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Title: Strategies targeting the innate immune response for the treatment of hepatitis C virus-associated liver fibrosis

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Abstract

Direct-acting antivirals eliminate hepatitis C virus (HCV) in more than 95% of treated individuals and may abolish liver injury, arrest fibrogenesis, and reverse fibrosis and cirrhosis. However, liver regeneration is usually a slow process that is less effective in the late stages of fibrosis. What is more, fibrogenesis may prevail in patients with advanced cirrhosis, where it can progress to liver failure and hepatocellular carcinoma. Therefore, the development of antifibrotic drugs that halt and reverse fibrosis progression is urgently needed.

Fibrosis occurs due to the repair process of damaged hepatic tissue, which eventually leads to scarring. The innate immune response against HCV is essential in the initiation and progression of liver fibrosis. HCV-infected hepatocytes and liver macrophages secrete proinflammatory cytokines and chemokines that promote the activation and differentiation of hepatic stellate cells (HSCs) to myofibroblasts that produce extracellular matrix (ECM) components. Prolonged ECM production by myofibroblasts due to chronic inflammation is essential to the development of fibrosis.

While no antifibrotic therapy is approved to date, several drugs are being tested in phase 2 and phase 3 trials with promising results. This review discusses current state-of-the-art knowledge on treatments targeting the innate immune system to revert chronic hepatitis C (CHC)-associated liver fibrosis. Agents that cause liver damage may vary (alcohol, virus infection, etc.), but fibrosis progression shows common patterns among them, including chronic inflammation and immune dysregulation, hepatocyte injury, HSC activation, and excessive ECM deposition. Therefore, mechanisms underlying these processes are promising targets for general antifibrotic therapies.

Key points:

- The development of liver fibrosis is related to life-threatening complications, which can end in liver failure and hepatocellular carcinoma.
- Strategies targeting the innate immune system to induce fibrosis regression include blocking chronic inflammation, hepatocyte injury, hepatic stellate cell activation, and excessive deposition of the extracellular matrix.
- Although several drugs are being tested in phase 2 and 3 trials with promising results, no antifibrotic therapy has been approved to date.
- New therapeutic strategies, such as combination therapies with different antifibrotics, novel techniques for drug testing and delivery, and the use of omics to decipher key signaling pathways involved in liver fibrosis, will aid in searching for an effective antifibrotic treatment.

1 Introduction

Hepatitis C virus (HCV) is a significant global health burden. The World Health Organization (WHO) estimates that there are about 71 million people with chronic hepatitis C (CHC) worldwide [1, 2]. CHC leads to hepatic inflammation, fibrosis, cirrhosis [3], and life-threatening complications that, even after HCV elimination, can end in hepatocellular carcinoma (HCC) [3, 4].

Liver fibrosis is a dynamic and potentially reversible process that attempts to repair damaged hepatic tissue but, ultimately, leads to the excessive accumulation of extracellular matrix (ECM). Liver fibrosis is induced by chronic liver injury of different etiologies, such as viral hepatitis, non-alcoholic fatty liver disease (NAFLD), alcohol-associated liver disease (AALD), or cholestatic, autoimmune, and genetic disorders [5]. Although these diseases are triggered by different agents [6-9], they all converge towards common mechanisms: chronic parenchymal injury, inflammatory/immunological responses, fibrogenesis, and portal hypertension [10]. Notably, molecular mechanisms and disease progression triggered by CHC resemble those causing NAFLD [11, 12].

The recent introduction of direct-acting antivirals (DAAs) has led to sustained virological response (SVR) rates greater than 95% in DAA-treated HCV patients [13]. SVR is associated with a reduced risk of hepatic decompensation, liver transplantation, and mortality [14, 15]. Moreover, many studies have shown that achieving SVR abolishes liver injury, arrests fibrogenesis, and helps reverse fibrosis and compensated cirrhosis, as discussed in various reviews and meta-analyzes on long-term outcomes in CHC patients who achieve SVR both with interferon (IFN)-based [16, 17] and IFN-free treatments [18]. However, despite the impressive efficacy of DAAs, some critical issues remain that would benefit from antifibrotic therapy. (i) Liver fibrosis is usually reversed in the early stages of infection after HCV clearance [19], but regression of liver fibrosis is not often observed in patients with advanced fibrosis or cirrhosis [20-27]. (ii) The spontaneous reversal of liver fibrosis is slow and could be accelerated by antifibrotic therapy [28]. (iii) Some degree of immune system activation may persist after SVR [29-33]. (iv) About 5% of CHC patients fail to clear the virus after DAA treatment [34-36]. (v) Many individuals become reinfected or relapse after DAA therapy, especially in high-risk behavior groups (people who inject drugs, men who have sex with men) [37-39]. In all these individuals, fibrosis regression is hampered.

Therefore, patients in all the above-mentioned situations are potential candidates for antifibrotic therapy. Although several drugs are currently in phase 2 and 3 trials [40-42], there is no currently approved antifibrotic treatment. Therefore, halting fibrosis progression continues to be a public health priority, and potent antifibrotic strategies are urgently needed [8, 43-45].

This review focuses on targets and molecular mechanisms involved in HCV-associated liver fibrosis to decrease inflammation and halt or reverse fibrosis progression. Additionally, advanced clinical trials involving treatments to these targets are briefly discussed as well. Studies on liver fibrosis regression from non-viral aetiologies are also examined since they may guide research on HCV-associated hepatic fibrosis due to shared mechanisms.

1.2 Methodology

A comprehensive search using MEDLINE (PubMed), Web of Science, SCOPUS, AdisInsight databases, clinical trial registries (ClinicalTrials.gov), and websites of manufacturers was conducted (the main search from 2017 to 2020, although previous studies are also mentioned). A search using combinations of the following terms was performed: ‘HCV’, ‘innate’, ‘therapy’, ‘antifibrotic’, ‘liver fibrosis’, ‘chronic liver disease’, ‘chronic hepatitis C’, and ‘liver cirrhosis’. All studies were included without language restriction.

2 Mechanisms underlying hepatic fibrosis

Liver fibrosis results from the activation of several complex pathways designed to repair damaged hepatic tissue but ultimately lead to uneven scarring of liver tissue [46]. Fibrosis begins with the damage of hepatocytes resulting in the release of inflammatory cytokines, which trigger the activation of resident liver macrophages (Kupffer cells [KCs]); the activation and differentiation of hepatic stellate cells (HSCs) into proliferative, contractile, and fibrogenic myofibroblasts (activated HSCs; aHSCs); and the migration of leukocytes to the site of injury [46]. The aHSCs secrete reactive oxygen species (ROS), cytokines and chemokines that promote inflammation [47, 48]. Leukocytes amplify the inflammatory response and activate more HSCs. The aHSCs produce fibers and large amounts of ECM proteins, such as collagen types 1 (Col1), 3 and 4, elastin, fibronectin, laminin, and proteoglycans [49, 50]. Additionally, ECM degradation (fibrinolysis) is reduced. Therefore, liver fibrosis is a dynamic and potentially reversible process characterized by an imbalance between fibrogenesis and fibrinolysis [51]. Eventually, progressive ECM accumulation results in a disruption of liver architecture, vascular changes, scarring, and organ dysfunction [52].

2.1 Inflammatory response

Inflammation is a defense mechanism against HCV infection and hepatic damage caused by the virus [53]. The mechanism responsible for CHC-associated fibrosis is a multifaceted process orchestrated by a broad spectrum of non-immune cells (hepatocytes, liver sinusoidal endothelial cells (LSECs), and HSCs) and professional immune cells (KCs, dendritic cells (DCs), natural killer (NK), and NK T cells) that are in the circulation or distributed within the hepatic compartment. There are multiple innate immune responses to HCV infection due to cellular diversity and the release of many immunological factors that activate HSCs, secrete ECM components, and promote fibrosis [49]. For clarity, the topic of inflammatory response to HCV infection is divided here into four sections. The reader is referred to recent seminal reviews for more details [52, 54-58].

(i) Activation of pattern recognition receptors (PRRs) and induction of the IFN response in infected hepatocytes. Infected hepatocytes express several PRRs that recognize viral pathogen-associated molecular patterns (PAMPs), such as single- and double-stranded RNA (ssRNA and dsRNA), by cytoplasmic sensors, such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5), and the endosomal toll-like receptor (TLR)3 [59, 60]. Viral recognition activates a signaling cascade that induces IFN, a primary response to the viral infection [61], proinflammatory chemokines (C-C motif chemokine ligand (CCL)3, CCL4, regulated on activation, normal T-cell expressed and secreted (RANTES), interleukin (IL)-8, and C-X-C motif chemokine ligand (CXCL)10) [59, 62, 63], and several IFN-stimulated genes (ISGs) [64-66], which establish an antiviral state in uninfected neighboring cells to control virus replication and spread.

