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Tungtungmadic Acid Isolated from *Salicornia herbacea* Suppresses the Progress of Carbon Tetrachloride-induced Hepatic Fibrosis in Mice

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ABSTRACT. Tungtungmadic acid (3-caffeoyl, 4-dihydrocaffeoyl quinic acid: CDCQ) is a new chlorogenic acid derivative isolated from the *Salicornia herbacea*. The suppressive effects of CDCQ on the progress of acute carbon tetrachloride ($CC1_4$)-induced hepatic fibrosis were investigated in mice. CDCQ significantly suppressed CCl_4-induced hepatic necrosis and inflammation, as determined by serum enzymatic activities of alanine and aspartate aminotransferase and serum TNF-α levels in a dose-dependent manner. In addition, increased hepatic lipid peroxidation and fibrosis after acute CCl_4 treatment were suppressed by the administration of CDCQ. CDCQ also significantly prevented the elevation of hepatic hydroxyproline and collagen content and α-smooth muscle actin (α-SMA) expression in the liver of $CC1_4$-intoxicated mice. These results suggest that the suppressive effects of CDCQ against the acute $CC1_4$-induced hepatic fibrosis possibly related to its ability to block both hepatic inflammation and the activation of hepatic stellate cells.

Keywords: Tungtungmadic acid, Carbon tetrachloride, Hepatic fibrosis, Collagen, Inflammation.

INTRODUCTION

Hepatic fibrosis is a common pathological feature of progressive chronic liver diseases, and is characterized by increased synthesis and deposition of newly formed extracellular matrix components (Friedman, 1993; Friedman, 2000). The increased extracellular matrices mechanically interfere with blood flow and reduce liver functions. The most important cells directly related to hepatic fibrosis are hepatic stellate cells (HSCs, referred to as Ito cells or fat-storing cells) (Friedman, 1993; Friedman, 2000). HSCs are non-parenchymal liver cells with a characteristic stellate morphology residing in the perisinusoidal space of Disse. When liver injury occurs, HSCs change their phenotypes from vitamin A-storing resting cells to myofibroblast-like cells expressing α-smooth muscle actin (α-SMA), which is considered to be the activated state (Ramadori *et al.*, 1990). These transformed (activated) HSCs enhance production of extracellular matrix components, especially collagens, and promote hepatic fibrosis (Brenner *et al.*, 2000). Following chemically-induced fibrosis, the HSCs is the main component of the fibrogenic cell population (Ramadori *et al.*, 1990; Pinzani and Gentilini, 1999). Oxidative stress, including oxygen-derived free radicals and lipid peroxidation, has also been shown to be implicated as a cause of hepatic fibrosis (Tsukamoto *et al.*, 1995; Lee *et al.*, 1995; Svegliati Baroni, 1998). It has been demonstrated that HSCs may be activated by free radicals as well as by malondialdehyde, a product of lipid peroxidation (Lee *et al.*, 1995). Antioxidant agents have been shown to exert protective effects against HSCs activation (Houglum *et al.*, 1997; Kawada *et al.*, 1998). Carbon tetrachloride ($CC1_4$) is widely used as inducer of hepatic fibrosis (Nakatsukasa *et al.*, 1990; Iredale *et al.*, 1996). Formation of the trichloromethyl free radicals ($CC1_3^{\cdot}$ and/or $CC1_3OO^{\cdot}$) is believed to be the basis for the toxic effects of $CC1_4$ (Brattin *et al.*, 1985).

Recently, there has been a global trend toward the use of natural phytochemicals present in natural resources as antioxidants, such as fruits, vegetables,

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oilseeds, and herbs (Kitts *et al.*, 2000). Herbs have begun to attract attention as health-beneficial foods (physiologically functional foods) and as a raw material for the development of drugs. Herbal medicines derived from plants are increasingly being utilized to treat a wide variety of clinical diseases, even though relatively little is known about their modes of action. *Salicornia herbacea*, which is commonly known as Tungtungmadi in Korea, is one of the halophytes that can grow in salt marshes, or salt fields along the coastline in Korea (Kim and Song, 1983; Lee *et al.*, 2004). Some people living in coastal areas have used it as both a traditional medicine as well as a seasoned vegetable. This plant has previously been shown to modulate the production of cytokines and the release of nitric oxide in macrophages (Im *et al.*, 2003). In an ongoing investigation into antioxidative compounds from natural products, we isolated a new chlorogenic acid derivative compound, tungtungmadic acid (3-caffeoyl, 4-dihydrocaffeoyl quinic acid: CDCQ) from *S. herbacea* and found its antioxidative activity (Chung *et al.*, 2005).

