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Diallyl trisulfide plays an antifibrotic role by inhibiting the expression of Bcl-2 in hepatic stellate cells

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Abstract

Hepatic fibrosis is an important early stage in the evolution of liver cirrhosis, and specific medicine and therapeutic measures are unavailable to date. Hepatic stellate cells (HSCs) are the main cells involved in the formation of hepatic fibrosis, and induction of the apoptosis of HSCs is an important strategy for the treatment of hepatic fibrosis. Diallyl trisulfide (DATS) is a natural product and is the main active ingredient in garlic. However, the exact molecular mechanisms underlying HSC apoptosis induced by DATS are not well understood. This study aimed to analyze the efficiency and mechanism of DATS in hepatic fibrosis. Different concentrations (25, 50, 100, and 200 μM) of DATS were used to treat HSCs. Changes in cell morphology and formation of apoptotic bodies were observed under an inverted microscope and an electric microscope. Bcl-2 signaling involving Bax, Caspase-3, Caspase-6, Caspase-8, Caspase-9, p53, Apaf-1, and Cyto-c in fibrosis were examined, which is a critical step in the evaluation of antihepatic fibrosis agents. We also evaluated the effect of DATS on the cellular morphology of HSCs and apoptosis-related factors under different Bcl-2 expression states. Our results suggest that DATS regulates hepatic fibrosis by blocking the Bcl-2 signaling pathway and upregulating the Bax/Bcl-2 ratio.

KEYWORDS

apoptosis, Bax, Bcl-2, diallyl trisulfide (DATS), hepatic stellate cells, signal transduction

Huai Pang, Cuizhe Wang, and Jing Ye contributed equally to the work.

1 | INTRODUCTION

Hepatic fibrosis is a worldwide clinical issue that can lead to cirrhosis and hepatocellular carcinoma.[1] It results due to the repairing process of the liver in a variety of chronic liver injuries such as viral hepatitis, alcoholism, cholestasis, drugs, and metabolic turbulence.[2] Although the hyperplasia of fibrous connective tissue can repair the defect, the repaired liver tissue does not have the structure and function of the original liver cells, and continuous progressive fibrosis can lead to the destruction of the liver structure and functions.[3] Many animal-based and clinical studies have shown that hepatic fibrosis and even early cirrhosis can be reversed, but their treatment remains a major challenge.[4]

Hepatic stellate cells (HSCs) are fibrogenic cells involved in the development of hepatic fibrosis and are the precursors of myofibroblasts. Myofibroblasts are the drivers of tissue fibrogenesis in multiple organs; they are responsible for the excessive deposition of extracellular matrix (ECM) proteins in the liver and the promotion of the hepatic fibrosis progression.[5,6] Therefore, it is vital to reduce the number of activated HSCs to reverse hepatic fibrosis.[7] Recent studies have shown that induction of HSC apoptosis is an effective method for the treatment of hepatic fibrosis.[8] Therefore, the identification of effective drugs that promote HSC apoptosis and molecular mechanisms responsible for the reversibility of hepatic fibrosis is imperative for developing diagnostic and therapeutic strategies to improve the quality of life of the patient.

Recently, natural products have received great attention for the development of new, safe, and economical medicaments. Garlic is a perennial herb in the Liliaceae family and has been used as traditional Chinese medicine. According to traditional Chinese medicine, garlic has a spicy and warm taste, and it warms the spleen and stomach, thus eliminating the accumulation of toxins caused by stagnation of Qi. Diallyl trisulfide (DATS) is the main active ingredient in garlic.[9,10] DATS has been shown to have anti-inflammatory, anti-infective, and antitumor effects.[11–13] Moreover, our previous studies have shown that apoptosis of rat hepatic stellate cells is induced by DATS in vitro.[14] However, the exact molecular mechanisms underlying HSC apoptosis induced by DATS remain unclear.

