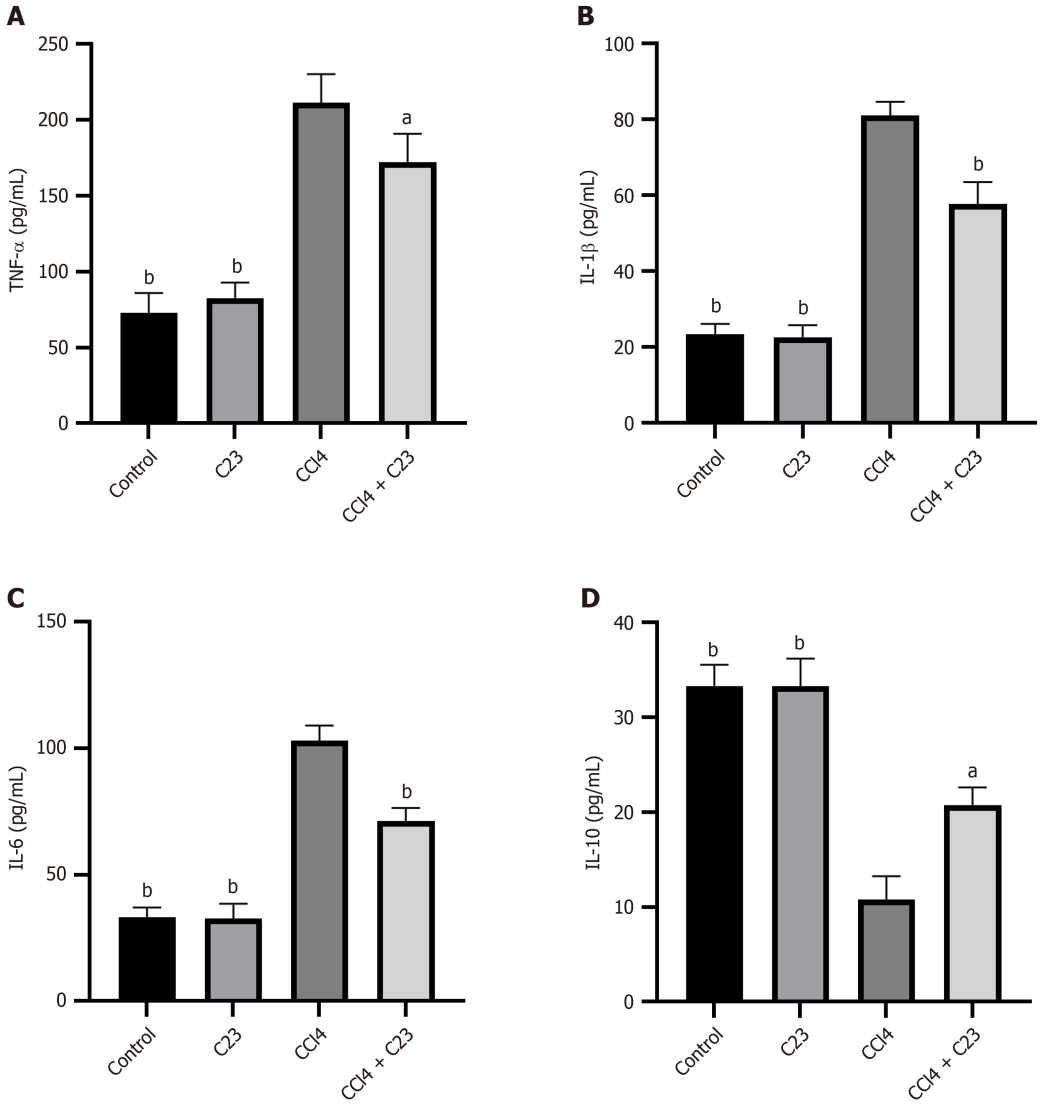

Figure 4 C23 inhibits carbon tetrachloride-induced liver inflammation. A: Enzyme-linked immunosorbent assay (ELISA) was used to examine changes in the expression of tumor necrosis factor alpha in the liver; B: ELISA was used to examine changes in the expression of IL (interleukin)-1β in the liver; C: ELISA was used to examine changes in the expression of IL-6 in the liver; D: ELISA was used to examine changes in the expression of IL-10 in the liver. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* carbon tetrachloride. CCl4: Carbon tetrachloride; TNF-α: Tumor necrosis factor alpha; IL: Interleukin.

important cause of liver fibrosis[11,12].

