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Article

CCN5-Derived Peptide Ameliorates Liver Fibrosis in Mice through Inhibiting the ERK1/2 and PI3K Signaling Pathways

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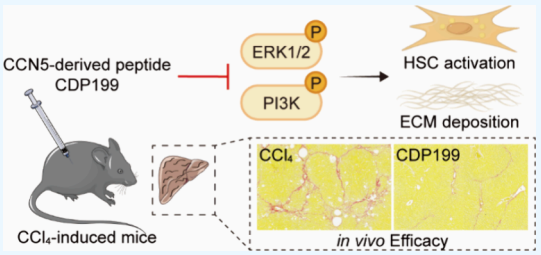
ABSTRACT: Liver fibrosis is a pathological process characterized by the activation of hepatic stellate cells (HSCs) and the excessive accumulation of extracellular matrix (ECM) proteins. Cellular communication network 5 (CCN5) has been recently recognized for its ability to counteract the profibrotic effects of CCN2 in fibrotic diseases. Herein, we report the discovery of a CCN5-derived peptide CDP199 that effectively suppresses HSC activation and reduces ECM protein deposition. Notably, CDP199 exhibits strong inhibitory effects on HSC proliferation and migration. The subsequent *in vivo* study demonstrated that peptide CDP199 significantly alleviates liver injury, enhances liver function, and mitigates liver fibrosis in a carbon tetrachloride-induced mouse model. Mechanistically, CDP199 inhibits the phosphorylation of ERK1/2 and PI3K both *in vitro* and *in vivo*. These findings highlight the therapeutic potential of CCN5-derived peptides, specifically CDP199, as a promising antifibrotic candidate for treating liver diseases through inhibition of ERK1/2 and PI3K signaling pathways.



INTRODUCTION

Liver fibrosis is characterized by the persistent accumulation of extracellular matrix (ECM) proteins, which causes tissue structural impairment and subsequent liver dysfunction.[1−5] During liver fibrosis, activated hepatic stellate cells (HSCs) serve as the primary producers of ECM proteins.[6−10] Mechanistically, inflammatory cytokines can activate quiescent HSCs, triggering their transdifferentiation into myofibroblasts.[6,11] These myofibroblasts secrete substantial amounts of collagens and other ECM components, including elastin and hyaluronan, which are crucial in the development of liver fibrosis.[12−14] Despite its potential to advance to severe complications like cirrhosis and hepatocellular carcinoma,[15−17] there are currently no approved pharmaceutical treatments for liver fibrosis. Therefore, there is a pressing need for the development of innovative antifibrotic therapies to prevent the progression of liver fibrosis.

Cellular communication network (CCN) proteins, a family of six highly conserved matricellular proteins, play a pivotal role in intercellular and cell-matrix communication, maintaining the homeostasis of parenchymal tissues.[18] Among these CCN proteins, CCN5 is unique due to its absence of the carboxyl-terminal fourth domain.[19,20] In addition, CCN5 has been demonstrated to inhibit cardiac hypertrophy and fibrosis.[21,22] Recently, a peptide (TAWGPCSTTCGLGMATRV, named CDP199) derived from the thrombospondin type 1 (TSP-1) domain of CCN5 has been first reported to inhibit the proliferation and migration of endothelial cells.[23] However, the effects of peptide CDP199 in the context of liver fibrosis remain insufficiently explored, posing a significant and important research question.

In this study, we synthesized the peptide CDP199 and explored its potential as an antifibrotic agent. The efficacy of peptide CDP199 against activation and ECM accumulation was first studied in a TGF-β1-induced LX-2 cell model. Meanwhile, the abilities of peptide CDP199 to reduce LX-2 cell proliferation and migration were evaluated. Furthermore, we assessed the impact of CDP199 on HSC activation and collagen accumulation in a CCl_4-induced liver fibrosis mouse model. To investigate its mechanism of action, we tested the ability of CDP199 to antagonize TGF-β1-induced ERK1/2 and PI3K activation. This work is among the first to demonstrate the role of CDP199 in mitigating liver fibrosis, laying the groundwork for its potential development as a therapeutic agent.

RESULTS

Peptide CDP199 Derived from CCN5 Inhibited TGF-β1-Induced LX-2 Cell Activation and ECM Accumulation. First, CCN5-derived peptide CDP199 (TAWGPCSTTCGLGMATRV) was synthesized using SPPS. The antifibrotic potential of peptide CDP199 was evaluated using the LX-2 cell model, which is derived from human HSCs. LX-2 cells were incubated with 10 ng/mL TGF-β1, a known

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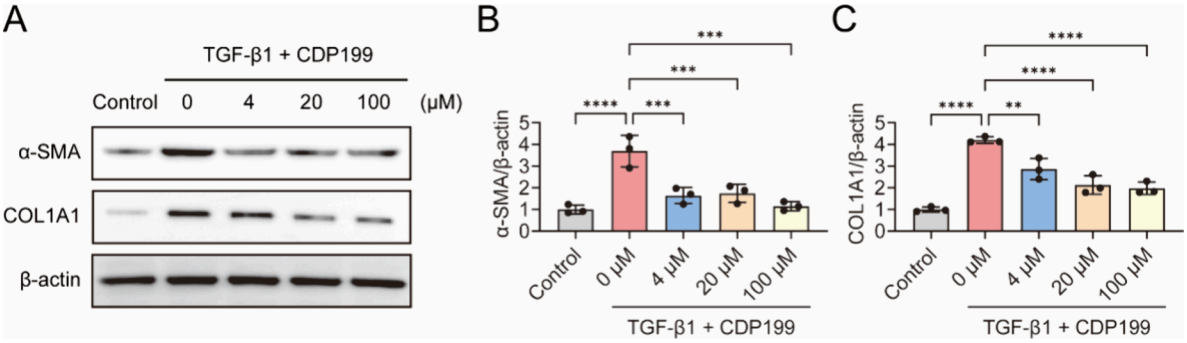

Figure 1. Peptide CDP199 derived from CCN5 inhibited the TGF-β1-induced activation of LX-2 cells and ECM accumulation. (A−C) Representative Western blot images and semiquantitative analyses of α-SMA and COL1A1 in TGF-β1-induced LX-2 cells treated with different concentrations of CDP199 (n = 3). β-actin level demonstrates equal loading. Data are presented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

activator of HSCs, in the presence of varying concentrations of the peptide CDP199 (4, 20, and 100 μM). Western blot analysis was conducted to assess the effect of peptide CDP199 on the expression of α-smooth muscle actin (α-SMA) and type I collagen (COL1A1), biomarkers indicative of HSC activation and ECM accumulation, respectively.[24,25] The results demonstrated that peptide CDP199 significantly reduced the levels of protein expression of α-SMA and COL1A1 (Figure 1A−C). These findings suggest that peptide CDP199 exhibits promising antifibrotic activity, highlighting its potential therapeutic application in the treatment of liver fibrosis.