Even though CDCQ showed antioxidative activity in our previous examinations, its effect on hepatic fibrosis have so far not been well elucidated. The present study was undertaken to evaluate the suppressive effects of CDCQ on the early phase of acute $CC1_4$-induced progress of hepatic fibrosis and to elucidate the mechanism(s) underlying these protective effects in the mice.

MATERIALS AND METHODS

Materials

CDCQ (Fig. 1) was isolated from *S. herbacea* as described previously (Chung *et al.*, 2005). Chemicals and cell culture materials were obtained from the following sources: $CC1_4$, diagnostic kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT), metrizamide, and antibodies to α-SMA from Sigma Co.; TNF-α enzyme linked immunosorbent assay (ELISA) kits from the R&D Systems Inc.; Western blotting detection reagents (ECL) from Amersham Pharmacia Biotech.; other chemicals were of the highest commercial grade available.

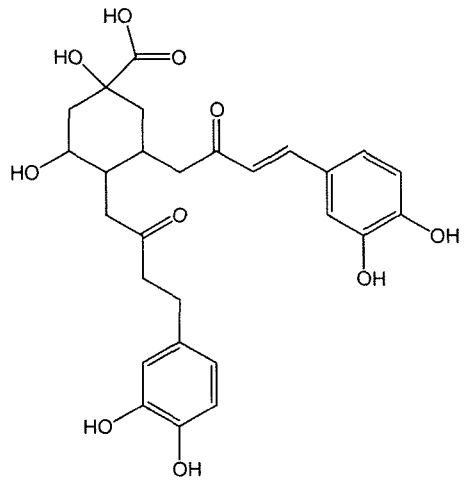

Fig. 1. Structure of tungtungmadic acid (3-caffeoyl, 4-dihydrocaffeoyl quinic acid: CDCQ).

Animals and treatment

Male ICR mice (25~30 g) were obtained from the Dae Han Laboratory Animal Research and Co. (Daejeon, Korea). The animals were allowed free access to Purina Rodent Chow and tap water, maintained in a controlled environment at 21 ± 2°C and 50 ± 5% relatively humidity with a 12 hr dark/light cycle, and acclimatized for at least I week before use. CDCQ in saline was administered intragastrically (i.g) at 5~20 mg/kg once daily. Three hours after the administration of CDCQ, mice were injected CCl_4 (50 mg/kg body weight intraperitoneally (i.p), dissolved in olive oil). Groups of control animals were given the respective vehicles. Twelve, 24, and 72 h after the CCl_4 treatment, mice were anesthetized with CO_2, blood was removed by cardiac puncture to determine the serum ALT and AST activities and serum TNF-α levels, and the animals were decapitated. After bleeding, livers were weighed and a thin slice preserved in a buffered formalin solution for obtaining histological sections. The remaining livers were frozen quickly in dry ice/methanol and stored at -70°C for lipid peroxidation, collagen and hydroxyproline analysis.

Measurement of serum ALT and AST activities and serum TNF-α levels

Hepatic necrosis was assessed by quantifying the serum activities of ALT and AST using a spectrophotometric diagnostic kit obtained from the Sigma Chemical Co. Hepatic inflammation was measured by quantifying the serum TNF-a levels using an ELISA kit obtained from the R&D Systems Inc.

Lipid peroxidation assay

The level of hepatic lipid peroxidation was measured by the formation of the thiobarbituric acid-reactive material, malondialdehyde (MDA) (Fairhurst *et al.*, 1982). The protein concentration was determined by the method of Bradford, using bovine serum albumin as the standard.