Some studies have investigated the use of other molecules for reducing liver fibrosis. Sappanone A alleviates liver fibrosis by regulating inflammation and macrophage polarization. Oxytrimene alleviates fibrosis by regulating inflammation and the TGF signaling pathway. However, these small molecules have demonstrated varying levels of pathological disruption in short-term toxicity tests and long-term toxicity data are lacking[13,14]. Extracellular CIRP, *via* the activation of IL-6Rα/Stat3/cdk5, aggravates the neuronal inflammatory response[15]. Extracellular CIRP induces cell proliferation, migration and invasion, increases the expression of N-cadherin and MMP-3, and promotes cellular IL-1β and the release of IL-33[16]. CIRP binds to the TLR4-MD2 complex or to TLR4 and MD2[17]. C23 is an oligopeptide derived from CIRP. C23 is synthesized by comparing the sequence of amino acid residues 111-125 of CIRP and has a high affinity for MD2. It can inhibit inflammation and alleviate tissue damage in a variety of tissues. Treatment of CLP mice with the specific CIRP inhibitor C23 attenuated GPX4 and MDA levels in lung tissue[18]. This evidence suggests that C23 may play an important role as an immune regulatory molecule in tissue damage caused by the inflammatory response. This hypothesis was confirmed in this study. C23 significantly inhibited CCl4-induced liver inflammation, TNF-α, IL-1β, and IL-6 were significantly downregulated and IL-10 was significantly increased.

In preclinical models of inflammatory diseases, *CIRP*$^{-/-}$ mice exhibited significant inhibition of inflammation and alleviation of tissue damage and the levels of IL-6, TNF-α, IL-1β and other inflammatory factors were attenuated. Moreover, TLR4 expression decreases with decreasing NF-κB activity[19]. In this context C23 has the same effect as CIRP knockout but with the technical difficulties and ethical problems associated with CIRP knockout, C23 has more application prospects. Other studies have shown that the lungs of *CIRP*$^{-/-}$ mice exhibit attenuated α-SMA and collagen (COL1A1 and COL3A1), decreased hydroxyproline levels, decreased fibrosis scores, and significantly improved lung fibrosis[7]. Other studies have attempted to intervene in liver fibrosis using rapamycin and have shown that rapamycin