Peptide CDP199 Inhibited TGF-β1-Induced Proliferation and Migration of LX-2 Cells. HSC proliferation and migration are important processes in the development of liver fibrosis. Given that peptide CDP199 has previously been reported to inhibit the proliferation and migration of human umbilical vein endothelial cells,[23] we assumed that peptide CDP199 might exert a similar inhibitory activity in TGF-β1-induced LX-2 cells. To verify this hypothesis, we first tested the ability of peptide on the proliferation of LX-2 cells using the cell counting kit-8 (CCK-8) assay.[24,26,27] As presented in Figure 2A, TGF-β1 stimulation significantly increased the proliferation of LX-2 cells, and treatment with peptide CDP199 resulted in a remarkable inhibition of LX-2 cell proliferation. Notably, the inhibitory effect at 20 and 100 μM restored the proliferation rate to levels comparable to the untreated control group. Subsequently, we assessed the migration of LX-2 cells using an *in vitro* wound-healing assay.[28] Following a 24 h incubation with peptide CDP199 in the presence of TGF-β1, there was a significant reduction in both cell migration and the wound closure area (Figure 2B,C). These results further confirmed the inhibitory effect of peptide CDP199 on LX-2 cell migration. Based on these promising *in vitro* results, peptide CDP199 was administered in a fibrosis mouse model to further evaluate its antifibrotic bioactivity *in vivo*.

Peptide CDP199 Ameliorated Liver Injury and Fibrosis in CCl_4-Treated Mice. To evaluate the *in vivo* antifibrotic effects of peptide CDP199, we utilized the CCl_4-induced mouse model of liver fibrosis.[29−31] Male C57BL/6 mice were injected intraperitoneally with either oil or CCl_4 diluted in oil three times per week for 6 weeks. Starting from the fourth week, peptide CDP199 was administered subcutaneously on a daily basis (Figure 3A). At the end of the experiment, liver histology was analyzed using hematoxylin and eosin (H&E) staining. In the oil-treated group, a normal

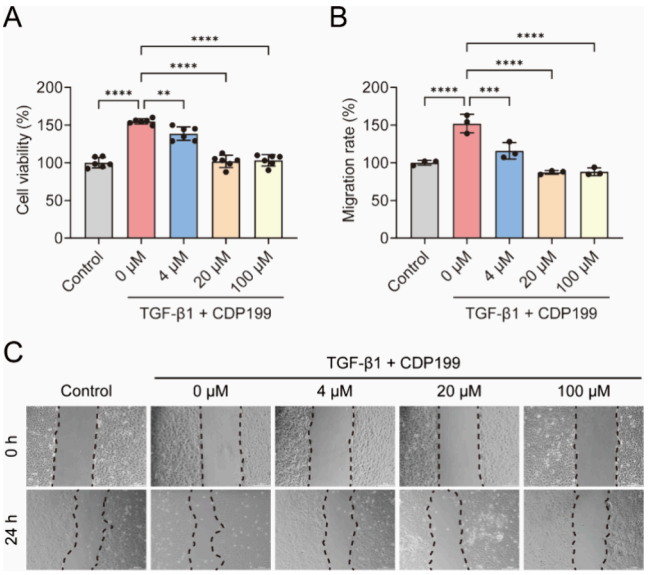

Figure 2. Peptide CDP199 inhibited TGF-β1-induced proliferation and migration of LX-2 cells. (A) Cell proliferation assay in TGF-β1-induced LX-2 cells treated with different concentrations of CDP199 (n = 6). (B, C) Representative bright-field images and semiquantitative analyses of the wound-healing assay in TGF-β1-induced LX-2 cells treated with different concentrations of CDP199 (n = 3). Data are presented as mean ± SD; **p < 0.01, ***p < 0.001, and ****p < 0.0001.

hepatic lobular architecture with central veins and radial hepatic cords was observed (Figure 3B). In contrast, the CCl_4-treated group displayed a disrupted liver structure characterized by hepatocyte enlargement, necrosis, cytoplasmic vacuolation, and pseudolobule formation. Remarkably, peptide CDP199 treatment significantly mitigated these pathological changes, as evidenced by reduced hepatocyte nuclear fragmentation and inflammatory cell infiltration (Figure 3B). Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), key biomarkers of liver function, were measured to assess hepatic injury.[32−34] The CCl_4-treated group exhibited elevated ALT and AST levels compared to the oil-treated group, indicating liver dysfunction caused by CCl_4 administration. However, peptide CDP199 treatment at doses of 80 and 240 μg/kg significantly reduced both ALT and AST levels, suggesting an improvement in liver function (Figure 3C,D). These data suggested that peptide CDP199 could

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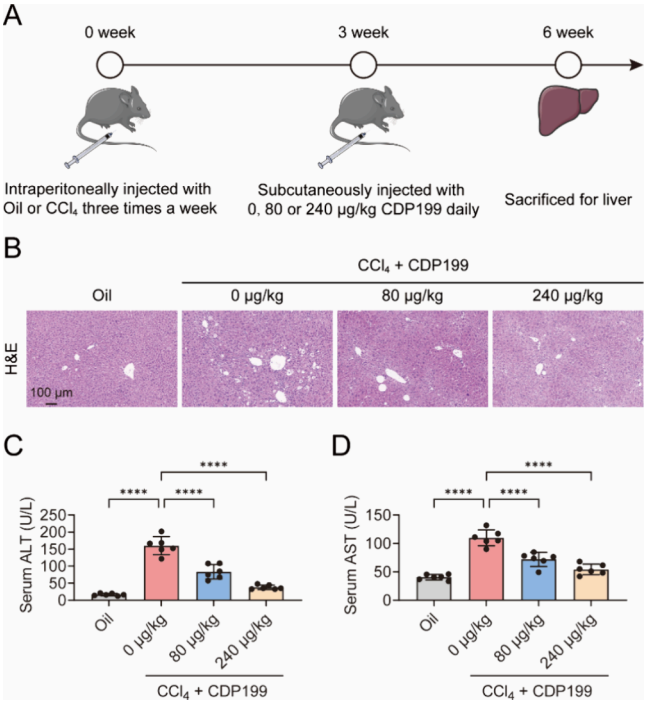

Figure 3. Peptide CDP199 ameliorated liver injury in CCl_4-induced mice. (A) Schematic diagram of the CCl_4-induced mice model of liver injury and CDP199 treatment. (B) Histological images of liver tissue stained with H&E (magnification of 10×, $n = 6$). (C, D) Serum levels of ALT and AST of CCl_4-induced mice treated with CDP199 ($n = 6$). Data are presented as mean ± SD; ***$p < 0.001$ and ****$p < 0.0001$.

alleviate liver injury and improve liver function in the CCl_4-induced mouse model.