(ii) Hepatocyte injury and inflammatory response. In response to hepatocyte injury, high levels of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), IL-18, IL-12, and IL-1β, are produced by KCs and other immune cells that are recruited to the liver. This is responsible for the amplification of the inflammatory response during CHC [67-72].

(iii) Inflammasome activation. HCV is phagocytosed by KCs where it activates the nucleotide-binding oligomerization domain-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome. The inflammasome is a cytoplasmic multiprotein complex that stimulates the activation of caspase-1, which cleaves pro-IL-1β and pro-IL-18 resulting in their mature forms [73]. The production of IL-1β is correlated with amplified inflammatory responses and liver fibrosis progression [67, 74, 75]. NLRP3-mediated activation of IL-18 induces IFN in monocytes, which inhibits HCV replication [76]. IL-18 activation is also a marker in acute hepatitis C infection and an indicator of persistent HCV infection [69].

(iv) Other cells involved in chronic inflammation. HCV's initial interaction with other innate immune cells is essential to understand the adaptive immune response and CHC. In addition to KCs,

infiltrating human monocyte-derived macrophages, defined in humans as CD14/CD16 cell subsets [77] and in the mouse as lymphocyte antigen 6 complex, locus C (Ly6C) [78, 79], contribute to inflammation and fibrogenesis [80, 81]. Mouse $Ly6C^{low}$ macrophages (homologous to human $CD14^{low}/CD16^{high}$) adopt a fibrinolytic phenotype that reduces inflammation and replaces resident tissue macrophages. $Ly6C^{high}$ macrophages (homologous to human $CD14^{high}/CD16^{low}$) are inflammatory and are recruited when the injury persists [81, 82].

In the early stages of liver injury, NK cells are activated by IFN-I and other cytokines (IL-12, IL-18) [83] and control HCV infection by killing HCV-infected hepatocytes and inducing T-cell responses [84, 85]. However, alterations in phenotype and function of NK cells are observed in liver disease's final advanced stages [86], namely increased cytotoxicity, which contributes to healthy hepatocyte death, and a decreased production of IFN-γ, which reduces HCV clearance [87, 88]. Similarly to NK cells, NK T cells inhibit HCV replication during the acute phase of infection through IFN-γ production [89]. However, the prevalence of NK T cells decreases during CHC [90]. At the same time, they produce profibrotic cytokines (IL-4, IL-13), promoting HSC activation [91].

DCs play a key role in controlling the antiviral response during CHC. Conflicting evidence in functionality and phenotype of DCs from CHC patients has been observed [92]. Some studies show that DCs exhibit normal maturation and proliferation markers, and preserve their ability to present antigens [93-95]. In contrast, other reports demonstrate that DC maturation and proliferation is altered in CHC patients, leading to an attenuated antiviral response [96-99].

HCV clearance coincides with strong and sustained T-cell responses, which deteriorate once CHC is established, leading to T-cell exhaustion [100]. CHC patients develop a lack of effective HCV-specific $CD4^{+}/CD8^{+}$ T-cells [101, 102], coincident with an increase in regulatory T cells (Treg) and a reduction in T-helper 17 cells (Th17) [103], leading to immune dysfunction and loss of immune control, which can only be partially restored [104].

2.2 HSC activation and ECM deposition

During normal repair/regeneration of damaged liver, healthy hepatocytes fill the gaps created by dead hepatocytes. When liver damage persists, there is an excessive replacement of healthy parenchyma by scar tissue (ECM) that interferes with normal liver function. Liver injury leads to the secretion of profibrotic and growth factor molecules (transforming growth factor-beta (TGF-β) [105-108], platelet-derived growth factor (PDGF) [109, 110], vascular endothelial growth factor (VEGF) [111-113], connective tissue growth factor (CTGF) [114-116], ROS [117-120], etc.) from infected-hepatocytes, activated KCs, infiltrating immune cells, LSECs, and cholangiocytes that activate HSCs [121-123]. The aHSCs phagocytose apoptotic bodies from HCV-infected hepatocytes [124, 125], triggering a profibrotic response [126]. The aHSCs cause an exaggerated wound scarring response through the excessive replacement of healthy parenchyma with ECM components [50]. Matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), are implicated in ECM degradation and HSC activation. The ECM increase is accompanied by the downregulation of MMPs and the upregulation of TIMPs, which are produced by several hepatic cells [127-133]. Therefore, an MMP/TIMP imbalance is associated with fibrosis. Further, the activation of HSCs leads to the loss of lipid droplets that contain retinoids (vitamin A and its metabolites) present in the cytoplasm of quiescent HSCs [134-136], which leads to homeostatic imbalance and chronic inflammation [137, 138].

In summary, all these findings support the idea that HSC activation, scar formation inhibition, and enhancement of ECM degradation are potential targets for remodeling ECM and reversing fibrosis.

3 Strategies for liver fibrosis regression

The elimination of the causal agent is not sufficient to induce a rapid reversal of advanced fibrosis or cirrhosis. After HCV clearance, the regression, or the resolution of fibrosis, involves eliminating inflammatory pathways, aHSCs, and degradation of excess ECM. Overall, strategies to induce fibrosis regression include: (i) reducing inflammation and immune responses, (ii) inhibiting hepatocyte injury, (iii) suppressing HSC activation and the underlying signaling pathways, and (iv) inducing scar ECM degradation [5, 139, 140]. Therefore, many mechanisms related to the innate immune response are involved in the regression of liver fibrosis, making them potential targets for therapy (**Figure 1**).

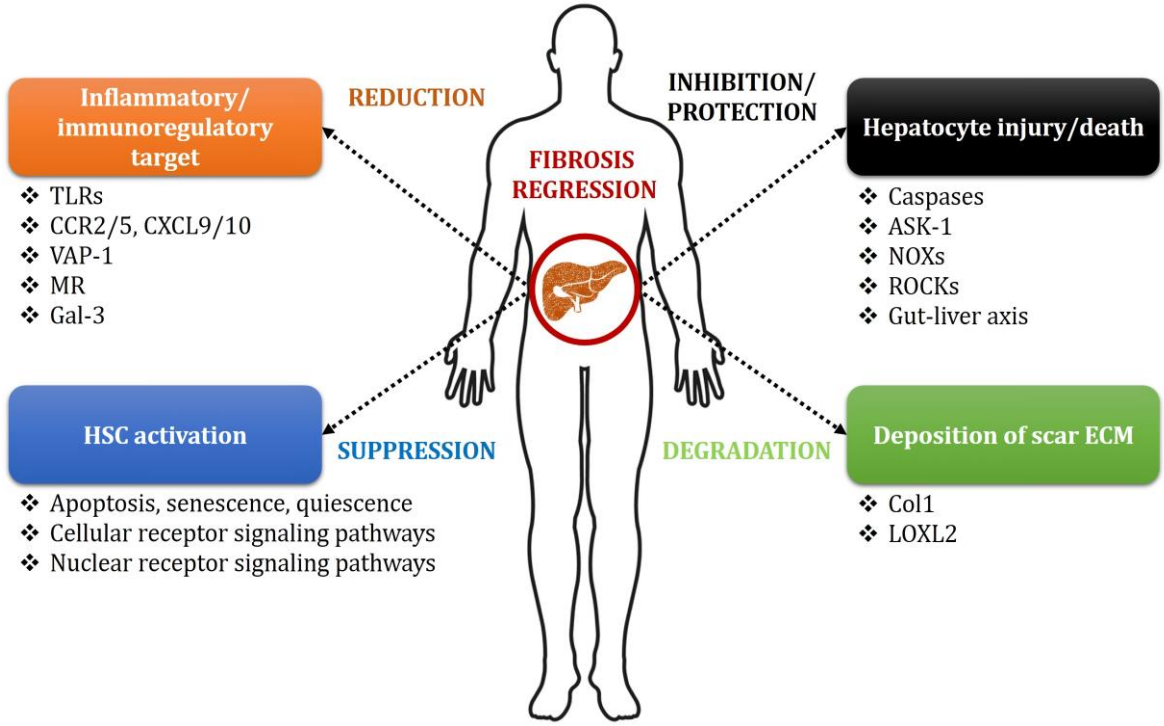

Figure 1. Antifibrotic strategies and targets for the treatment of hepatitis C virus-associated liver fibrosis. See the text for a full description of each therapeutic approach. *ASK-1* apoptosis signal-regulating kinase 1, *CCR* C-C motif chemokine receptor, *Col1* collagen type 1, *CXCL* C-X-C motif chemokine ligand, *Gal-3* galectin 3, *HSCs* hepatic stellate cells, *LOXL2* lysyl oxidase-like 2, *MR* mineralocorticoid receptor, *NOXs* nicotinamide adenine dinucleotide phosphate oxidases, *ROCKs* Rho-associated protein kinases, *TLRs* toll-like receptors, *VAP-1* vascular adhesion protein 1