Measurement of tissue collagen and hydroxyproline content

For measurement of collagen, each liver was homogenized in 0.5 M acetic acid at 4°C. After acid extraction, the gelatin content of the acid extract was assayed using the Sircol collagen assay kit (Biocolor, Belfast, Ireland) according to the manufacturer's instructions (Shiba *et al.*, 1998). For measurement of hydroxyproline, each liver was homogenized in saline at 4°C. The hepatic hydroxyproline content was determined using a modification of the method of Jamall *et al.* (1981), as previously described (Fort *et al.*, 1998).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis for type I collagen mRNA

Total RNA was prepared from frozen livers by the acidic phenol extraction procedure of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). cDNA synthesis, semiquantitative RT-PCR for rat αI(I) collagen (sense; 5'-CTGACTGGAAGAGCGGAGAG-3', antisense; 5'-TGAGTTTGGGTTGTTGGTCT-3'; 676 bp) and glycerol aldehyde 3-phosphate dehydrogenase (GAPDH, sense; 5'-GATGAATTCTGAAGGTCGGAGTCAACGGA TTTGGT-3', antisense; 5'-GATAAGCTTCATGTGGGCCATGAG-GTCCACCAC-3'; 980 bp) mRNA, and the analysis of the results were all performed as described previously (Brandsten *et al.*, 1999). PCR reactions were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Western blot analysis for α-SMA

Whole tissue extracts were prepared by treating the cells with lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin). The lysates were sonicated for 20 s on ice and centrifuged at 10,000 × g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Bradford (Bradford, 1976). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels. The resolved proteins were transferred onto polyvinylidene difluoride membranes. After blocking, the membrane was incubated with monoclonal antibody to α-SMA. The secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

Statistical analysis

Means ± SD were calculated for each group and Dunnet's 't' test was used to calculate statistical significance. Differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Hepatic fibrosis represents the main complication of most chronic liver disorders and, regardless of its etiology, is characterized by excessive deposition of extracellular matrix components (Friedman, 2000). Hepatic fibrogenesis is accompanied by hepatocellular necrosis and inflammation. HSCs are regarded as the primary target cells for inflammatory stimuli in the injured liver (Li and Friedman, 1999), and activated HSCs have been identified as the primary source of excess accumulation of extracellular matrix components in liver fibrosis (Friedman, 1993, 2000).

The $CC1_4$ is frequently used as an experimental model to study hepatic fibrosis (Davis Nakatsukasa *et al.*, 1990; Iredale *et al.*, 1996). The effects of CDCQ on the liver necrosis and inflammation early phase in the liver following acute $CC1_4$ intoxication were investigated to address whether CDCQ suppresses the early phase of acute $CC1_4$-induced progress of hepatic fibrosis. A single dose of $CC1_4$ caused necrotic hepatotoxicity in mice, as indicated by the increase in ALT and

Table 1. Effects of CDCQ on $CC1_4$-induced serum ALT and AST activities and hepatic lipid peroxidation

Treatment (mg/kg)	Serum ALT (U/liter)	Serum AST (U/liter)	Lipid Peroxidation (MDA: nmole/g liver)
Control	65 ± 8*	48 ± 6*	2.32 ± 0.34*
CDCQ 20	59 ± 7*	52 ± 7*	2.21 ± 0.36*
CCl_4	2,821 ± 431	1,774 ± 258	6.26 ± 0.79
CDCQ 5 + CCl_4	2,535 ± 383	1,458 ± 235	6.12 ± 0.83
CDCQ 10 + CCl_4	1,164 ± 182*	728 ± 97*	4.26 ± 0.58*
CDCQ 20 + CCl_4	523 ± 68*	316 ± 45*	3.22 ± 0.42*

Mice were treated with CCl_4 and/or CDCQ simultaneously. Necrotic hepatotoxicity was determined 24 h later by quantifying the serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and hepatic lipid peroxidation. Each value represents the mean ± SD of five mice. *$P < 0.05$, significantly different from the CCl_4.