In this study, we cultured HSC-T6 cells and detected the expression of Bcl-2 and apoptosis-related factors after treatment with DATS. This study aimed to clarify the molecular mechanisms of HSC apoptosis by DATS, which will provide a molecular basis for the clinical treatment of hepatic fibrosis.

2 | MATERIALS AND METHODS

2.1 | Reagents

Diallyl trisulfide (DATS) (MW: 178.34, C6H10S3) was purchased from ChromaDex.

2.2 | Cell culture and DATS treatment

The HSC-T6 cell line was purchased from the Cancer Hospital Chinese Academy of Medical Science. Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2. After reaching 40%–60% confluence, the cells were treated with 0.1% dimethylsulfoxide containing different concentrations of DATS.

2.3 | Morphological study of apoptotic cells

The ultrastructural changes in HSC-T6 cells treated with DATS for 12 h were observed by transmission electron microscopy using previously described methods.[14]

2.4 | MTS assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Exponentially growing HSCs were seeded in 96-well culture plates at 1×10^4 cells per well and treated with or without DATS for 0, 6, 12, 24, 48, and 72 h respectively. After the treatment, 10 μl of CellTiter96AQ (Promega, Cat. No. G3582) was added into each well and the plates were incubated at 37°C for 4 h. A 96-well microplate reader (Thermo Multiskan Mk3) was used to measure the absorbance at 490 nm (A490). At least three independent experiments were conducted.

2.5 | Analysis of cell apoptosis by flow cytometry

Flow cytometric analysis of apoptosis of HSC-T6 cells treated with 100 μM DATS for 48 h under different Bcl-2 expression conditions was carried out using previously described methods.[1]

2.6 | Western blot analysis

Total cellular proteins of HSC-T6 cells were extracted using RIPA buffer (Solarbio). Protein content was quantified using a Pierce-23225BCA protein assay kit (Beyotime Biotechnology) according to the manufacturer's instructions. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking, the membranes were incubated overnight at 4°C with specific primary antibodies. The membranes were probed with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and detection was performed by exposure development in a dark room.

2.7 | Statistical analysis

SPSS17.0 software was used for statistical analysis. All measurement data are expressed as the mean ± standard deviation (X ± SD). An unpaired two-sided *t* test was used to assess the differences between the control and experimental groups. The statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Effects of DATS on HSC apoptosis

To explore the effect of DATS on the apoptosis of HSCs, different concentrations (25, 50, 100, and 200 μM) of DATS were used to treat the cells. With the increase in the concentration and treatment time, we found a reduction in the number of HSCs with disappeared polygonal pseudopodia and smaller or elliptical morphology (Figure 1A,B). Similarly, the apoptotic phenotype of HSCs was observed using an electric microscope, demonstrating that 100 μM DATS caused apoptotic cell death characterized by nuclear condensation, nuclear fragmentation, chromatin condensation, and apoptotic body formation (Figure 1C). Moreover, results from MTS assay showed that with the increase in DATS concentration and prolongation of DATS treatment duration, the proliferative ability of cells gradually and significantly decreased, indicating that DATS led to concentration and time-dependent inhibition of HSC proliferation (Figure 1D).

3.2 | Effects of DATS on the expression of Bcl-2 and apoptosis-related factors in HSC-T6 cells

To clarify the underlying mechanism of DATS-induced cell apoptosis, different concentrations of DATS (25, 50, 100, and 200 μM) were used to stimulate HSC-T6 cells for 48 h to detect the expression levels of BCL-2 and apoptosis-related factors by western blot analysis. The results showed that with the increase in DATS concentration, the expression level of antiapoptotic protein Bcl-2 gradually and significantly decreased, while those of apoptosis-related proteins Bax, p53, and Caspase-3/6/8/9 gradually and significantly increased (Figure 2A–C). In addition, the ratio of Bax to Bcl-2 increased with increasing DATS concentration (Figure 2D). Thus, DATS markedly affected HSC-T6 cell apoptosis through Bcl-2-mediated signaling pathways.