Masson and Sirius Red stainings were used to analyze the inhibitory effect of peptide CDP199 on collagen deposition. Masson's trichrome staining revealed that CCl_4 administration resulted in connective tissue proliferation, distorted liver architecture, and fibrous collagen accumulation between the portal vein and liver lobules, confirming the establishment of liver fibrosis (Figure 4A,B). In contrast, peptide CDP199 treatment significantly reduced collagen fiber accumulation. Sirius Red staining also demonstrated prominent collagen deposition in the livers of CCl_4-treated mice, which was reversed following peptide CDP199 administration (Figure 4A,C). To understand the mechanism behind the reduction in collagen deposition, Western blot analysis was performed to evaluate the protein levels of α-SMA and COL1A1 in mouse livers. Consistent with the histological findings, CCl_4 administration upregulated α-SMA and COL1A1 protein expression, whereas peptide CDP199 decreased their protein contents in mouse livers (Figure 4D−F). These data collectively suggest that the peptide CDP199 alleviates liver fibrosis in the CCl_4-induced mouse model by inhibiting HSC activation and reducing collagen deposition.

Peptide CDP199 Inhibited the Activation of the ERK1/2 and PI3K Signaling Pathways in LX-2 Cells and CCl_4-Induced Mice. The extracellular signal-regulated kinase 1/2 (ERK1/2) pathway plays a pivotal role in the activation of HSCs and the progression of liver fibrosis.[35] Previous studies have shown that inhibiting the activation of the ERK1/2 pathway can reduce HSC activation and the expression of ECM-related proteins, thereby alleviating liver fibrosis. Given the TSP-1 domain of CCN5 inhibiting ERK1/2

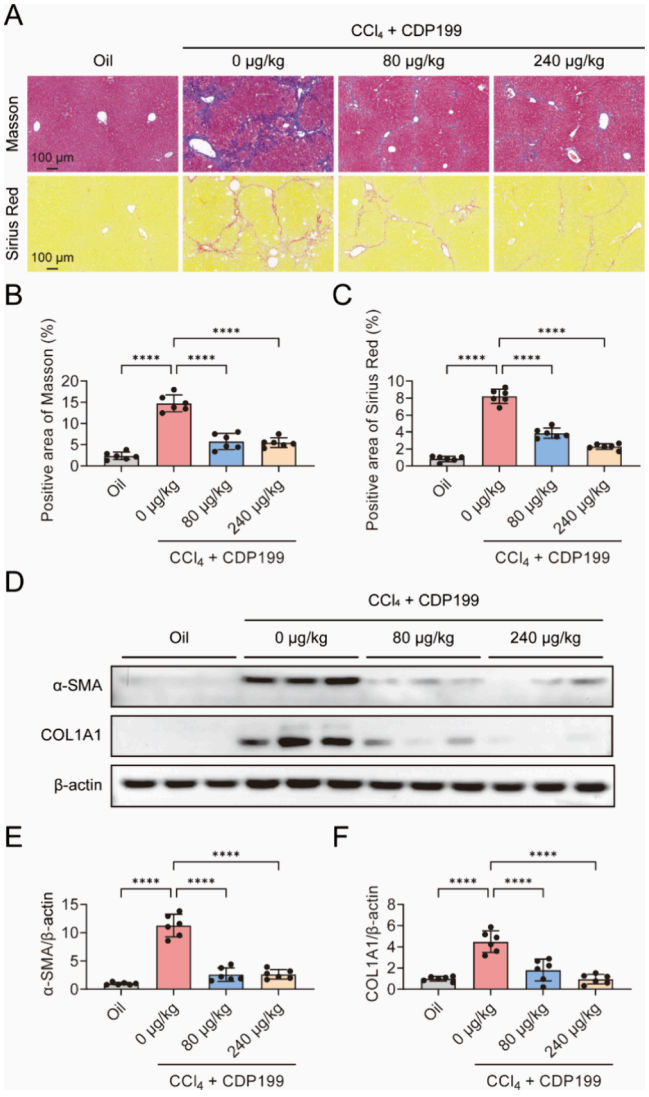

Figure 4. Peptide CDP199 ameliorated liver fibrosis in CCl_4-induced mice. (A−C) Histological images and semiquantitative analyses of liver tissue stained with Masson and Sirius Red stainings (magnification of 10×, $n = 6$). (D−F) Representative Western blot images and semiquantitative analyses of α-SMA and COL1A1 of liver tissue in a CCl_4-induced mice liver injury model ($n = 6$). β-actin level demonstrates equal loading. Data are presented as mean ± SD; ****$p < 0.0001$.

phosphorylation, we hypothesized peptide CDP199 could suppress ERK1/2 phosphorylation in TGF-β1-induced LX-2 cells. To test this hypothesis, we assessed the expression levels of phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 proteins in LX-2 cells by using Western blot analysis. As expected, TGF-β1 stimulation led to significant activation of the ERK1/2 pathway, as indicated by a remarkable upregulation of p-ERK1/2 expression compared to the control group. In addition, treatment with peptide CDP199 significantly reduced the phosphorylation of ERK1/2 (Figure 5A,B). Cross-talk occurred between the ERK1/2 and PI3K signaling pathways during the activation of HSC in liver fibrosis progression.[36] Based on this regulatory network, we subsequently investigated the effect of the peptide CDP199 on the PI3K signaling pathway. Western blot analysis demonstrated that TGF-β1 stimulation markedly enhanced phosphorylated PI3K (p-PI3K) protein expression in LX-2 cells, whereas