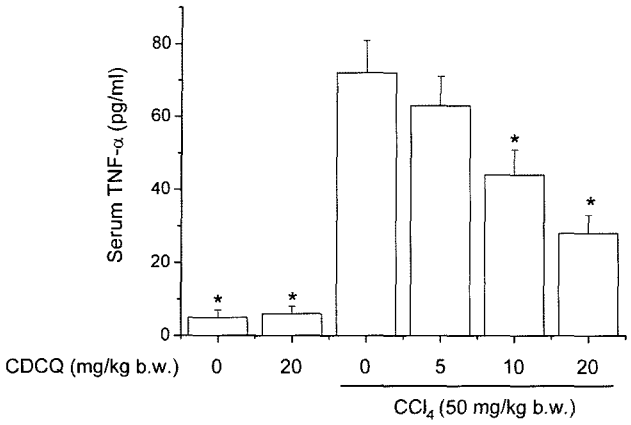

Fig. 2. Effects of CDCQ on $CC1_4$-induced serum TNF-α levels. Mice were treated with CCl_4 and/or CDCQ simultaneously. Serum TNF-α levels were determined 12 h later by ELISA. Each value represents the mean ± SD of four mice. *$P < 0.05$, significantly different from the CCl_4.

AST serum levels and lipid peroxidation 24 h after $CC1_4$. CDCQ treatment prevented the $CC1_4$-induced elevation ALT and AST serum levels and lipid peroxidation (Table 1) in a dose-dependent manner. Histopathological studies showed that $CC1_4$, compared to the control, induces degeneration in hepatocytes and hepatic cords and focal necrosis (data not shown). By microscopic examination, the severe hepatic lesions induced by $CC1_4$ were remarkably reduced by CDCQ, and this was in good agreement with the results of the serum aminotransferases activities and lipid peroxidation.

The effects of CDCQ on the changes in inflammatory responses caused by $CC1_4$ were assessed by measuring the serum TNF-α levels. A single dose of $CC1_4$ treatment increased serum TNF-α levels at 12 h when determined by ELISA (Fig. 2). CDCQ significantly prevented $CC1_4$-induced increases in serum TNF-α levels. Taken together, these results suggest that CDCQ reduced necrosis and inflammatory responses following acute $CC1_4$ treatment. Studies have shown that expansion and activation of the HSCs population follows that of the monocyte/macrophage population, and both cell types accumulate exclusively within areas of necrosis. It induces extracellular matrix deposition by simultaneously stimulating the synthesis of new matrix components, increasing the synthesis of enzymes that inhibit extracellular matrix degradation, and decreasing the synthesis of matrix degrading proteases. Soluble factors, such as TNF-α and IL-1β, released by Kupffer cells and hepatocytes lead to HSCs proliferation and promote their synthesis of collagen, proteoglycans, and hyaluronate. Preventing the production of TNF-α can block the induction of collagen. Kupffer cells could be activated by toxic metabolite(s) from $CC1_4$, which might result in acute liver toxicity. Kupffer cell-derived stimulatory factors such as TNF-α are responsible for liver injury and subsequent fibrogenesis. TNF-α is the principal mediator of inflammatory responses, and is closely associated with the acute hepatotoxicity induced by immunological and chemical toxicants (Kunstle *et al.*, 1999). In the current study, CDCQ inhibited increase in the serum TNF-α levels following acute $CC1_4$ intoxication. These results raised the possibility that CDCQ inhibits activation of mononuclear phagocytes (e.g. Kupffer cells) and hence the expression of TNF-α. Inhibition of TNF-α production by CDCQ may contribute to its therapeutic efficacy against chemical hepatitis as well