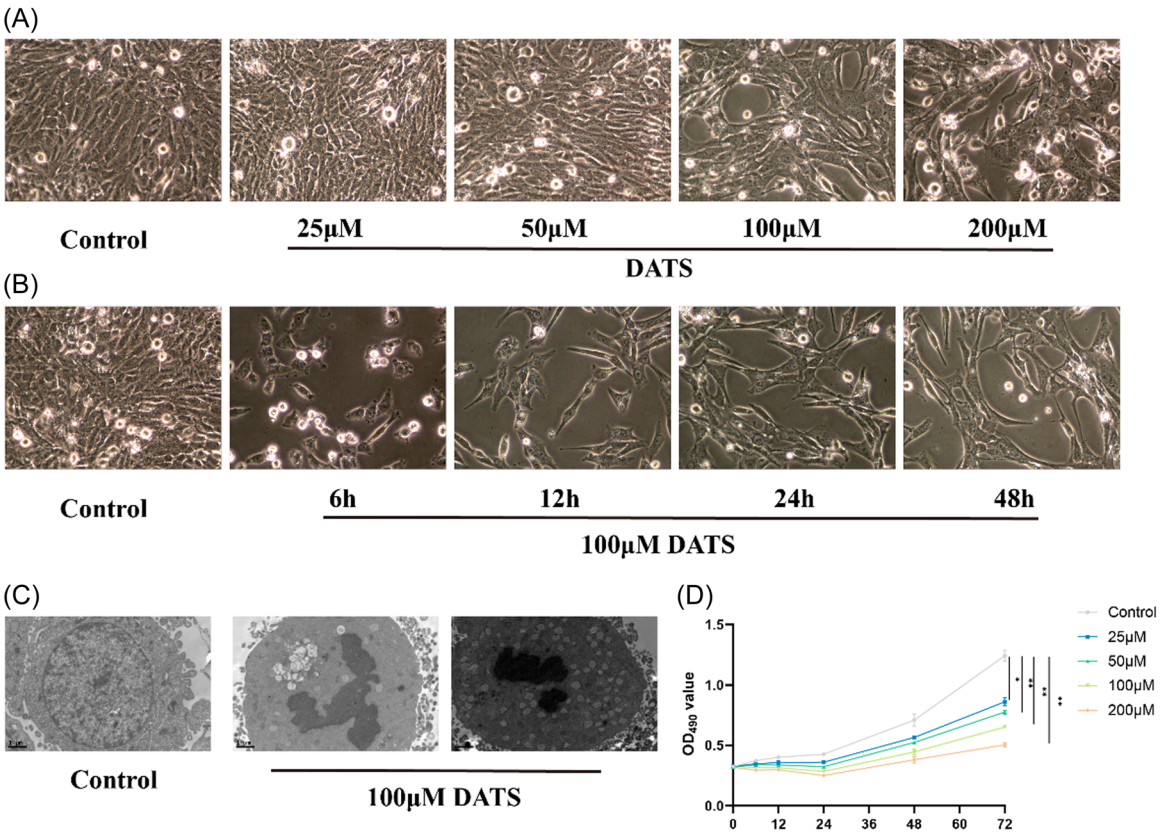

FIGURE 1 Effects of DATS on HSC apoptosis. (A) Morphology of HSC-T6 cells after the treatment with different concentrations of DATS for 48 h (200X magnification). (B) Morphology of HSC-T6 cells after the treatment with 100 μM DATS at different times (200X magnification). (C) Ultrastructure of HSC-T6 cells was observed using transmission electron microscopy after the treatment with 100 μM DATS. (D) The proliferative ability of cells was determined by MTS assay. *t* test, $^{*}p < 0.05$ and $^{**}p < 0.01$; the difference was statistically significant. DATS, diallyl trisulfide; HSC, hepatic stellate cell; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