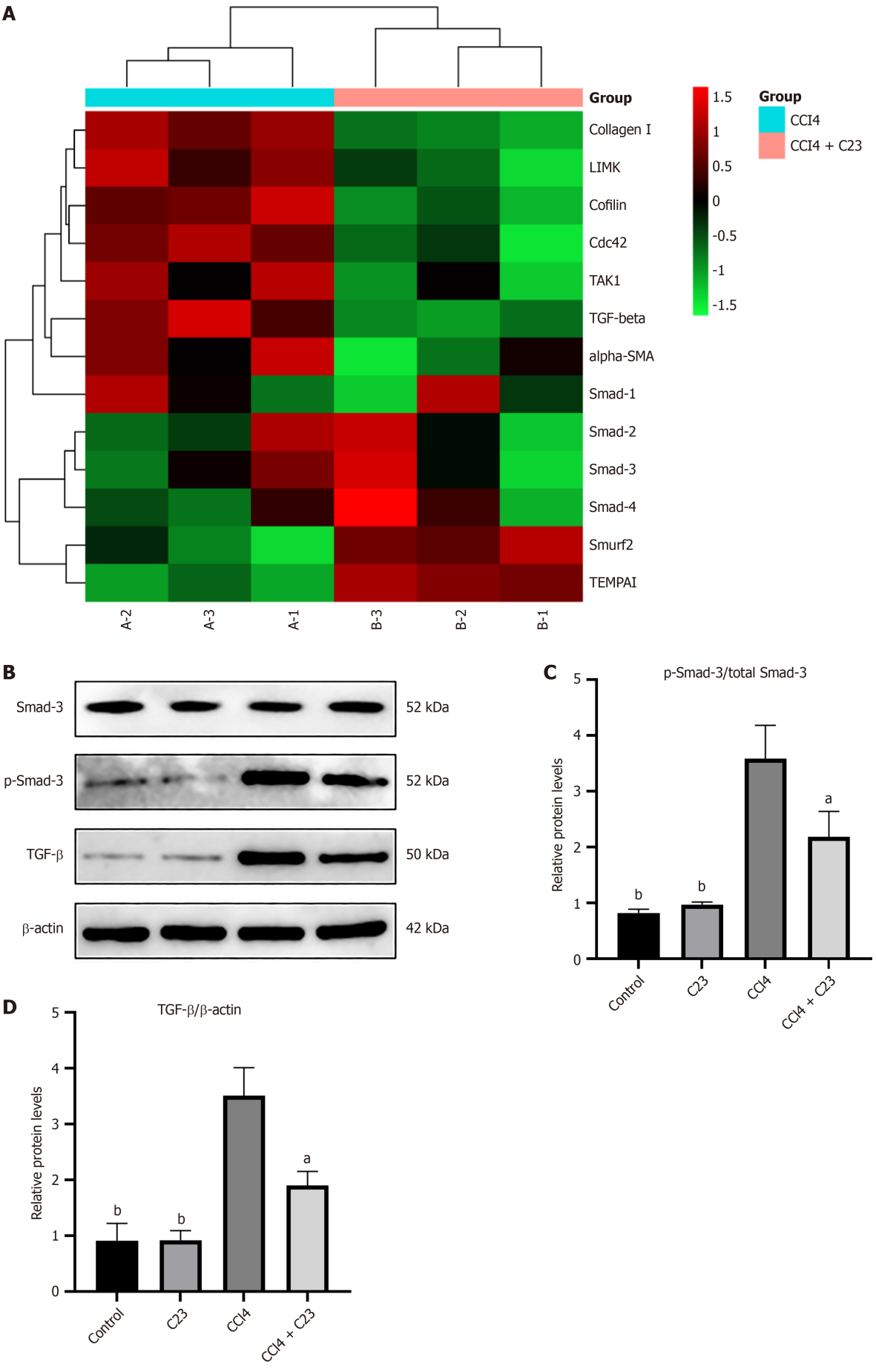

Figure 5 C23 inhibits transforming growth factor beta/Smad3 activation in the fibrotic pathway. A: Heatmap of the transcriptome array comparing

the expression of genes involved in the regulation of fibrosis function between carbon tetrachloride (CCl4)-treated and CCl4+C23-treated mouse livers harvested 30 days after the procedure, $n = 3$; B: Western blotting was used to examine changes in the expression of factors involved in the transforming growth factor beta (TGF-β)/Smad3 signaling pathway during liver fibrosis. β-Actin was used as an internal reference protein; C: The gray value was calculated for p-Smad-3/Smad-3; D: The gray value was calculated for TGF-β/β-actin. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* carbon tetrachloride. CCl4: Carbon tetrachloride; TGF-β: Transforming growth factor beta.