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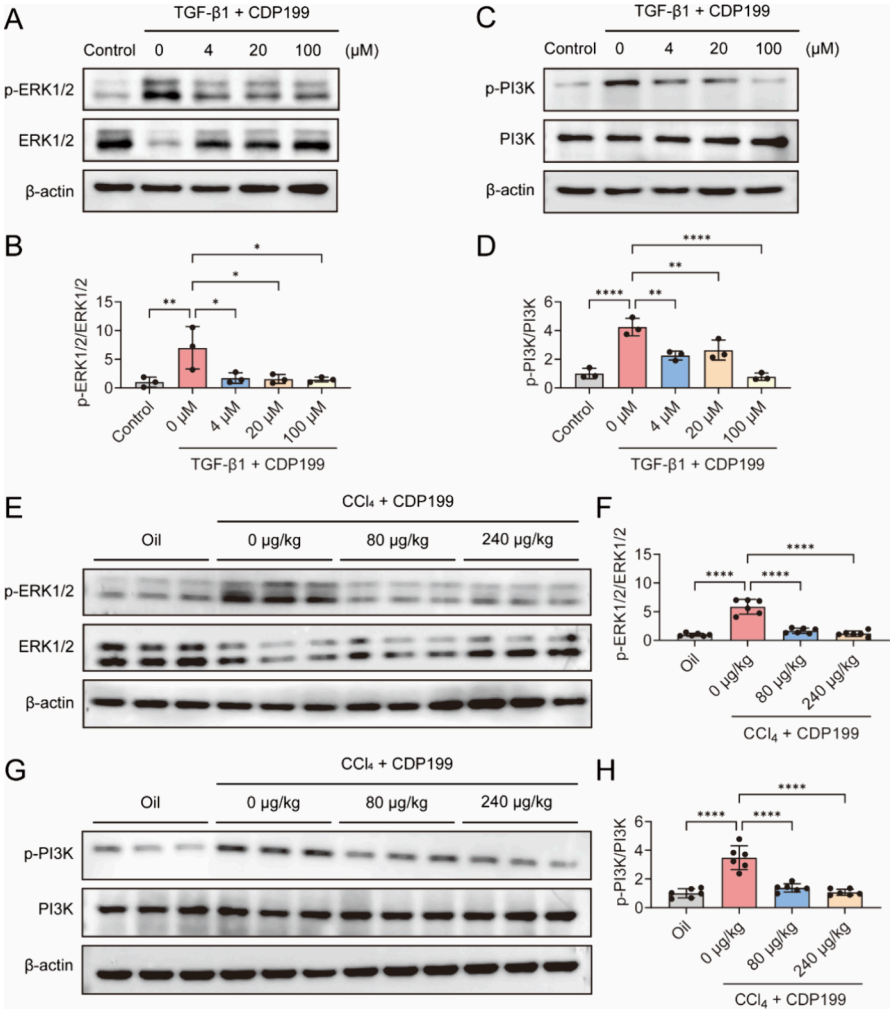

Figure 5. Inhibitory effects of peptide CDP199 on ERK1/2 and PI3K phosphorylation pathways *in vitro* and *in vivo*. (A−D) Representative Western blot images and semiquantitative analyses of p-ERK1/2, ERK1/2, p-PI3K, and PI3K expression in TGF-β1-stimulated LX-2 cells following treatment with varying concentrations of CDP199 (n = 3). (E−H) Representative Western blot images and semiquantitative analyses of p-ERK1/2, ERK1/2, p-PI3K, and PI3K expressions in CCl_4-induced fibrotic livers (n = 6). β-actin level demonstrates equal loading. Data are presented as mean ± SD; *p < 0.05, **p < 0.01, and ****p < 0.0001.

CDP199 treatment significantly attenuated this activation, suggesting its potential inhibitory effect on PI3K signaling (Figure 5C,D). Similarly, in liver tissues from CCl_4-treated mice, administration of peptide CDP199 resulted in a significant decrease in ERK1/2 and PI3K phosphorylation, suggesting that the peptide inhibits ERK1/2 and PI3K signaling pathways *in vivo* (Figure 5E−H). Taken together, these results indicate that peptide CDP199 attenuates the development and progression of liver fibrosis in both the TGF-β1-induced LX-2 cell model and the CCl_4-induced mouse model by inhibiting the activation of the ERK1/2 and PI3K signaling pathways.

DISCUSSION AND CONCLUSIONS

Liver fibrosis is a key feature of chronic liver diseases, characterized by hepatocyte injury, inflammation, and the activation of HSCs, which leads to the excessive accumulation of ECM proteins.[2−4] Numerous studies have demonstrated that part of peptide drugs are effective in inhibiting HSC activation and reducing ECM protein deposition.[37−39] Compared to small-molecule drugs, peptides offer several advantages, including higher efficacy, increased potency, greater selectivity, and enhanced safety profiles.[40,41] Additionally, the ease of chemical synthesis and modification makes peptides a more practical and preferred option over recombinant proteins or antibodies.[42,43] Given these benefits, peptide drugs present a promising therapeutic approach for treating liver fibrotic disorders and other chronic liver diseases. The peptide CDP199 was derived from the TSP-1 domain of CCN5 and has been identified to have antiangiogenic properties, inhibiting the proliferation and migration of endothelial cells.[23] However, the effects of CDP199 have not been studied in the liver fibrosis area. In this study, we demonstrated that peptide CDP199 effectively inhibited TGF-β1-induced HSC activation and ameliorated CCl_4-induced liver fibrosis via suppressing ERK1/2 and PI3K signaling pathways.

Liver fibrosis is a complex process that occurs in chronic liver disease stemming from factors such as viral infections, cholestasis, alcohol abuse, nonalcoholic fatty liver disease, drug-induced injuries, and various other causes.[44] The pathogenesis involves persistent hepatocyte damage, which triggers the release of proinflammatory cytokines from activated Kupffer cells, subsequently initiating the activation

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of HSCs. Substantial experimental evidence has established that HSC activation serves as the central driving force in the initiation, progression, and maintenance of hepatic fibrogenesis. In the present study, we demonstrated that peptide CDP199 significantly inhibited the activation, proliferation, and migratory capacity of TGF-β1-stimulated LX-2 cells, indicating its potential as a therapeutic agent for liver fibrosis. These *in vitro* findings were further validated in a CCl_4-induced mouse model of hepatic fibrosis. However, considering that CCl_4-induced fibrosis primarily reflects toxin-mediated fibrogenesis and may not fully recapitulate the complex pathophysiology of fibrosis induced by other etiologies, other preclinical models of liver fibrosis should be incorporated to comprehensively evaluate the therapeutic efficacy of peptide CDP199 across different fibrotic mechanisms.