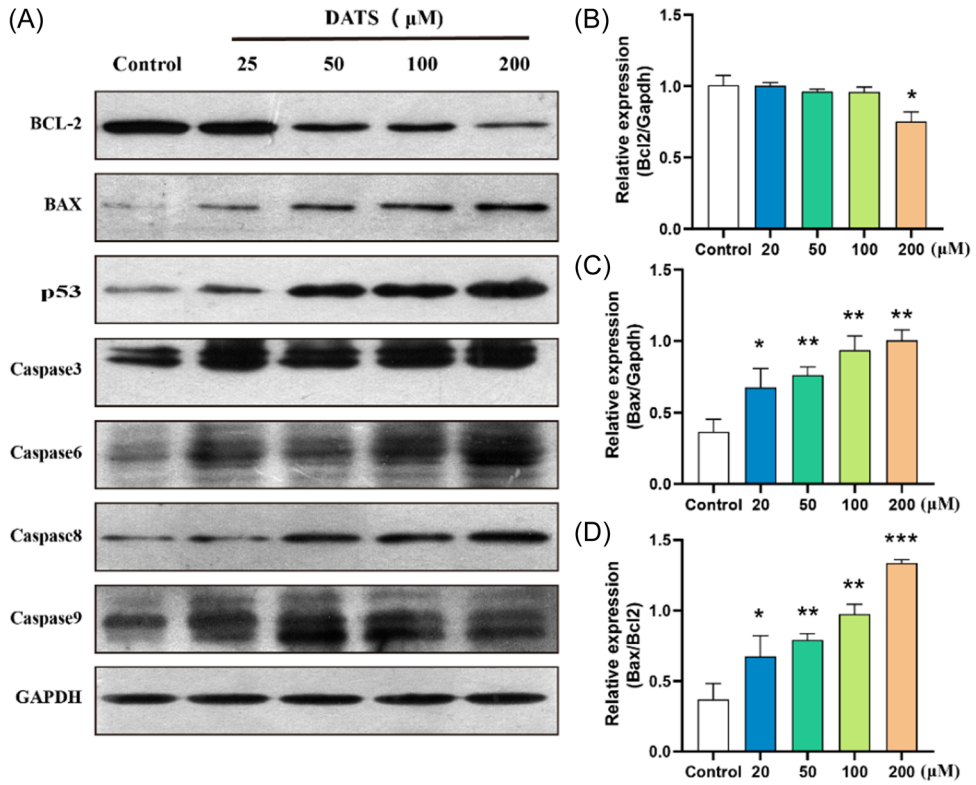

FIGURE 2 Effects of DATS on the expression of Bcl-2 and apoptosis-related factors in HSC-T6 cells. (A) The expression levels of Bcl-2, Bax, p53, and Caspase-3/6/8/9 proteins were detected using western blot analysis (B–D). Quantitation of the data presented in panel (A). *t* test, $^{*}p < 0.05$ and $^{**}p < 0.01$; the difference was statistically significant. DATS, diallyl trisulfide; HSC, hepatic stellate cell

3.3 | Effect of DATS on morphology and apoptosis of HSC-T6 cells under different Bcl-2 expression states

Since Bcl-2 is important for cell apoptosis, we further explored whether the function of DATS is dependent on Bcl-2. The morphology of HSC-T6 cells under different Bcl-2 expression states after 72 h was observed using an inverted optical microscope. We found that when Bcl-2 was inhibited, HSC-T6 cells became smaller and smoother, with polygonal pseudopodia decreasing or disappearing gradually; the inhibition of cell proliferation was apparent, and with the overexpression of Bcl-2, cells grew adherent to the wall rapidly with pleomorphism. In addition, the cellular morphology of HSC-T6 cells with overexpression of Bcl-2 could be reversed upon the treatment with DATS at a concentration of 100 μM for 48 h. Other cellular morphologies of HSC-T6 cells under different Bcl-2 expression status did not change significantly after treatment with DATS (Figure 3A). Meanwhile, apoptosis was detected in HSC-T6 cells with different Bcl-2 expression states upon treatment with DATS (100 μM) for 48 h by flow cytometry. The apoptosis rate of HSC-T6 cells with deregulated Bcl-2 treated with DATS was similar to that of HSC-T6 cells treated with DATS, while the apoptosis rate of HSC-T6 cells overexpressing Bcl-2 treated with DATS declined; this was similar to the results obtained upon treatment with DMSO (Figure 3B,C).