3.1 Inflammation and immunoregulatory targets

Despite the successful elimination of HCV infection after DAA treatment, both residual liver disease and immune activation persist in many cases. For example, after viral clearance several immunological traits remain altered, such as elevated markers of macrophage activation [30], decreased NK cell repertoire diversity [32], an altered milieu of soluble inflammatory mediators [141], the appearance of Tregs [31], and dysfunction of mucosal-associated invariant T-cells [33]. Therefore, the impact of the cellular immune system could persist after achieving SVR, and modulation of the proinflammatory response that leads to liver injury is a potential target for antifibrotic treatments. Other strategies, mostly targeting the innate inflammatory system, have also been discussed. The most advanced antifibrotic candidates developed to interfere with inflammation/immunomodulation processes are summarized in **Table 1**.

Table 1 **Clinical studies focusing on antifibrotics targeting inflammation/immunoregulatory mechanisms**

Compound	Target	Patients	Clinical trial identifier / Trial stage	Ref.
Nalmefene (JKB-121)	TLR4	Biopsy-proven NASH	NCT02442687 Phase 2 completed	[142]
Cenicriviroc	CCR2/CCR5	NASH with fibrosis	NCT02217475 (CENTAUR) Phase 2 completed	[143-145]
		NASH with fibrosis	NCT03028740 (AURORA) Phase 3 recruiting	[146]
		NASH from CENTAUR/ AURORA studies	NCT03059446 Phase 2 enrolling by invitation	-
Cenicriviroc + Tropifexor (LJN-452)	CCR2/CCR5 + FXR	NASH with fibrosis	NCT03517540 (TANDEM) Phase 2 active, not recruiting	[147]
BI-1467335 (PXS-4728A)	VAP-1	NASH with fibrosis	NCT03166735 Phase 2 completed	[148]
TERN-201	VAP-1	NASH with fibrosis	Phase 2 recruiting	[149]
Belapectin (GR-MD-02)	Gal-3	NASH with advanced bridging fibrosis	NCT02421094 (NASH-FX) Phase 2 completed	[150]
		NASH with cirrhosis and portal hypertension	NCT02462967 (NASH-CX) Phase 2 completed	[151]
		NASH with cirrhosis, portal hypertension and not esophageal varices	NCT04365868 (NASH-RX) Phase 2/3 recruiting	[152]
Apararenone (MT-3995)	MR	NASH	NCT02923154 Phase 2 completed	-

CCR2 C-C motif chemokine receptor 2, *CCR5* C-C motif chemokine receptor 5, *FXR* farnesoid X receptor, *Gal-3* galectin 3, *MR* mineralocorticoid receptor, *NASH* non-alcoholic steatohepatitis, *TLR4* toll-like receptor 4, *VAP-1* vascular adhesion protein 1

3.1.1 Toll-like receptors (TLRs)

TLRs are PRRs that play a fundamental role in innate immunity [153, 154]. TLRs recognize HCV PAMPs (viral RNA and proteins), mediate cytokine production, lead to liver damage, and are associated with CHC pathogenesis [155-157]. TLR4 is activated by HCV NS5A and increases IFN-β and IL-6 production [158]. In CHC patients, there is increased TLR4 expression, which leads to high levels of serum and intrahepatic TNF-α that contribute to chronic inflammation [155, 159, 160]. Therefore, TLR4 antagonists are potential therapeutic agents for the management of liver fibrosis (**Table 1**). JKB-121 is the only small molecule that has progressed as a TLR4 receptor antagonist to a phase 2 trial (NCT02442687) in patients with biopsy-proven non-alcoholic steatohepatitis (NASH). Despite the promising results obtained in *in vitro* and preclinical studies [161], JKB-121 did not improve endpoints compared to the control group and did not show a beneficial effect on liver disease [142].

Other TLRs are also potential targets for antifibrotic therapy. HCV core and NS3 proteins activate TLR2, which forms a heterodimer complex with TLR1 or TLR6 in monocytes and KCs [162]. In CHC patients, TLR2-TLR1/6 dimers stimulate the production of inflammatory cytokines, such as TNF-α, IL-10, and IL-8, which alter DC function and antiviral activity of KCs [98, 163, 164].

NS3/4A disrupts TLR3 signaling by cleaving toll-IL-1-receptor domain-containing adaptor-inducing IFN-β (TRIF), which hampers IFN antiviral activity [165, 166] and promotes persistent HCV infection [167]. Notably, polyinosinic:polycytidylic acid (usually abbreviated poly I:C) is a TLR3 ligand that has been shown to reduce liver fibrosis by killing aHSCs in a mouse model of liver fibrosis [168]. Therefore, TLR3 agonists may be useful in counteracting liver fibrosis.

Phagocytosed HCV ssRNA stimulates TLR7/8, which leads to inflammatory mediators via NLRP3-dependent inflammasomes [67]. TLR9 recognizes unmethylated cytosine-phosphate-guanine (CpG) DNA motifs from apoptotic cells, and acts as a critical mediator of HSC differentiation [169]. In this case, TLR7/8/9 antagonists may also work as potential antifibrotic treatments.

3.1.2 Chemokines and chemokine receptors

CHC progression is associated with proinflammatory macrophage recruitment via C-C motif chemokine receptor (CCR)2 [170-173], and recruitment of HSCs and leukocytes via CCR5 [173, 174]. Cenicriviroc (TAK-652) is a dual CCR2/CCR5 antagonist initially used as a treatment against human immunodeficiency virus (HIV) infection [175]. A phase 2 study (CENTAUR; NCT02217475) evaluated cenicriviroc's efficacy and safety in NASH patients with liver fibrosis [143], showing an improvement in liver fibrosis and attenuated inflammatory signaling in treated patients [144, 145]. Cenicriviroc is currently in a phase 3 trial (AURORA; NCT03028740) in NASH patients with liver fibrosis to evaluate the improvement in fibrosis and long-term clinical outcomes related to cirrhosis progression [146]. There is currently a phase 2 rollover study (NCT03059446) in patients who participated in CENTAUR or AURORA studies to assess the long-term safety of continuous cenicriviroc treatment. A phase 2 trial (TANDEM, NCT03517540), testing the combination of cenicriviroc and tropifexor (LJN-452; a farnesoid X receptor (FXR) agonist) in NASH patients with liver fibrosis is currently ongoing to evaluate the safety, tolerability, and efficacy of the combination therapy compared to monotherapy [147] (**Table 1**).

Other chemokines, such as CXCL9, 10, and 11, are ligands of the C-X-C motif chemokine receptor 3 (CXCR3), which are highly expressed during chronic inflammation and CHC progression [176-178]. In a mouse model of chronic liver inflammation [179] and CHC patients [180, 181], there is a downregulation of C-X3-C motif chemokine receptor 1 (CX3CR1) and its ligand CX3CL1. These chemokines are, therefore, potential targets for antifibrotic therapies.

3.1.3 Vascular adhesion protein 1 (VAP-1)

Vascular adhesion protein 1 (VAP-1) is a sialoglycoprotein that facilitates leukocyte recruitment and promotes oxidative stress [182]. Increased levels of the soluble form of VAP-1 (sVAP-1) have been reported in different chronic liver diseases, such as NAFLD, primary sclerosing cholangitis [182, 183], and recently, CHC [184]. Therefore, VAP-1 is being investigated in different chronic liver diseases and has been suggested as a potential therapeutic target for CHC [185].