In addition, our results demonstrated that peptide CDP199 administration at both 80 and 240 μg/kg doses effectively ameliorated CCl_4-induced hepatic injury and fibrotic progression. Therefore, further studies are needed to fully explore the potential of peptide CDP199, including identifying the optimal dosage and administration routes. Notably, despite its promising antifibrotic effects, the applications of linear peptides like CDP199 face several pharmacological challenges, including inherent proteolytic instability, rapid plasma clearance, and limited bioavailability.[39,45] To address these limitations, future research should focus on elucidating the structure−activity relationship of peptide CDP199 and developing structural modifications,[39,46−48] such as cyclization or incorporation of non-natural amino acids, to enhance its metabolic stability and therapeutic efficacy while maintaining its biological activity.

Extensive research has demonstrated that ERK1/2 signaling can be activated through phosphorylation mediated by various growth factors, subsequently regulating HSC activation and ECM production.[36] In addition, downregulating the ERK1/2 signaling pathway has been shown to effectively suppress HSC activation and attenuate liver fibrosis.[35,49] Building upon a previous finding that the TSP-1 domain of CCN5 exerted antifibrotic effects through antagonizing profibrotic CCN2 and suppressing ERK1/2 signaling activation,[19] we first sought to determine whether peptide CDP199 could modulate ERK1/2 phosphorylation. As observed, the peptide CDP199 inhibited ERK1/2 phosphorylation, thus preventing activation, proliferation, and migration in the TGF-β1-induced LX-2 cell model. Consistently, peptide CDP199 inhibited ERK1/2 activation, decreased collagen deposition, and attenuated liver fibrosis in the CCl_4-induced mouse model.

Accumulating evidence has established that PI3K signaling plays a crucial role in regulating HSC activation and ECM synthesis during liver fibrogenesis.[39,45] Previous pharmacological studies have demonstrated that targeted inhibition of the PI3K signaling pathway effectively suppresses HSC activation, attenuates excessive ECM deposition, and ameliorates liver fibrosis progression *in vivo*, establishing PI3K as a promising therapeutic target for antifibrotic intervention.[50] In the present study, peptide CDP199 significantly inhibited the PI3K signaling pathway in CCl_4-induced liver fibrosis, indicating that peptide CDP199 improved liver fibrosis through inhibiting the PI3K signaling pathway. Notably, emerging evidence suggests a functional cross-talk between PI3K and ERK1/2 signaling pathways in HSC activation, as PI3K inhibition has been shown to concurrently reduce ERK1/2 activity.[36,51] Thus, the potential involvement of the cross-talk between ERK1/2-PI3K in the antifibrotic mechanism of peptide CDP199 remains to be investigated in future studies.

In summary, our study demonstrates that the peptide CDP199 displayed potent inhibition activity against activation, ECM accumulation, proliferation, and migration in a TGF-β1-induced LX-2 cell model. Notably, peptide CDP199 could effectively inhibit HSC activation and ameliorate collagen accumulation in a CCl_4-induced liver fibrosis mouse model. Mechanistically, peptide CDP199 could effectively inhibit ERK1/2 activation *in vitro* and *in vivo*. This work provides an effective and promising pharmacological strategy targeting TGF-β1-induced ERK1/2 and PI3K activation, further supporting the potential of CCN5-derived peptides as antifibrotic therapeutics for liver diseases.

MATERIALS AND METHODS

Peptide Synthesis. Peptide CDP199 (TAWGPCSTTCGLGMATRV) was produced according to standard procedures for solid-phase peptide synthesis (SPPS) using 4-methylbenzylbenzylamine resin (GL Biochem, Shanghai, China). A reversed-phase liquid chromatography−mass spectrometer (RP-HPLC-MS, Agilent Technologies, USA) was used for peptide purification and atomic deposition. Analysis results can be found in the Supporting Information.

Cell Culture and Treatment. The human HSC cell line LX-2 was procured from Procell Life Science and Technology Co., Ltd. (Wuhan, China). These cells were cultivated in RPMI 1640 medium (Thermo Fisher Scientific, USA), which was added with 10% fetal bovine serum (Thermo Fisher Scientific, USA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, USA). The culturing conditions were maintained at 37 °C under suitable humidity containing 5% CO_2. The LX-2 cells were seeded onto six-well plates and incubated until reaching 80% confluence. Following an 8 h period of starvation, the LX-2 cells were exposed to 10 ng/mL TGF-β1 (Novoprotein, Suzhou, China) for a duration of 24 h.

Western Blot Analysis. To extract proteins from cells and liver samples, RIPA lysis buffer (Beyotime Biotechnology, China) supplemented with phosphatase and protease inhibitors was utilized. Protein concentrations were quantified by the BCA kit (Pierce, Thermo Fisher Scientific, USA). The extracted proteins were separated by SDS-PAGE and subsequently transferred to an equilibrated PVDF membrane (Merck Millipore, Germany). After blocking the membranes with 5% skim milk for 1 h, the PVDF membranes were incubated with the appropriate primary antibodies against COL1A1 (1:1000, Cell Signaling Technology, American, 7206S), α-SMA (1:1000, Boster Biological Technology, China, BM0002), p-ERK1/2 (1:1000, Cell Signaling Technology, American, 4370S), ERK1/2 (1:1000, Cell Signaling Technology, American, 4695S), p-PI3K (1:1000, Cell Signaling Technology, American, 4228S), PI3K (1:1000, Cell Signaling Technology, American, 4257S), and β-actin (1:5000, TransGen Biotech, China, HC201) at 4 °C overnight. Then, incubation was performed with an HRP-labeled secondary antibody antirabbit IgG (1:5000, Cell Signaling Technology, American, 7074S) or an antimouse IgG (1:5000, Cell Signaling Technology, American, 7076S) at room temperature for 1 h. The blots were visualized through enhanced chemiluminescence (Glarity Western ECL Substrate, Bio-Rad, USA), and the signals were documented and

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ACS Omega 2025, 10, 14221−14228

quantified by Tanon-Image Software (Shanghai, China). β-actin was used as a reference antibody for data normalization.