3.4 | Effect of DATS on apoptosis-related factors under different Bcl-2 expression states

The protein expression levels of major molecules in the Bcl-2 pathway were detected by western blot analysis after treatment with 100 μM DATS for 48 h. To begin with, the upregulation or downregulation of Bcl-2 was successful (Figure 4A). The protein expression levels of Bax, cleaved-Caspase-3/8/9, Apaf-1, and Cyto-c increased after treatment with DATS, while upregulation of Bcl-2 counteracted the effect of DATS (Figure 4B). Consistently, downregulation of Bcl-2 enhanced the effect of DATS (Figure 4C). These data suggest that DATS exerts its proapoptotic effect via Bcl-2-mediated signaling pathways.

4 | DISCUSSION

The mechanisms of liver fibrosis and cirrhosis have been investigated and studied for a long time, and few prophylactic and therapeutic remedies have been reported. DATS has been shown to have extensive anti-inflammatory, antitumor, antioxidation effects.[15–17] Recently great attention has been given to the hepatoprotective role of DATS against fibrosis.

The present results demonstrate that DATS promotes HSC apoptosis by inhibiting Bcl-2 signaling pathway. We observed that

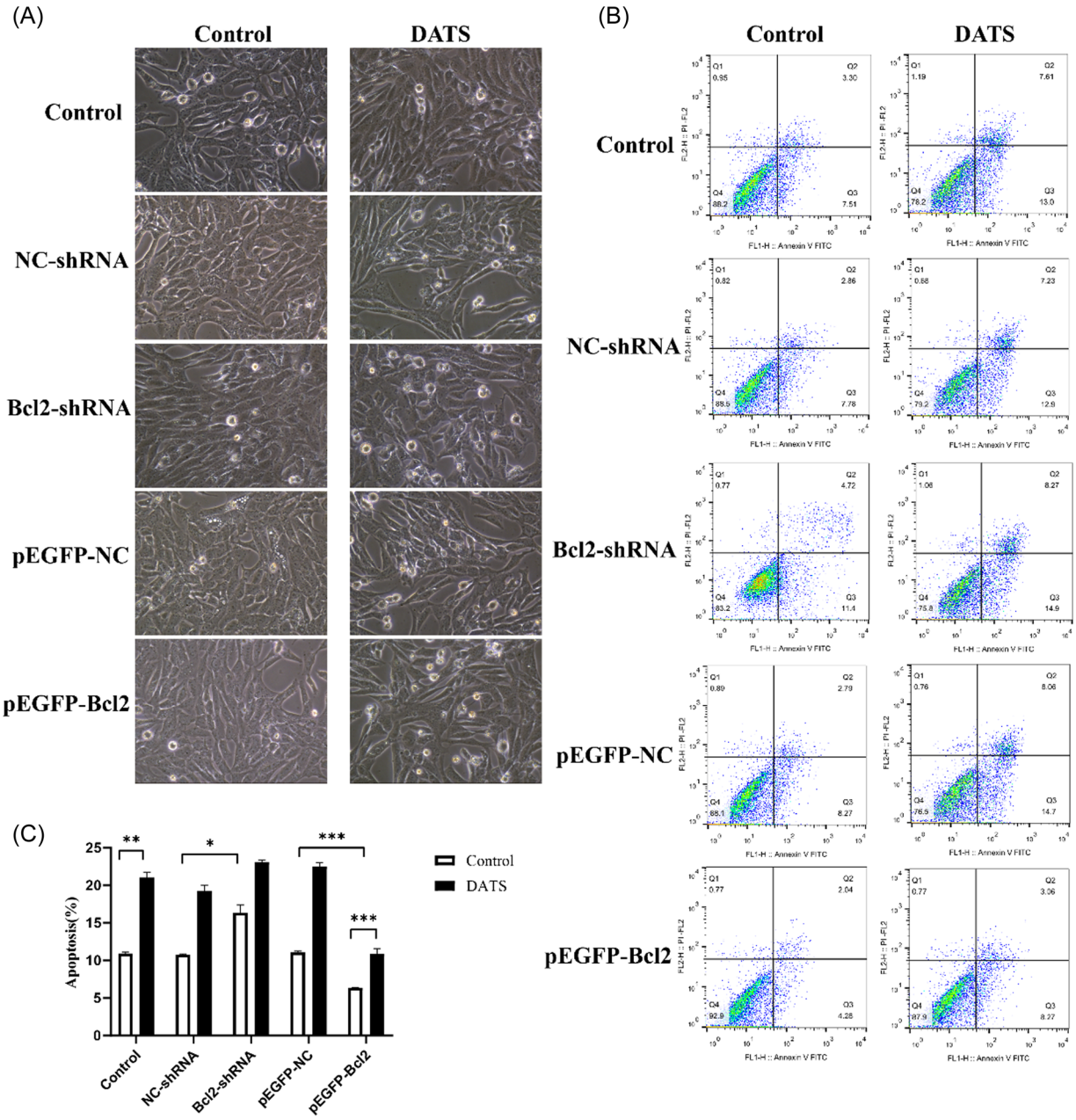

FIGURE 3 Effect of DATS on cellular morphology and apoptosis of HSC-T6 cells under different Bcl-2 expression states. (A) Morphology of HSC-T6 cells under different Bcl-2 expression states and treatment with DATS (200X). (B) The apoptosis of HSC-T6 cells was detected under