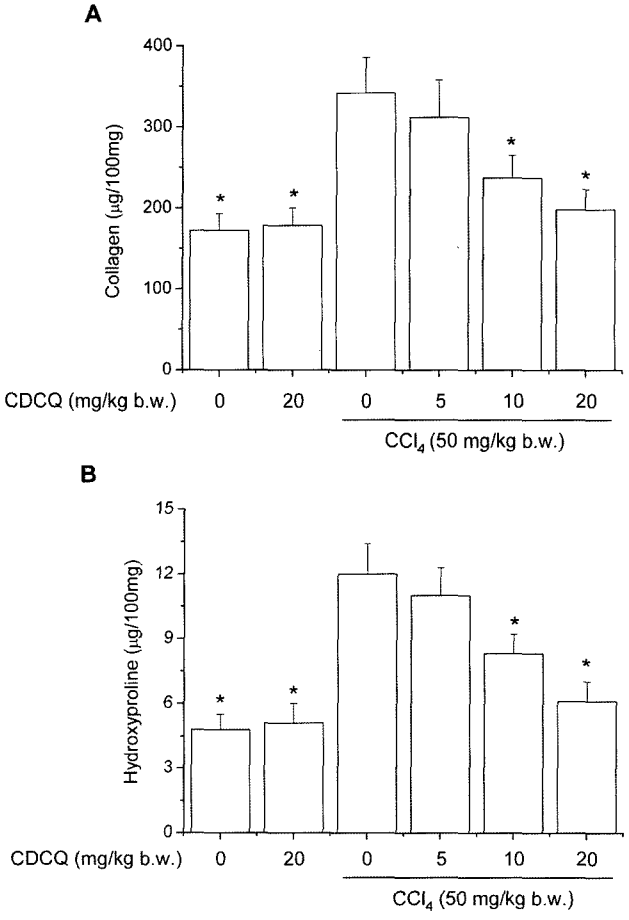

Fig. 3. Effect of CDCQ on $CC1_4$-induced collagen and hydroxyproline contents in the liver. Mice were treated with CCl_4 and/or CDCQ simultaneously. Subsequently, CDCQ or vehicles alone as controls were treated once daily for 3 consecutive days, and liver samples were obtained 72 h after the CCl_4 treatment. Hepatic collagen (A) and hydroxyproline (B) content were measured as described in Materials and methods. Each value represents the mean ± SD of four mice. *$P < 0.05$, significantly different from the CCl_4.

as against hepatic fibrosis.

Hepatic fibrosis is a fundamental, clinically serious problem in various types of chronic liver diseases. To investigate the suppressive effect of CDCQ on the progress of acute CCl_4-induced hepatic fibrosis, mice were given a single treatment of CCl_4. The effects of CDCQ on the extent of the early phase of hepatic fibrosis were evaluated by measurement of hepatic collagen and hydroxyproline contents after 3 days of CCl_4 treatment. Treatment with CCl_4 increased the level of tissue collagen (Fig. 3A) and hydroxyproline contents (Fig. 3B). These were almost reversed when the mice were treated with CDCQ. These results suggest that CDCQ suppresses collagen production, and thus contributes to decreasing the early progress of fibrogenic responses in the liver following acute CCl_4 treatment. We therefore determined the effect of CDCQ on type I collagen by assessing the mRNA levels of αI(I) procollagen by RT-PCR. Treatment with CCl_4 increased the level of expression of αI(I) procollagen mRNA (Fig. 4). This was almost reversed when the rats were treated with CDCQ. The amount of GAPDH, which was present as an internal control, remained unchanged. These results suggest that CDCQ suppresses type I collagen expression at the transcriptional level, and thus contributes to decreasing the early progress of fibrogenic responses in the liver following acute CCl_4 treatment. Hepatic fibrogenesis is a fundamental, clinically serious problem in various types of chronic liver diseases. HSCs are believed to play a pivotal role in fibrogenesis in the liver (Friedman, 1993; Friedman, 2000). Because activation of HSCs is a key event in the early phase of fibrogenesis in the liver, expression of α-SMA, a typical marker of activation of HSCs, was detected by immunoblotting in the acute CCl_4-treated liver after 3 days. Considerable α-SMA expression was detected in CCl_4-treated mice compared with control livers. In contrast, treatment with CDCQ markedly reduced the α-SMA expression in the livers of rats treated with CCl_4 (Fig. 5). This suppression of α-SMA expression suggests that CDCQ may have blocked the synthesis of type 1 collagen.