Scratch Assay. A two-well ibidi silicone culture insert, which was designed with a precisely defined cell-free gap and was appropriate for migration assays, served as the migration barrier. Initially, the two-well ibidi silicone culture insert was positioned precisely in the center of the 24-well plate. Subsequently, LX-2 cells were seeded into each well of the inset at 8×10^3 and permitted to proliferate until reaching 90% confluence under suitable conditions. After adequate time for cell attachment, the silicone insert was meticulously removed to create a cell-free zone. Subsequently, diverse concentrations of drugs were added separately to the respective wells of the plate. Finally, a Nikon TS100 inverted microscope (20× Nikon, Japan) was utilized to record the migration distance of LX-2 cells into the cell-free zone at both 0 and 24 h post insert removal. ImageJ software (https://imagej.nih.gov/ij/) was used to compute the migration areas at these two time points, thereby enabling the quantitative assessment of the migratory behavior of LX-2 cells under the influence of the applied drugs.

Cell Proliferation Assay. The assessment of cell proliferation was performed employing the CCK-8 (Beyotime, Shanghai, China) in strict conformity with the manufacturer's prescribed protocol. Cells were plated at a seeding density of 5×10^3 cells per well in 100 μL of medium within a 96-well microplate (Corning, USA). Subsequently, the cells were exposed to 10 ng/mL TGF-β1 and treated with peptide CDP199 at the concentrations of 20 and 100 μM. After a 24 h incubation interval, 10 μL of the CCK-8 reagent was dispensed into each well, followed by an additional 2 h incubation phase. All experimental trials were carried out in triplicate to enhance the statistical robustness. Absorbance measurements were taken at 450 nm using a microplate reader (Bio-Rad, Hercules, USA), with wells lacking cells utilized as blank controls for calibration.

Animal and Ethics Statement. Male C57BL/6J mice at 6−8 weeks old (20−24 g) were bought from Guangdong Medical Laboratory Animal Center and kept under a light/dark cycle of 12:12 h in cages at an ambient temperature of 20−25 °C. Food and water were available without restriction. Efforts have been made to minimize the suffering and the number of mice used. All experimental procedures followed the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health and were approved by the Animal Care and Use Committee of Sun Yat-sen University.

CCl_4-Induced Liver Fibrosis Model and Tissue Collection. To induce liver fibrosis, mice were given 20% CCl_4 (Sigma) three times a week for 6 weeks (5 mL/kg of body weight) via intraperitoneal injections. Mice that were injected with a volume of corn oil equivalent to that of the experimental group were utilized as the control group. Mice with liver fibrosis that had been treated with peptides during the final 3 weeks were employed to assess the pharmacological effects (80 or 240 μg/kg daily subcutaneous injection).

Upon completion of the experiments, the mice were sacrificed after an 8 h fasting period. Liver tissues were either flash-frozen in liquid nitrogen and subsequently stored at −80 °C for later analysis or fixed in 10% paraformaldehyde for histological evaluations. Blood samples were collected from the posterior venous plexus of the eyes in anesthetized mice. Subsequently, the blood was subjected to centrifugation at 1000 *g* for 15 min for separation. Thereafter, the serum was preserved at −80 °C for future assays.

Determination of ALT and Aspartate Transaminase. The ALT and aspartate transaminase (AST) levels in mouse serum were measured to assess liver damage in each group of mice. The ALT assay kit and the AST assay kit (Nanjing Jiancheng Bioengineering Institute, China) were used for measurement; then experiments were conducted according to the instructions.

Histopathology. Formalin-fixed liver tissues that had been embedded in paraffin were sectioned. After undergoing deparaffinization and rehydration procedures, the sectioned slices were subjected to Hematoxylin and Eosin (H&E) staining, Sirius Red staining, and Masson staining, which are standard methods for evaluating collagen accumulation. Subsequently, the positive area of stained tissue sections was measured by ImageJ.

Statistical Analysis. Statistical analysis was determined by GraphPad Prism 9.0 (https://www.graphpad.com/). Measurement data followed a normal distribution, exhibited homogeneity of variance, and were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by a Tukey post hoc test was used for comparison between multiple groups. *P* value less than 0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

Data Availability Statement

All data are included either as figures or as tables within the manuscript and Supporting Files.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c00128.

Analysis of Western blot; analysis of cell viability and migration rate; analysis of serum ALT and AST; positive area of Masson and Sirius Red; and chromatogram and mass spectrometry of peptide CDP199 (PDF)