different Bcl-2 expression states and treatment with DATS. (C) Quantitation of the data presented in panel (B) control, treatment with DMSO; NC-shRNA. a control for downregulation of Bcl-2; Bcl2-shRNA, downregulation of Bcl-2; pEGFP-NC, a control for upregulation of Bcl-2; pEGFP-Bcl2, upregulation of Bcl-2. *t* test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; the difference was statistically significant. DATS, diallyl trisulfide; HSC, hepatic stellate cell; DMSO, dimethyl sulfoxide; shRNA, short hairpin RNA

DATS promoted apoptosis in HSC-T6 cells. This was accompanied by a decrease in Bcl-2 expression and an increase in Bax expression. In addition, we found that a series of proapoptotic factors, including Caspase-3, Caspase-6, Caspase-8, Caspase-9, MMP13, p53, Apaf-1, Cyto-c, glutaminase, GLUD-1, and Col-1 were elevated after the treatment with DATS, as many studies have shown an important role in the apoptotic pathway.[18–22] In addition, DATS significantly promoted HSC-T6 apoptosis when combined with sh-RNA of Bcl-2, while overexpression of Bcl-2 weakened the proapoptotic effect of DATS.

Members of the B-cell lymphoma 2 (BCL-2) family are essential for regulating programmed cell death by controlling proapoptotic and antiapoptotic intracellular signals, including proapoptotic proteins Bcl-2, Bcl-xL, and Bcl-w, and the antiapoptotic proteins Bax and Bak.[23,24] The Bcl-2 protein is a membrane-binding protein that is mainly distributed in the mitochondrial outer membrane, endoplasmic