The VAP-1 inhibitor BI-1467335 (PXS-4728A) has been in a phase 2 clinical trial (NCT03166735) to evaluate the liver infiltration of immune cells, reduction of alanine aminotransferase (ALT) levels, and fibrosis in NASH patients (**Table 1**). This trial has already been completed, and despite meeting the targets, the study has been halted due to possible undesired drug interactions [148]. To avoid potential drug-drug interactions of PXS-4728A, the semicarbazide-sensitive amine oxidase inhibitor TERN-201 is currently in a phase 2 trial to treat non-cirrhotic NASH patients. In healthy volunteers, TERN-201 is well-tolerated and inhibits VAP-1 after single and multiple doses [149] (**Table 1**).

3.1.4 Galectin (Gal)

Galectins (Gal) are galactose-binding proteins expressed and released by several cells involved in numerous biological processes, including innate immune responses [186]. Gal-3 is an important marker of chronic liver fibrosis because Gal-3 stimulates aHSCs to produce Col1 and TGF-β [187-190]. Gal-3 is elevated in patients with chronic liver diseases, and its expression is 3-fold higher in

alcoholic cirrhosis than in HCV-mediated cirrhosis due to different injury mechanisms [191]. Thus, Gal-3 could also be a target for antifibrotic therapy in CHC patients.

Belapectin (GR-MD-02) is a Gal-3 polysaccharide polymer inhibitor [192] that has been in two phase 2 trials to evaluate safety and efficacy in NASH patients with cirrhosis (NASH-CX; NCT02462967) and advanced fibrosis (NASH-FX; NCT02421094) [150, 151]. Belapectin improved hepatocyte ballooning, reduced the development of esophageal varices (a marker of reduced blood flow to the liver), and showed a favorable safety profile. However, these findings were not significant [150, 151]. Belapectin announced patient enrollment to phase 2b/3 (NASH-RX; NCT04365868) to assess the safety, tolerability, and efficacy in NASH patients with cirrhosis, clinical signs of portal hypertension, and without esophageal varices (**Table 1**). The study is expected to start in late 2020, and data readout is expected in late 2023 [152].

3.1.5 Mineralocorticoid receptor (MR)

The mineralocorticoid receptor (MR) is a nuclear receptor expressed in LSECs and HSCs, whose activation induces inflammation by stimulating ROS and collagen deposition [193, 194]. It is relevant in the NAFLD setting [193, 195], but few studies are currently evaluating MR expression in the progression of CHC [196]. However, preliminary investigations point to MR receptor blockade as a potential antifibrotic strategy to explore in patients with CHC.

To date, the non-steroidal MR antagonist apararenone (MT-3995) has already completed a phase 2 trial (NCT02923154) to evaluate the efficacy, safety, tolerability, and pharmacokinetics in NASH patients (**Table 1**). However, this study analyzes ALT levels and adverse changes, not a direct improvement in liver fibrosis, but no published data are available yet.

3.2 Inhibition of hepatocyte injury/death

Inhibition of hepatocyte apoptosis may be an approach for potential antifibrotic therapies in CHC patients. Caspase inhibition or reduction of oxidative stress by blocking apoptosis signal-regulating kinase-1 (ASK-1), nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), or Rho-associated protein kinases (ROCKs) are promising strategies. Antifibrotics undergoing clinical trials based on inhibition of hepatocyte injury/death are listed in **Table 2**.

Table 2 **Clinical studies focusing on antifibrotics inhibiting hepatocyte injury/death**

Compound	Target	Patients	Clinical trial identifier / Trial stage	Ref.
Emricasan (IDN-6556, PF-03491390)	Caspase	CHC [(1)] with compensated cirrhosis	Phase 1/2 completed	[197]
		CHC [(2)] without cirrhosis	NCT00088140 Phase 2 completed	[198]
		CHC [(3)] with compensated cirrhosis and portal hypertension	NCT02230683 Phase 2 completed	[199]
		CHC [(4)], AALD, or NASH with cirrhosis	NCT02230670 Phase 2 completed	[200]
		CHC [(5)] with advanced cirrhosis and acute or chronic liver failure	NCT01937130 Phase 2 terminated	[201]
		NASH with decompensated cirrhosis	NCT03205345 (ENCORE-LF) Phase 2b active, not recruiting	[202]

		NASH with compensated cirrhosis and portal hypertension	NCT02960204 (ENCORE-PH) Phase 2b completed	[202]
		NASH with fibrosis	NCT02686762 (ENCORE-NF) Phase 2b completed	[151]
		CHC [6] with liver transplantation but still with fibrosis	NCT02138253 (POLT-HCV-SVR) Phase 2b completed	[203]
Nivocasan (GS-9450)	Caspase	Biopsy-proven NASH	NCT00740610 Phase 2 completed	[204]
		CHC [7] with fibrosis	NCT00874796 NCT00725803 Phase 2 terminated and completed	[205, 206]
Selonsertib (GS-4997)	ASK-1	NASH with fibrosis	NCT02466516 Phase 2 completed	[207, 208]
		NASH with bridging fibrosis	NCT03053050 (STELLAR-3) Phase 3 terminated	[209-211]
		NASH with compensated cirrhosis	NCT03053063 (STELLAR-4) Phase 3 terminated	[209-211]
Selonsertib in dual combination	ASK-1 + FXR ASK-1 + ACC	NASH with bridging fibrosis or compensated cirrhosis	NCT03449446 (ATLAS) Phase 2 completed	[212]
Selonsertib in dual and triple combinations	ASK-1 + ACC ASK-1 + FXR ASK-1 + FXR + ACC	NASH with advanced fibrosis	NCT02781584 Phase 2 active, not recruiting	[213]
Fasudil	ROCK	NASH and CHC [8] with cirrhosis and portal hypertension	Phase 2 completed	[214]
IMM 124-E	Gut-liver axis	NASH	NCT02316717 Phase 2 completed	-

[1] HCV patients had HCV RNA (PCR) >10^5 IU/mL, [2] Patients with HCV infection who were previously intolerant to treatment or failed to achieve an SVR during anti-HCV treatment; [3] Excluded HCV infected subjects receiving or planning on receiving antiviral therapy during the course of the study; [4] Excluded HCV infected subjects who are receiving or are planed to receive antiviral therapy during the study; [5] Active HCV infection; [6] HCV was eliminated by antiviral therapies prior to the study; [7, 8] Not specified whether the individuals included are with active HCV or not. *ACC* acetyl-CoA carboxylase, *AALD* alcohol-associated liver disease, *ASK-1* apoptosis signal-regulating kinase 1, *CHC* chronic hepatitis C, *FXR* farnesoid X receptor, *HCV* hepatitis C virus, *NASH* non-alcoholic steatohepatitis, *PCR* polymerase chain reaction, *ROCK* Rho-associated protein kinases, *SVR* sustained virological response.

3.2.1 Caspases

Caspases are proteases involved in the apoptosis of HCV-infected hepatocytes via the extrinsic pathway (a death receptor-dependent pathway that activates caspase 8/10) or the intrinsic pathway (triggered by intracellular stress resulting in mitochondrial membrane perturbation, activation of caspases 9, 3, 6, and 7, and subsequent degradation of cellular components) [215]. Moreover,

hepatocyte apoptosis via caspase results in HSC activation, Col1 production [216, 217], and is associated with inflammation in CHC [218, 219].

Emricasan (IDN-6556, PF-03491390) is a pan-caspase inhibitor that reduces aspartate aminotransferase (AST) and ALT activity in CHC patients [197]. Two clinical trials in phase 1 and phase 2 have shown emricasan to be safe and well-tolerated in CHC patients [197, 198]. Emricasan was also effective in CHC patients (NCT00088140) and patients with compensated cirrhosis and portal hypertension (NCT02230683). Both studies showed significant reductions in AST and ALT levels [198, 199]. Moreover, in a phase 2 trial (NCT02230670), emricasan improved liver function in cirrhotic patients with CHC, AALD, or NASH using the Model for End-Stage Liver Disease (MELD) score [200], a predictor of survival in decompensated cirrhotic patients. However, in another phase 2 trial (NCT01937130) in CHC patients with advanced cirrhosis and acute or chronic liver failure, its efficacy was not confirmed [201]. Recently, three phase 2b trials evaluating emricasan in NASH patients with preexisting liver fibrosis (ENCORE-NF; NCT02686762), decompensated cirrhosis (ENCORE-LF; NCT03205345), or compensated/early decompensated cirrhosis (ENCORE-PH; NCT02960204), also failed because they did not reach the endpoints related to improvement of liver inflammation or hepatic fibrosis [151, 202]. On the other hand, a phase 2 trial in CHC patients who had liver transplantation and achieved SVR following anti-HCV therapy but still had fibrosis and/or incomplete cirrhosis (POLT-HCV-SVR; NCT02138253) has shown a significant improvement in liver fibrosis [203] (**Table 2**).