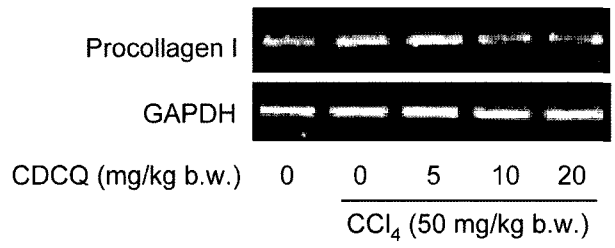

Fig. 4. Effect of CDCQ on CCl_4-induced expression of α1(I)procollagen mRNA in the liver. Mice were treated with CCl_4 and/or CDCQ simultaneously. Subsequently, CDCQ or vehicles alone as controls were treated once daily for 3 consecutive days, and liver samples were obtained 72 h after the CCl_4 treatment. Total RNA was prepared and RT-PCR was performed as described in the Materials and Methods. The PCR products were separated on agarose gel and stained with ethidium bromide. GAPDH, the housekeeping gene, was used as an internal control.

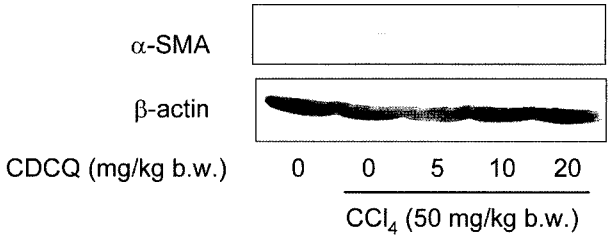

Fig. 5. Effects of CDCQ on CCl_4-induced a-SMA expression in the liver. Mice were treated with CCl_4 and/or CDCQ simultaneously. Subsequently, CDCQ or vehicles alone as controls were treated once daily for 3 consecutive days, and liver samples were obtained 72 h after the CCl_4 treatment. The expression of α-SMA in the liver was detected by Western blot analysis.

In the present study, we demonstrated that CDCQ has preventive effects against necrosis and inflammatory responses and subsequent suppressed progress of the early-phase of acute CCl_4-induced fibrotic responses in the liver. CDCQ significantly inhibited increases in serum transaminases and lipid peroxidation and TNF-α levels following a single CCl_4 treatment. It is likely therefore that CDCQ inhibits the activation of Kupffer cells during CCl_4 intoxication, thereby decreasing subsequent fibrogenic progress in the liver. In addition, we confirmed that CDCQ suppresses not only the induction of α-SMA, but also the expression of type I collagen. During hepatic fibrosis, HSCs in the necrotic area are probably activated by locally excreted cytokines and/or conformational changes in Disse's cavity (Pinzani and Gentilini, 1999; Friedman, 2000). After activation, HSCs proliferate and transform into myofibroblasts expressing collagen and a-SMA, resulting in liver fibrosis (Knittel *et al.*, 1992; Friedman, 1993, 2000). Therefore, agents that inhibit HSCs activation might be candidates for the therapeutic prevention of chronic liver injury and/or liver fibrosis (Friedman, 1993, 2000). In the previously study, we showed that the CDCQ isolated from *S. herbacea* has a potent antioxidant effects (Chung *et al.*, 2005). Since oxidative stress has been recently associated with HSCs activation (Tsukamoto *et al.*, 1995; Lee *et al.*, 1995; Houglum *et al.*, 1997; Kawada *et al.*, 1998; Svegliati Baroni *et al.*, 1998) and

has been shown to modulate collagen gene expression (Casini *et al.*, 1991), the CDCQ could be prevent the HSCs activation and inhibit the progress of $CC1_4$-induced hepatic fibrosis. However, further studies are needed in order to clarify the exact mechanism(s).

In conclusion, CDCQ suppresses the progress of $CC1_4$-induced hepatic fibrosis and these effects of CDCQ may be due to its ability to block the activation of Kupffer cells and HSCs. Recently, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on their mechanism of action. This study therefore provides biological evidence supporting the use of CDCQ for the prevention and/or treatment of liver disorders, such as hepatic fibrosis.

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