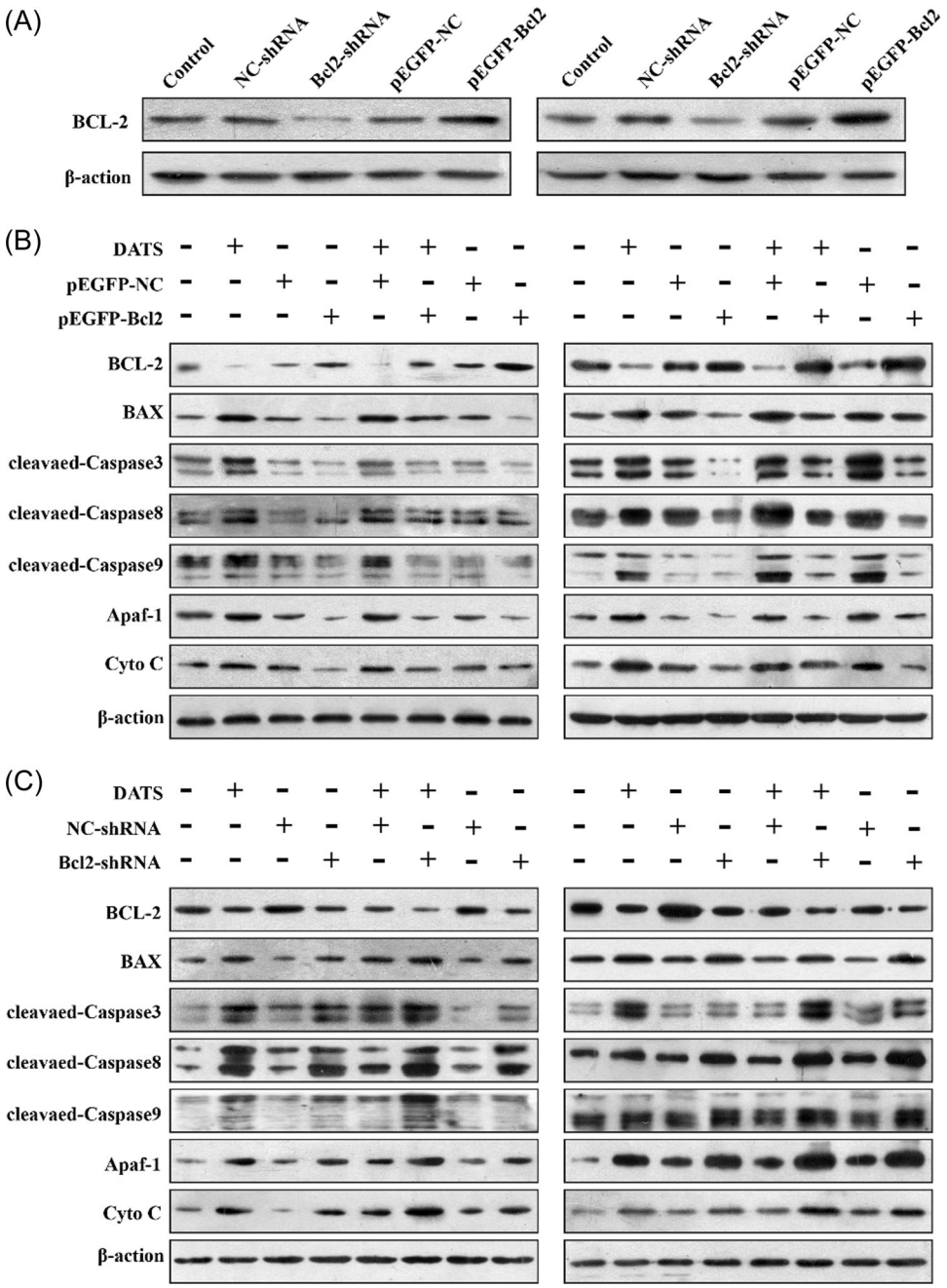

FIGURE 4 Effect of DATS on apoptosis-related factors under different Bcl-2 expression states. (A) The protein expression levels of Bcl-2 under different Bcl-2 expression states are presented. (B) The protein expression levels of Bcl-2, Bax, cleaved-Caspase-3/8/9, Apaf-1, and Cyto-c under upregulation of Bcl-2 or treatment with DATS are presented. (C) The protein expression levels of Bcl-2, Bax, cleaved-Caspase-3/8/9, Apaf-1, and Cyto-c under downregulation of Bcl-2 or treatment with DATS are presented. DATS, diallyl trisulfide; HSC, hepatic stellate cell; shRNA, short hairpin RNA