The safety and activity of another caspase inhibitor, nivocasan (GS-9450), was evaluated in patients with CHC (NCT00874796, NCT00725803) and NASH (NCT00740610). Nivocasan reduced ALT levels in both types of patients, but only CHC patients showed a reduction in cytokeratin 18 (a marker for liver cell apoptosis) [204, 220]. Nevertheless, the phase 2 trial was stopped due to significant abnormalities and adverse events in several individuals [205] (**Table 2**).

In summary, there is evidence that targeting cell death may be beneficial for liver fibrosis resolution. Despite the disappointing results, the knowledge gained will guide the search for other caspase inhibitor-based alternatives to treat chronic liver disease [221].

3.2.2 Apoptosis signal-regulating kinase 1 (ASK-1)

ASK-1 is a serine/threonine-protein kinase that is primarily activated in response to oxidative stress and regulates cell death through p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) intracellular pathways [222]. In CHC patients, TGF-β, VEGF, and ROS production mediate the development of angiogenesis via ASK-1 [113].

The only ASK-1 compound that has successfully entered the clinical stage is selonsertib (GS-4997) (**Table 2**). In a phase 2 trial (NCT02466516), selonsertib was safe, effective, and improved fibrosis in NASH patients [207, 208]. Furthermore, reduced fibrosis was associated with decreased liver stiffness and collagen content, but these results must be taken with a grain of salt due to the absence of a control group. Selonsertib progressed to a phase 3 trial for NASH-induced bridging fibrosis (STELLAR-3, NCT03053050) or compensated cirrhosis (STELLAR-4, NCT03053063), but unfortunately, neither fibrosis regression nor reduction of disease progression was observed [209].

A phase 2 trial (ATLAS, NCT03449446) has recently evaluated the safety and efficacy of dual combinations of selonsertib/cilofexor (GS-9674, a non-steroidal FXR agonist) and selonsertib/firsocostat (GS-0976, an acetyl-CoA carboxylase inhibitor) in NASH patients with advanced fibrosis, including bridging fibrosis and cirrhosis. Both dual combinations showed fibrosis improvement at lower doses compared with the higher doses used in monotherapy. Moreover, fewer side effects of cilofexor were observed in the combination group compared with cilofexor as monotherapy [212]. Another phase 2 trial also evaluated dual and triple combinations with selonsertib, cilofexor, firsocostat, fenofibrate (a peroxisome proliferator-activated receptor (PPAR)-α specific inhibitor) and vascepa (a diacylglycerol acyltransferase inhibitor) in NASH patients with advanced fibrosis (**Table 2**). Preliminary data presented at the 2019 American Association for the

Study of Liver Diseases (AASLD) annual meeting showed that only firsocostat/fenofibrate combination led to a fibrosis improvement [213]. Nevertheless, these results on combination therapies should be interpreted with caution due to the small sample size.

3.2.3 Nicotinamide adenine dinucleotide phosphate oxidases (NOXs)/Rho-associated protein kinases (ROCKs)

NOXs are enzymes that mediate electron transfer from NADPH to molecular oxygen, producing superoxide radicals. NOXs promote oxidative stress, which leads to hepatocyte apoptosis, HSC activation, and ECM deposition [223]. The role of NOXs in CHC patients has been increasingly recognized and involves dysregulation of T-cell response and hepatocyte injury [224-228].

As with NOXs, Ras homolog gene member A (RhoA) and its downstream effector, ROCKs, promote oxidative stress. ROCKs are serine/threonine kinases that act as effectors of the small GTPase Rho, enhancing fiber formation, HSC contractility, and promoting hepatocyte apoptosis [229, 230]. Although few studies relate ROCKs and HCV infection [231], ROCKs and NOXs share oxidative stress as the mechanism that causes the fibrosis progression. Therefore, ROCKs are targets to consider as antifibrotic therapy in CHC patients in the future. Interestingly, the ROCK inhibitor fasudil reduces portal venous pressure in cirrhotic rats [232] and decreases portal venous and arterial pressure in CHC and NASH patients with cirrhosis and portal hypertension [214] (**Table 2**).

3.2.4 Hepatocyte protection via gut-liver axis

The importance of the gut-liver axis in CHC has been recently revised [233-236]. The gut and the liver are interconnected, both anatomically and physiologically. The gut-liver imbalance in CHC patients can be responsible for several cirrhosis-related complications [237] and HCC development [238, 239]. These studies are consistent with those of patients with other chronic liver diseases [233, 240]. The liver harbors translocated bacteria and a repertoire of gut-derived microbial products (lipopolysaccharide (LPS) is the most studied PAMP) that traverse the intestinal epithelium and activate the innate and inflammatory immune response in the liver [241, 242]. This bacterial translocation from the gut occurs during CHC, particularly in the cirrhotic stage [243]. The exacerbated cellular activation in CHC leads to impaired intestinal permeability with an increased translocation of bacteria and bacterial products that activate TLR4 [244]. Additionally, CHC patients show an altered intestinal microbial composition associated with liver fibrosis, which is characterized by an abundance of *Enterobacteriaceae* and *Bacterioidetes* and a slight decrease in *Firmicutes* [245]. Thus, therapies that prevent bacterial translocation into systemic circulation and the liver are of interest. However, further studies should be performed to show whether there are more factors in addition to the bacterial composition that modulate the gut-liver axis, liver function, and fibrosis progression.

A phase 2 trial with IMM 124-E (hyperimmune bovine colostrum enriched with IgG anti-LPS) has currently been completed in patients with severe alcoholic hepatitis (NCT02316717) (**Table 2**). The results are expected soon, although the improvement in fibrosis is not one of the endpoints.

3.3 Inhibition of HSC activation

There are numerous potential targets to inhibit HSC activation and its fibrogenic response due to the complexity and multitude of pathways involved in their activation and functionality and the number of substances released during liver damage. Here, we focus on antifibrotic strategies aimed at the inactivation/elimination of aHSCs or any pathways involved in their activation [246] (**Figure 2**). Candidates that are currently in clinical trials are summarized in **Table 3.**