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Author Contributions

R.F. and J.D. conceived and designed the research. R.F., C.Q., C.X., and J.D. performed experiments and analyzed the data. R.F., J.D., Y.Z., and X.J. interpreted the results of the experiments. C.X. and J.D. prepared the figures. R.F. and J.D. drafted the manuscript. R.F., C.Q., C.X., J.D., Y.Z., and X.J. edited and revised the manuscript and approved the final version of the manuscript. R.F. and C.Q. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Parola, M.; Pinzani, M. Liver fibrosis: Pathophysiology, pathogenetic targets and clinical issues. *Mol. Aspects Med.* **2019**, *65*, 37−55.
(2) Zhang, J.; Liu, Q.; He, J.; Li, Y. Novel Therapeutic Targets in Liver Fibrosis. *Front Mol. Biosci* **2021**, *8*, No. 766855.
(3) Zhang, S.; Wang, Z.; Zhu, J.; Xu, T.; Zhao, Y.; Zhao, H.; Tang, F.; Li, Z.; Zhou, J.; Gao, D.; et al. Carnosic Acid Alleviates BDL-Induced Liver Fibrosis through miR-29b-3p-Mediated Inhibition of the High-Mobility Group Box 1/Toll-Like Receptor 4 Signaling Pathway in Rats. *Front Pharmacol* **2018**, *8*, 976.
(4) Liu, Z.; Zhu, P.; Zhang, L.; Xiong, B.; Tao, J.; Guan, W.; Li, C.; Chen, C.; Gu, J.; Duanmu, J.; et al. Autophagy inhibition attenuates the induction of anti-inflammatory effect of catalpol in liver fibrosis. *Biomed Pharmacother* **2018**, *103*, 1262−1271.
(5) Matsuda, M.; Seki, E. Hepatic Stellate Cell-Macrophage Crosstalk in Liver Fibrosis and Carcinogenesis. *Semin Liver Dis* **2020**, *40* (3), 307−320.
(6) Kisseleva, T.; Brenner, D. Molecular and cellular mechanisms of liver fibrosis and its regression. *Nat. Rev. Gastroenterol Hepatol* **2021**, *18* (3), 151−166.
(7) Arab, J. P.; Cabrera, D.; Sehrawat, T. S.; Jalan-Sakrikar, N.; Verma, V. K.; Simonetto, D.; Cao, S.; Yaqoob, U.; Leon, J.; Freire, M.; et al. Hepatic stellate cell activation promotes alcohol-induced steatohepatitis through Igfbp3 and SerpinA12. *J. Hepatol* **2020**, *73* (1), 149−160.
(8) Friedman, S. L. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev.* **2008**, *88* (1), 125−172.
(9) Lee, U. E.; Friedman, S. L. Mechanisms of hepatic fibrogenesis. *Best Pract Res. Clin Gastroenterol* **2011**, *25* (2), 195−206.
(10) Friedman, S. L. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J. Biol. Chem.* **2000**, *275* (4), 2247−2250.
(11) Rachfal, A. W.; Brigstock, D. R. Connective tissue growth factor (CTGF/CCN2) in hepatic fibrosis. *Hepatol Res.* **2003**, *26* (1), 1−9.
(12) Trauner, M.; Fuchs, C. D. Novel therapeutic targets for cholestatic and fatty liver disease. *Gut* **2022**, *71* (1), 194−209.
(13) Loomba, R.; Friedman, S. L.; Shulman, G. I. Mechanisms and disease consequences of nonalcoholic fatty liver disease. *Cell* **2021**, *184* (10), 2537−2564.
(14) Badylak, S. F.; Freytes, D. O.; Gilbert, T. W. Extracellular matrix as a biological scaffold material: Structure and function. *Acta Biomater* **2009**, *5* (1), 1−13.
(15) Zhou, W. C.; Zhang, Q. B.; Qiao, L. Pathogenesis of liver cirrhosis. *World J. Gastroenterol* **2014**, *20* (23), 7312−7324.
(16) Dhar, D.; Baglieri, J.; Kisseleva, T.; Brenner, D. A. Mechanisms of liver fibrosis and its role in liver cancer. *Exp Biol. Med. (Maywood)* **2020**, *245* (2), 96−108.
(17) Affo, S.; Yu, L. X.; Schwabe, R. F. The Role of Cancer-Associated Fibroblasts and Fibrosis in Liver Cancer. *Annu. Rev. Pathol* **2017**, *12*, 153−186.
(18) Barkin, J. M.; Jin-Smith, B.; Torok, K.; Pi, L. Significance of CCNs in liver regeneration. *J. Cell Commun. Signal* **2023**, *17* (2), 321−332.
(19) Zolfaghari, S.; Kaasbøll, O. J.; Monsen, V. T.; Sredic, B.; Hagelin, E. M. V.; Attramadal, H. The carboxyl-terminal TSP1-homology domain is the biologically active effector peptide of matricellular protein CCN5 that counteracts profibrotic CCN2. *J. Biol. Chem.* **2023**, *299* (1), No. 102803.
(20) Bork, P. The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett.* **1993**, *327* (2), 125−130.
(21) Song, M. H.; Yoo, J.; Oh, J. G.; Kook, H.; Park, W. J.; Jeong, D. Matricellular Protein CCN5 Gene Transfer Ameliorates Cardiac and Skeletal Dysfunction in mdx/utrn (±) Haploinsufficient Mice by Reducing Fibrosis and Upregulating Utrophin Expression. *Front Cardiovasc Med.* **2022**, *9*, No. 763544.
(22) Xu, H.; Liu, C.; Sun, Z.; Guo, X.; Zhang, Y.; Liu, M.; Li, P. CCN5 attenuates profibrotic phenotypes of fibroblasts through the Smad6-CCN2 pathway: Potential role in epidural fibrosis. *Int. J. Mol. Med.* **2015**, *36* (1), 123−129.
(23) Karagiannis, E. D.; Popel, A. S. Peptides derived from type I thrombospondin repeat-containing proteins of the CCN family inhibit proliferation and migration of endothelial cells. *Int. J. Biochem. Cell Biol.* **2007**, *39* (12), 2314−2323.
(24) Xu, S.; Chen, Y.; Miao, J.; Li, Y.; Liu, J.; Zhang, J.; Liang, J.; Chen, S.; Hou, S. Esculin inhibits hepatic stellate cell activation and CCl(4)-induced liver fibrosis by activating the Nrf2/GPX4 signaling pathway. *Phytomedicine* **2024**, *128*, No. 155465.
(25) Wang, R.; Zhang, D.; Tang, D.; Sun, K.; Peng, J.; Zhu, W.; Yin, S.; Wu, Y. Amygdalin inhibits TGFβ1-induced activation of hepatic stellate cells (HSCs) in vitro and CCl(4)-induced hepatic fibrosis in rats in vivo. *Int. Immunopharmacol* **2021**, *90*, No. 107151.
(26) Xiu, A. Y.; Ding, Q.; Li, Z.; Zhang, C. Q. Doxazosin Attenuates Liver Fibrosis by Inhibiting Autophagy in Hepatic Stellate Cells via Activation of the PI3K/Akt/mTOR Signaling Pathway. *Drug Des Devel Ther* **2021**, *15*, 3643−3659.
(27) Wynn, T. A.; Ramalingam, T. R. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat. Med.* **2012**, *18* (7), 1028−1040.
(28) Li, W.; Lv, R.; Zou, T.; Chen, M. Tricetin protects against liver fibrosis through promoting autophagy and Nrf2 signaling in hepatic stellate cells. *Life Sci.* **2024**, *351*, No. 122798.
(29) Bao, Y. L.; Wang, L.; Pan, H. T.; Zhang, T. R.; Chen, Y. H.; Xu, S. J.; Mao, X. L.; Li, S. W. Animal and Organoid Models of Liver Fibrosis. *Front Physiol* **2021**, *12*, No. 666138.
(30) Scholten, D.; Trebicka, J.; Liedtke, C.; Weiskirchen, R. The carbon tetrachloride model in mice. *Lab. Anim.* **2015**, *49* (1 Suppl), 4−11.
(31) Faccioli, L. A. P.; Dias, M. L.; Paranhos, B. A.; Dos Santos Goldenberg, R. C. Liver cirrhosis: An overview of experimental models in rodents. *Life Sci.* **2022**, *301*, No. 120615.
(32) Xiong, X.; Ren, Y.; Cui, Y.; Li, R.; Wang, C.; Zhang, Y. Obeticholic acid protects mice against lipopolysaccharide-induced liver injury and inflammation. *Biomed Pharmacother* **2017**, *96*, 1292−1298.
(33) Yuan, R.; Tao, X.; Liang, S.; Pan, Y.; He, L.; Sun, J.; Wenbo, J.; Li, X.; Chen, J.; Wang, C. Protective effect of acidic polysaccharide from Schisandra chinensis on acute ethanol-induced liver injury