reticulum, and nuclear membrane and is synergistic with other apoptosis-related factors. It affects the stability of the common regulation of mitochondrial structure and function, but has no effect on cell differentiation and cell cycle. Bax proteins (including Bax-α, Bax-β, and Bax-γ) are found in the study which promotes apoptosis, and studies have found that increased expression of Bax-α can accelerate cell apoptosis.[25,26] In recent years, studies concerning the treatment of liver fibrosis have found that downregulation of Bcl-2 protein expression and upregulation of Bax protein expression can lead to HSC apoptosis, and the ratio of Bax/Bcl-2 is considered to be an important determinant of apoptosis. The increased Bax/Bcl-2 ratio promoted apoptosis, while decreased Bax/Bcl-2 ratio inhibited apoptosis.[27–29] Our study is consistent with the finding that DATS increased the Bax/Bcl-2 ratio. Accordingly, we assessed the effect of Bcl-2 on the apoptosis of HSCs. Overexpression of Bcl-2 inhibited

apoptosis and diminished the proapoptotic role of DATS, while suppression of Bcl-2 had the opposite effect. These findings verify that DATS can be considered as a hepatoprotective agent.

The cysteinyl aspartate-specific proteinase (caspase) family is an executor of apoptosis. Caspases are thought to be closely related to eukaryotic cell apoptosis. There are two main types of caspases: the promoter enzyme and the effector enzyme, which play an upstream and a downstream role in the death signal transduction, respectively. Caspase-3, Caspase-6, Caspase-8, Caspase-9, and other effector enzymes are activated by cleavage under the action of foreign protein signals. The activated promoter enzyme cleaves and activates the effector enzyme, and the activated effector enzyme leads to programmed cell death by hydrolyzing the target protein.[30–32] In this study, we found that upon treatment with DATS, the expression levels of Caspase-3, Caspase-6, Caspase-8, and Caspase-9 in HSC-T6 cells increased significantly, consistent with the increased ratio of Bax/Bcl-2, which further indicated that DATS may activate caspases and induce apoptosis of HSC-T6 cells by regulating the expression of Bcl-2 and Bax proteins.

DATS is a natural organic sulfide obtained from garlic. Our study found that DATS can promote HSC apoptosis and inhibit HSC proliferation; however, there is a debate about the role of organic sulfides in liver cells. Caro et al. found that garlic-derived organosulfur compounds directly impair mitochondrial functions and integrity in isolated mouse liver mitochondria,[33] while Heidari et al. found that organosulfur chemicals extended their protective effects against methimazole-induced toxicity by attenuating oxidative stress caused by this drug and preventing the adverse effects of methimazole and/or its metabolite(s) on subcellular components such as mitochondria.[34] However, all these studies have been carried out in vitro, and the role of DATS in vivo needs to be studied further. In our study, we have found that DATS acted in a concentration-dependent manner. Therefore, we believe that determining the appropriate concentration of DATS is a key issue, and the next in vivo experiment should actively explore the appropriate concentration of DATS and evaluate its effect on the liver, which will provide a basis for the clinical study of DATS against hepatic fibrosis. In addition, DATS and other organic sulfides have poor targeting performance as drugs, which may limit their administration in future research; an increase in the targeting performance of these drugs will help expand their clinical applications. In this study, we found that DATS plays an anti-fibrotic role by inhibiting Bcl-2 expression and promoting HSC apoptosis, which will provide a theoretical basis for in vivo experiments and clinical studies analyzing the role of DATS against hepatic fibrosis. Limited time, funding, and technology have prevented us from performing further investigations currently; nevertheless, these issues will be addressed in our future endeavors to provide more solid evidence for the clinical application of DATS against hepatic fibrosis.

5 | CONCLUSION

In conclusion, DATS can induce HSC apoptosis and inhibit cell proliferation, which may be related to the regulation of the Bcl-2 signaling pathway. Therefore, DATS is expected to be an effective treatment for liver fibrosis.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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