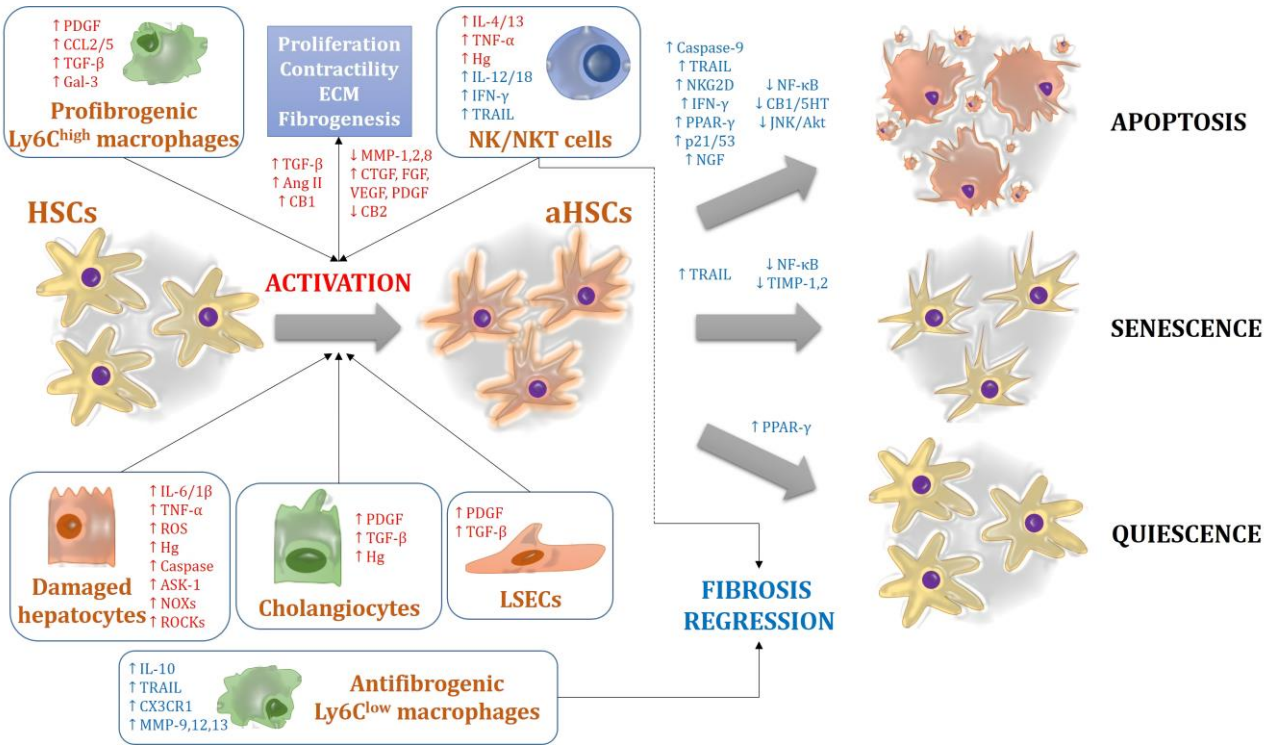

Figure 2. Hepatic stellate cell (HSC) activation/inactivation in liver disease. When chronic hepatitis C-mediated liver injury occurs, HSC activation is triggered by different cytokine stimuli from neighboring cells. If liver damage persists, many changes in HSC physiology occur. Activated HSCs (aHSC) proliferate, acquire a contractile phenotype, and produce ECM components. Inactivation or elimination of aHSCs is achieved by apoptosis, senescence, or reversion to quiescence. See the text for a full description of the different processes. *5HT* serotonin 5-hydroxytryptamine, *Akt* serine/threonine-protein kinase B, *Ang II* angiotensin II, *ASK-1* apoptosis signal-regulating kinase 1, *CB1/2* cannabinoid receptor type 1/2, *CCL* C-C motif chemokine ligand, *CTGF* connective tissue growth factor, *CX3CR1* C-X3-C motif chemokine receptor 1, *ECM* extracellular matrix, *FGF* fibroblast growth factor, *Gal-3* galectin 3; *Hg* hedgehog, *IFN-γ* interferon-gamma, *IL* interleukin, *JNK* c-Jun N-terminal kinase, *Ly6C* lymphocyte antigen 6 complex locus C, *MMP* matrix metalloproteinase, *NF-κB* nuclear factor κ-light-chain-enhancer of activated B cells, *NGF* nerve growth factor, *NK* natural killer, *NKG2D* natural killer group 2 member D, *NOXs* nicotinamide adenine dinucleotide phosphate oxidases, *PDGF* platelet-derived growth factor, *PPAR-γ* peroxisome proliferator-activated receptor-gamma, *ROCKs* Rho-associated protein kinases, *ROS* reactive oxygen species, *TFG-β* transforming growth factor-beta, *TIMP* tissue inhibitor of metalloproteinase, *TNF-α* tumor necrosis factor-alpha, *TRAIL* tumor necrosis factor-related apoptosis-inducing ligand, *VEGF* vascular endothelial growth factor, ↑ increased, ↓ decreased

Table 3 **Clinical studies focusing on antifibrotics inhibiting hepatic stellate cell activation**

Compound	Target	Patients	Clinical trial identifier / Trial stage	Ref.
Tipelukast (MN-001)	Leukotriene receptor	NASH and NAFLD with hypertriglyceridemia	NCT02681055 Phase 2 completed	-
Pirfenidone	TGF-β	CHC [(1)] with cirrhosis	NCT02161952 Phase 2 completed	[247]
Pirfenidone	TGF-β	CHC [(2)] with advanced fibrosis	NCT04099407 (PROMETEO) Phase 2 recruiting	[248]
Losartan	ATR1	CHC [(3)] with fibrosis	NCT00298714 Phase 4 completed	[227]

Losartan, valsartan and irbesartan	ATR1	CHC [4] with portal hypertension and liver biopsy	Phase 2 completed	[249]
Irbesartan	ATR1	CHC [5] with fibrosis	NCT00265642 (FIBROSAR) Phase 3 completed	-
Candesartan and ramipril	ATR1	CHC [6] with fibrosis	NCT03770936 Phase 3 recruiting	-
OCA	FXR	NASH without cirrhosis	NCT01265498 (FLINT) Phase 2 completed	[250]
		NASH with compensated cirrhosis	NCT03439254 (REVERSE) Phase 3 active, not recruiting	[251]
		NASH with fibrosis	NCT02548351 (REGENERATE) Phase 3 active, not recruiting	[251, 252]
Nor-UDCA	FXR	Primary sclerosing cholangitis	NCT01755507 (NUC-3) Phase 2 completed	[253]
Tropifexor in dual combination	FXR + CCR2/CCR5	NASH with fibrosis	NCT03517540 (TANDEM) Phase 2 active, not recruiting	[147]
Cilofexor in dual combination	FXR + ASK-1	NASH with bridging fibrosis or compensated cirrhosis	NCT03449446 (ATLAS) Phase 2 completed	[212]
Cilofexor in dual/triple combinations	FXR + ASK-1 FXR + ACC FXR + ASK-1 + ACC FXR + ACC + DGAT FXR + ACC + PPAR	NASH with advanced fibrosis	NCT02781584 Phase 2 active, recruiting	[213]
Pioglitazone	PPAR-γ	NASH with fibrosis	NCT00063622 (PIVENS) Phase 3 completed	[148, 254]
Farglitazar	PPAR-γ	CHC [7] with fibrosis	NCT00244751 Phase 2 completed	[255]
Elafibranor (GFT-505)	PPAR-α/δ	NASH without cirrhosis	NCT01694849 (GOLDEN-505) Phase 2b completed	[256]
		NASH with fibrosis	NCT02704403 (RESOLVE-IT) Phase 3 active, not recruiting	[167]
Lanifibranor (IVA-337)	PPAR-α/γ/δ	NASH with steatosis and necroinflammation	NCT03008070 (NATIVE) Phase 2b completed	[257]

[1] Patients with positive anti-HCV antibodies and detectable serum HCV RNA; [2] Patients with SVR; [3] Active HCV infection; [4] Positive serology for HCV and detectable HCV RNA; [5] Patients without antiviral therapy, and non-responders or patients with relapse after antiviral treatment; [6] Not specified whether the individuals included are with active HCV or not; [7] Serum HCV RNA positive, and failure to achieve SVR with previous

treatment. *ACC* acetyl-CoA carboxylase, *ASK-1* apoptosis signal-regulating kinase 1, *ATR1* angiotensin II type 1 receptor, *CCR2* C-C motif chemokine receptor 2, *CCR5* C-C motif chemokine receptor 5, *CHC* chronic hepatitis C, *DGAT* diglyceride acyltransferase, *FXR* farnesoid X receptor, *HCV* hepatitis C virus, *NAFLD* non-alcoholic fatty liver disease, *NASH* non-alcoholic steatohepatitis, *nor-UDCA* nor-ursodeoxycholic acid, *OCA* obeticholic acid, *PPAR* peroxisome proliferator-activated receptor, *SVR* sustained virological response, *TGF-β* transforming growth factor-beta.

3.3.1 Clearance of activated HSCs

Removing aHSCs can be achieved through three well-known mechanisms [246] (**Fig. 2**), which are described below:

(i) Apoptosis. In contrast to hepatocytes, HSC apoptosis is required for fibrosis regression in CHC patients [258]. Hepatocytes, KCs, NK, and NK T cells can initiate HSC apoptosis through different signaling pathways [259], such as the inhibition of leukotriene receptors, a class of arachidonic acid-derived bioactive molecules, and the blockade of JNK phosphorylation [260, 261]. In this regard, tipelukast (MN-001) is a promising compound demonstrating antifibrotic and antiinflammatory activity in preclinical models. However, new therapies promoting aHSC apoptosis are still not available in clinical trials, although current studies with tipelukast are being carried out in NASH and NAFLD individuals with hypertriglyceridemia (NCT02681055) (**Table 3**).

(ii) Senescence. Senescence is a physiological mechanism that restricts cell division to avoid the accumulation of damaged cells. Senescent HSCs adopt a more inflammatory but less fibrogenic phenotype [262-265]. Therefore, inducing HSC senescence may be a potential antifibrotic therapy.

(iii) Quiescence. Monocyte-derived macrophages direct the reversal of aHSC to an inactive phenotype. Nearly 50% of aHSCs can revert to a quiescent phenotype with a lower threshold to reactivation by exposure to fibrogenic agents [266]. Therefore, this process is another potential antifibrotic target.