through reducing CYP2E1-dependent oxidative stress. *Biomed Pharmacother* **2018**, *99*, 537−542.
(34) Xu, L.; Yu, Y.; Sang, R.; Li, J.; Ge, B.; Zhang, X. Protective Effects of Taraxasterol against Ethanol-Induced Liver Injury by Regulating CYP2E1/Nrf2/HO-1 and NF-κB Signaling Pathways in Mice. *Oxid Med. Cell Longev* **2018**, *2018*, No. 8284107.
(35) Xie, G.; Jiang, R.; Wang, X.; Liu, P.; Zhao, A.; Wu, Y.; Huang, F.; Liu, Z.; Rajani, C.; Zheng, X.; et al. Conjugated secondary 12α-hydroxylated bile acids promote liver fibrogenesis. *EBioMedicine* **2021**, *66*, No. 103290.
(36) Li, H. G.; You, P. T.; Xia, Y.; Cai, Y.; Tu, Y. J.; Wang, M. H.; Song, W. C.; Quan, T. M.; Ren, H. Y.; Liu, Y. W.; et al. Yu Gan Long Ameliorates Hepatic Fibrosis by Inhibiting PI3K/AKT, Ras/ERK and JAK1/STAT3 Signaling Pathways in CCl(4)-induced Liver Fibrosis Rats. *Curr. Med. Sci.* **2020**, *40* (3), 539−547.
(37) Lin, Y.; Yan, M.; Bai, Z.; Xie, Y.; Ren, L.; Wei, J.; Zhu, D.; Wang, H.; Liu, Y.; Luo, J.; et al. Huc-MSC-derived exosomes modified with the targeting peptide of aHSCs for liver fibrosis therapy. *J. Nanobiotechnology* **2022**, *20* (1), 432.
(38) Li, B.; Huang, Y.; Zou, Q. Peptide-Based Nanoarchitectonics for the Treatment of Liver Fibrosis. *Chembiochem* **2023**, *24* (9), No. e202300002.
(39) Song, N.; Li, H.; Tang, Q.; Luo, S.; Shi, Z.; Zhao, Q.; Li, R.; Chen, Y.; Cai, X.; Jiang, X. Design and Discovery of Novel Cyclic Peptides as EDPs-EBP Interaction Inhibitors for the Treatment of Liver Fibrosis. *J. Med. Chem.* **2023**, *66* (7), 4689−4702.
(40) Craik, D. J.; Fairlie, D. P.; Liras, S.; Price, D. The future of peptide-based drugs. *Chem. Biol. Drug Des* **2013**, *81* (1), 136−147.
(41) Sato, A. K.; Viswanathan, M.; Kent, R. B.; Wood, C. R. Therapeutic peptides: technological advances driving peptides into development. *Curr. Opin Biotechnol* **2006**, *17* (6), 638−642.
(42) Kent, S. B. Total chemical synthesis of proteins. *Chem. Soc. Rev.* **2009**, *38* (2), 338−351.
(43) Ma, W.; Liu, H.; Li, X. Chemical Synthesis of Peptides and Proteins Bearing Base-Labile Post-Translational Modifications: Evolution of the Methods in Four Decades. *Chembiochem* **2023**, *24* (20), No. e202300348.
(44) Zhang, F.; Song, J.; Wu, H.; Lin, K.; Wang, C.; Zeng, L.; Kong, X.; Zou, K.; Diao, H.; Wang, Z.et al.A novel deacetylase inhibitor KLX suppresses liver fibrosis by deacetylating PPARgamma through promoting ubiquitination-mediated HDAC1 degradation. *Sci. China Life Sci.* **2025**. .
(45) Xu, H.; Zhao, Q.; Song, N.; Yan, Z.; Lin, R.; Wu, S.; Jiang, L.; Hong, S.; Xie, J.; Zhou, H.; et al. AdipoR1/AdipoR2 dual agonist recovers nonalcoholic steatohepatitis and related fibrosis via endoplasmic reticulum-mitochondria axis. *Nat. Commun.* **2020**, *11* (1), 5807.
(46) Dong, J.; Xiao, J.; Li, J.; Yu, H.; Zhao, Q.; Tang, Q.; Chen, H.; Liu, H.; Wu, K.; Lei, J.; et al. Discovery and Design of Novel Cyclic Peptides as Specific Inhibitors Targeting CCN2 and Disrupting CCN2/EGFR Interaction for Kidney Fibrosis Treatment. *J. Med. Chem.* **2023**, *66* (12), 8251−8266.
(47) Shi, Z.; Liu, X.; Wu, S.; Song, N.; Tang, Q.; Li, H.; Luo, S.; Chan, A. S. C.; Cai, X.; Liu, H.; et al. Discovery of Novel Peptide Antagonists Targeting GPR55 for Liver Inflammation and Fibrosis. *J. Med. Chem.* **2024**, *67* (14), 12085−12098.
(48) Zhao, Q.; Dong, J.; Liu, H.; Chen, H.; Yu, H.; Ye, S.; Yu, S.; Li, Y.; Qiu, L.; Song, N.; et al. Design and discovery of a highly potent ultralong-acting GLP-1 and glucagon co-agonist for attenuating renal fibrosis. *Acta Pharm. Sin B* **2024**, *14* (3), 1283−1301.
(49) Lin, Q.; Lei, D.; Zhong, T.; Zhang, Y.; Da, Q.; Chen, X.; Li, X.; Liu, J.; Yan, Z. Inactivation of ERK1/2 in cancer-associated hepatic stellate cells suppresses cancer-stromal interaction by regulating extracellular matrix in fibrosis. *Am. J. Cancer Res.* **2024**, *14* (3), 1015−1032.
(50) Son, G.; Hines, I. N.; Lindquist, J.; Schrum, L. W.; Rippe, R. A. Inhibition of phosphatidylinositol 3-kinase signaling in hepatic stellate cells blocks the progression of hepatic fibrosis. *Hepatology* **2009**, *50* (5), 1512−1523.
(51) McCubrey, J. A.; Steelman, L. S.; Chappell, W. H.; Abrams, S. L.; Wong, E. W.; Chang, F.; Lehmann, B.; Terrian, D. M.; Milella, M.; Tafuri, A.; et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim. Biophys. Acta* **2007**, *1773* (8), 1263−1284.

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