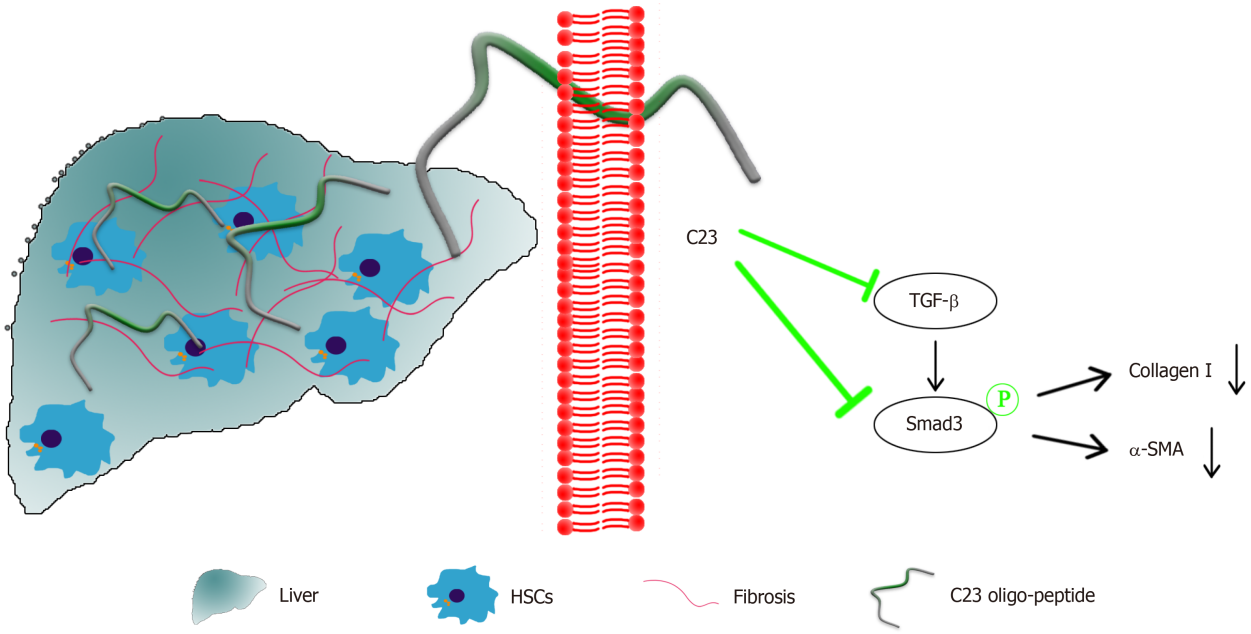

Figure 6 C23 ameliorates carbon tetrachloride-induced liver fibrosis in mice. CCl4: Carbon tetrachloride; HSCs: Hematopoietic stellate cells; TGF-β: Transforming growth factor beta; α-SMA: α-smooth muscle actin.

can reduce liver inflammation and liver fibrosis by inducing autophagy; however, the activation of HSCs has been less well-explored[20]. In this study, we demonstrate that C23 can significantly inhibit CCl4-induced hepatic α-SMA and collagen I expression, suggesting that C23 can inhibit HSC activation. On the other hand, the activation of HSCs leads to the release of a variety of inflammatory mediators, inducing macrophage activation and neutrophil infiltration. This study demonstrates that C23 inhibited the CCl4-induced inflammatory response in liver tissue and alleviated inflammatory cell infiltration.

TGF-β is a recognized profibrotic cytokine that exerts its biological and pathological effects through the Smad signaling pathway. A variety of inhibitors that inhibit the TGF signaling pathway have been found to block liver fibrosis[21,22]. To clarify the relationship between the TGF-β/Smad3 signaling pathway and C23 we explored the molecular mechanism by which C23 alleviates liver fibrosis. Experiments showed that C23 significantly inhibits TGF-β expression and Smad3 phosphorylation. These findings suggest that C23 may inhibit the proliferation and activation of HSCs through the TGF-β/Smad3 signaling pathway. Whether C23 can also affect liver fibrosis through other pathways needs further in-depth study.

CONCLUSION

Our investigation demonstrated that C23 could act as a negative regulator to inhibit inflammation and alleviate liver fibrosis by blocking the activation of the TGF-β/Smad3 signaling pathway. These findings provide theoretical evidence that C23 is a molecular target for the treatment of liver fibrosis.

FOOTNOTES

Author contributions: Wang YG and Tang RX conducted conceptualization, Writing; Xie XJ conducted data curation; Xie XJ, Xiong Y and Li S conducted formal analysis; Xiong Y and Li S conducted investigation; Li S and Lou C conducted methodology. All authors have read and agreed to the published version of the manuscript.

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