3.3.2 Inhibition of cellular receptor signaling pathways

Several growth factors and signaling pathways are involved in the development of liver fibrosis:

(i) TGF-β. The most potent profibrotic cytokine in the liver is TGF-β, which is stored as an inactive latent complex in the ECM [267, 268]. TGF-β triggers several signaling pathways to control the epithelial-to-mesenchymal transition involved in chronic liver disease [269, 270]. Several studies have shown high levels of TGF-β in CHC patients [271, 272] that decrease after SVR [273, 274]. TGF-β can have apoptotic or cancerogenic effects during CHC [275, 276]. A phase 2 trial (NCT02161952) with pirfenidone, a TGF-β inhibitor, showed an improvement in liver inflammation and fibrosis in cirrhotic CHC patients [247]. Pirfenidone is currently in a phase 2 trial (PROMETEO; NCT04099407) in patients with advanced liver fibrosis from diverse chronic liver disease aetiologies, including CHC patients. This study showed an improvement in inflammation and liver stiffness through the administration of extended-release pirfenidone, but these results must be interpreted carefully due to the absence of a control group [248]. Therefore, targeting TGF-β1 as an antifibrotic therapy will be a challenge, and additional studies are required to understand the mechanisms involved in CHC.

(ii) Other growth factors. Liver injury is further exacerbated by growth factors and signaling pathways, such as PDGF, VEGF, CTGF, epidermal growth factor (EGF), and Wnt/β-catenin, which are strongly implicated in CHC and HCC [116, 277-280]. PDGF is the most potent mitogen and chemoattractant factor that stimulates HSC proliferation [281, 282] and contributes to the development of CHC [109, 110]. VEGF is released from LSECs and HSCs to form new blood vessels by playing a key role in angiogenesis [113]. Several reports have shown that HCV core can upregulate VEGF expression in chronic patients with HCV-related HCC [280, 283-285]. CTGF is a potent fibrogenic protein expressed at low levels in normal liver, but it is produced at high levels in hepatocytes and aHSCs during CHC [114-116]. EGF is overexpressed in aHSCs and contributes to

fibrosis development and HCC [278, 286, 287]. EGF is also elevated in CHC patients with advanced stages of fibrosis [288, 289].

(iii) Wnt pathway. The Wnt pathway plays a crucial role in cellular differentiation and development and is associated with both the induction and inhibition of HSC activation [290]. When Wnt activation occurs, β-catenin translocates to the nucleus recruiting cyclic adenosine monophosphate (cAMP)-response element-binding protein (CREB), which activates HSCs and promotes macrophage-mediated inflammation [291]. Inhibition of canonical Wnt pathway activation using CREB/β-catenin inhibitors was shown to reduce aHSC and liver fibrosis in mice [292]. In contrast, β-catenin-dependent canonical Wnt activation seems necessary to maintain the quiescent state of HSCs [205]. Non-canonical Wnt pathway activation increased HSC survival in a rat model [293]. It has recently been reported that the inhibition of the activation of canonical and non-canonical Wnt signaling pathways can prevent NASH-related liver fibrosis in mice on a methionine-choline-deficient diet [294]. Therefore, the Wnt pathway is a highly complex process, but its careful modulation could be a promising antifibrotic strategy.

(iv) Angiotensin II (Ang II). Ang II is secreted by HSCs and binds to Ang II type 1 receptor (AT1R) [295]. Ang II/AT1R interactions induce HSC activation, proliferation, contraction, and increased deposition of Col1. Therefore, blocking Ang II by Ang-converting enzyme inhibitors or AT1R blockers may be an effective antifibrotic strategy [203, 296]. In a phase 4 trial (NCT00298714), the long-term administration of losartan (an AT1R blocker) decreased inflammation, fibrogenic mediators, and Col1 deposition in CHC patients [227] (**Table 3**). However, this study lacked randomization. A phase 3 clinical trial (NCT03770936) is also evaluating the efficacy of two AT1R antagonists, candesartan and ramipril, in CHC patients, and results are expected in 2027. A mixture of ATR1 antagonists (losartan, valsartan, and irbesartan) led to reduced fibrosis in CHC patients with portal hypertension [249]. A phase 3 study examined the efficacy of irbesartan on liver fibrosis progression in CHC patients (NCT00265642) (**Table 3**), but these results have not been published yet.

(v) Hedgehog pathway. The Hedgehog pathway is a critical modulator of liver repair [297]. During CHC, HCV activates Hedgehog signaling in hepatocytes to promote fibrogenesis [298, 299], which is enhanced by the accumulation of profibrotic and growth factors, such as TGF-β, PDGF, and EGF [300-302]. Thus, Hedgehog signaling inhibitors could attenuate liver fibrosis, as has been observed in different animal models [303-305].

(vi) Neurotransmitters. HSC activation is also influenced by neurotransmitters expressed in myofibroblasts, such as cannabinoids (CB), opioids, and serotonin 5-hydroxytryptamine (5HT). The CB system is involved in neuromodulatory functions through a profibrogenic receptor (CB1) and an antifibrotic and hepatoprotective receptor (CB2) [212]. Curcumin and derivatives can inhibit CB1 and the cellular pathways involved in HSC activation [306, 307]. There are currently ongoing trials using CB1 antagonists for metabolic liver diseases, but the goal of fibrosis improvement is not listed as an endpoint. Conversely, CB2 agonists have been seen to reduce collagen by inducing HSC quiescence/apoptosis [308, 309]. Moreover, a germline genetic variant in the CNR2 gene (encoding CB2) is associated with necro-inflammation in CHC patients with HIV/HCV coinfection [310]. Therefore, antifibrotic therapies should be aimed at using mainly CB1 antagonists and, to a lesser extent, CB2 agonists. Opioid signaling increases HSC proliferation, and the opioid antagonist naltrexone and other opioid-like compounds attenuate liver fibrosis [214, 311]. 5HT has a profibrotic effect, and its receptors are upregulated in HSCs. Moreover, 5-HT2A and 5-HT2B receptor antagonism reduces inflammation and aHSCs, and increases aHSC apoptosis in mice and rat models [312].

3.3.3 Inhibition of nuclear receptor signaling pathways

HSCs express a diverse group of nuclear receptors, mainly the FXR and PPARs, which are potential targets for antifibrotic agents [49, 313].

(i) FXR. FXR is a nuclear receptor present in the liver that plays a role as a regulator of hepatic bile acid homeostasis, HSC activation, and Col1 production [314, 315]. It is important for bile acid-mediated HCV replication [316]. Thus, FXR could also represent a therapeutic target for the treatment of liver fibrosis [313]. However, most FXR ligands failed clinical assessment due to poor pharmacokinetics or toxicity. Obeticholic acid (OCA) is a potent whole-body bile acid FXR agonist that, in a phase 2 study (FLINT; NCT01265498), was well-tolerated, improved necroinflammation, and reduced fibrosis in non-cirrhotic NASH patients [250]. OCA is currently being conducted in two phase 3 studies in NASH patients with compensated cirrhosis (REVERSE; NCT03439254) and non-cirrhotic patients (REGENERATE; NCT02548351) [251]. The results of the REGENERATE interim analysis showed a clinically significant improvement in liver fibrosis [252] (**Table 3**). However, OCA has some side effects, such as elevation of low-density lipoprotein (LDL) levels ('bad' cholesterol) and pruritus [317]. Other bile acid FXR agonists are being investigated, such as nor-ursodeoxycholic acid (nor-UDCA), which is the most advanced drug in a phase 2 trial for primary sclerosing cholangitis (NUC-3; NCT01755507), but the goal of improvement of liver fibrosis is not listed as an endpoint [253] (**Table 3**). Several non-bile acid synthetic FXR agonists have been developed to enhance tolerability and avoid the drawbacks of OCA [318], such as cilofexor (ATLAS, NCT03449446, and NCT02781584) and tropifexor (**Table 3**). Tropifexor has entered a phase 2 trial (TANDEM, NCT03517540) combined with cenicriviroc [147]. INT-767, a dual agonist on FXR/G-protein-coupled bile acid receptor (Gpbar1), has shown to modulate KC activation and improve liver function by reducing steatosis and fibrosis in preclinical studies. Unfortunately, currently there are no ongoing clinical trials [319].

(ii) PPARs. PPARs belong to the steroid/thyroid hormone receptor superfamily and are mainly expressed in hepatocytes [320]. Three PPAR isoforms have been identified (PPAR-γ, PPAR-α, and PPAR-δ), which vary in tissue distribution and are potential antifibrotic therapy targets. PPAR-γ and PPAR-α are decreased during HCV infection [321, 322], while PPAR-δ has not been investigated relating to HCV infection. Thiazolidinediones (TZDs) are PPAR-γ agonists that reduce aHSC and collagen deposition [323]. A phase 2 study with pioglitazone (PIVENS, NCT00063622) showed significant reductions in liver enzyme levels, steatosis, inflammation, and hepatocellular ballooning, but with substantial adverse effects [148, 254]. Clinical trials in both CHC and NASH patients have been unsuccessful regarding evidence of reduced fibrosis [148, 255] (**Table 3**). TZDs can suppress HCC recurrence in HCV-infected patients [324] and help to improve steatosis in the context of HIV/HCV co-infection [325]. Nevertheless, TZD treatments have several clinical concerns, such as a higher risk of prostate and pancreas cancer, body weight gain, and increased cardiovascular events, among others. Elafibranor (GFT-505), a hepatotropic dual PPAR-α/δ agonist tested in a phase 2b trial (GOLDEN-505; NCT01694849), was well-tolerated, improved liver enzymes, and reduced liver fibrosis in non-cirrhotic NASH patients [256]. A phase 3 trial is currently underway to evaluate histological improvement and all-cause mortality and liver-related outcomes in NASH patients (RESOLVE-IT; NCT02704403), but results from an interim analysis were disappointing [167] (**Table 3**).

Other PPAR drugs are also in development, including lanifibranor (IVA-337; a pan-PPAR agonist), saroglitazar (a PPAR-α/γ dual agonist), and seladelpar (a PPAR-δ agonist). IVA-337 is an agonist that activates all three PPARs (PPAR-γ, PPAR-α, and PPAR-δ) and is currently being tested in a phase 2b trial (NATIVE; NCT03008070) in non-cirrhotic NASH patients with liver steatosis and moderate to severe necroinflammation (**Table 3**). The clinical trial has already been completed and showed a favorable tolerability profile with a significant reduction in steatosis and fibrosis. These results support the idea of entering into phase 3 development [257].

3.4 Reduction of fibrosis

Drugs directly targeting fibrosis are promising candidates. Scarring is a dynamic process whose regression depends on its duration and scar factors. Since aHSCs are in the injured liver, not in the healthy liver, their apoptosis may facilitate scar removal and fibrosis regression [326]. Here, we focus

on potential antifibrotic therapies based on inhibiting collagen synthesis and ECM cross-linking enzymes that directly affect scar tissue. In this regard, nanotechnology could have a crucial role [327]. Examples of antifibrotic candidates that reduce fibrotic scar evolution undergoing clinical trials are listed in **Table 4.**

Table 4 **Clinical studies focusing on antifibrotics promoting fibrosis degradation**

Compound	Target	Patients	Clinical trial identifier / Trial stage	Ref.
ND-L02-s0201	Col1	CHC [(1)] with moderate to extensive fibrosis	NCT02227459 Phase 1b/2 completed	[328-330]
		CHC [(2)] with cirrhosis	NCT03420768 Phase 2 completed	[328-330]
Simtuzumab	LOXL2	Unknown etiology with fibrosis	NCT01452308 Phase 2 completed	[331]
		HIV and/or CHC [(3)] with fibrosis	NCT01707472 Phase 2 completed	[331]
		NASH with advanced fibrosis, but not cirrhosis	NCT01672866 Phase 2 terminated	[332]
		NASH with compensated cirrhosis	NCT01672879 Phase 2 terminated	[332]
PAT-1251	LOXL2	Healthy volunteers	NCT02852551 Phase 1 completed	-
PXS-5153A	LOXL2	Healthy volunteers	Two phases 1 completed	[240, 318]

[(1)] Patients with SVR; [(2)] Patients with a SVR for at least one year before the date of screening are included, and patients with detectable HCV RNA at screening are excluded; [(3)] Patients must have HCV RNA ≥ 2000 IU/ml, and failed therapy or are unwilling to receive or have contraindications to interferon therapy for HCV. *CHC* chronic hepatitis C, *Col1* collagen type 1, *HCV* hepatitis C virus, *HIV* human immunodeficiency virus, *LOXL2* lysyl oxidase-like 2, *NASH* non-alcoholic steatohepatitis, *SVR* sustained virological response.

3.4.1 Collagen type 1 (Col1)

The major ECM components produced by aHSCs are collagens, especially Col1, which represent the major structural component of the fibrotic scaffold (more than 50% of the scar protein) [333, 334] with levels being 10-fold higher in advanced fibrosis and cirrhosis [335]. Therefore, targeting Col1 could be a potent antifibrotic strategy. Total hepatic collagen content and proinflammatory cells in the liver were significantly reduced after treatment with specific small interfering RNAs (siRNAs) targeting the procollagen α1(I) gene in three *in vivo* models of liver fibrosis progression and an *in vivo* model of advanced fibrosis regression [327]. Similar results were found in transgenic mice with inducible knockdown of Col1, 3, 4, or 6 [336]. Promising results were also obtained with Col1 siRNA-loaded cationic nano-hydrogel particles [337, 338].

Human heat shock protein 47 (hsp47) is a Col1 chaperone expressed in HSCs that is essential for the maturation and secretion of collagen [49]. Targeted conjugates like vitamin A-coupled liposomes containing hsp47-siRNA (ND-L02-s0201) can be used to block collagen synthesis in different rodent models [339]. Currently, ND-L02-s0201 is being investigated in two clinical trials in phase 1b/2 and 2, respectively, in patients with fibrosis (NCT02227459) and cirrhosis after clearing the HCV infection (NCT03420768) (**Table 4**). These studies have shown that ND-L02-s0201 was well-tolerated [329, 330] and was not immunogenic [328]. Therefore, Col1, and other ECM molecules that play essential roles in the fibrotic matrix organization could also be targets of antifibrotic therapy.

3.4.2 Lysyl oxidase-like 2 (LOXL2)

The lysyl oxidases are a family of enzymes secreted by HSCs involved in collagen cross-linking and ECM stabilization. The imbalance in this process leads to excessive cross-linking characterized by

liver scarring and stiffness, which leads to liver failure [340]. Moreover, ECM stiffness promotes HSC proliferation via integrins. Of the five members, lysyl oxidase-like-2 (LOXL2) is the most widely studied in chronic liver diseases including CHC [341, 342]. LOXL2 is a matrix enzyme overexpressed by aHSCs that stabilizes ECM, making it more resistant to protease degradation. Therefore, ECM cross-linking and remodeling can be regulated by LOXL2 inhibitors [343].

The IgG4 monoclonal antibody simtuzumab (SIM, GS-6624) is a LOXL2 inhibitor that has shown poor results in a clinical trial (**Table 4**) [341]. Two pilot phase 2 trials (NCT01452308, NCT01707472) in patients with liver fibrosis of variable etiology (i.e., HIV and/or HCV-infected patients) showed that simtuzumab was well-tolerated and had no serious adverse events [331]. However, two phase 2 studies in NASH patients with bridging fibrosis or compensated cirrhosis (NCT01672866, NCT01672879) were stopped due to a lack of efficacy in decreasing liver fibrosis or liver-related clinical events in cirrhotic patients [332]. These disappointing results using antibodies have opened the way for small molecules, which can maximize inhibition by more easily penetrating the fibrotic matrix and intracellular compartments, and some of which are now in the early stages of clinical trials [344].

PAT-1251 is a potent irreversible inhibitor of LOXL2 that has shown high specificity in preclinical studies [345]. Consequently, PAT-1251 was the first small-molecule LOXL2-inhibitor to enter into clinical trials (**Table 4**). The phase 1 trial (NCT02852551) in healthy volunteers has already been completed, but results are not publicly available. PXS-5153A (BI-1467335), another LOXL2 inhibitor, improved liver function by diminishing collagen content and collagen cross-links in a mouse model [343]. Two phase 1 trials with PXS-5153A have been completed showing good safety and pharmacokinetic profile and a substantial and highly significant reduction of LOXL2 levels [240, 318] (**Table 4**). These results support the use of small molecules targeting LOXL2, or other lysyl oxidases, as a tool for treating liver diseases with abnormal increases in collagen cross-linking.

4 Conclusions

After HCV clearance by successful DAA treatments, liver fibrosis may persist. Even if the cure of HCV infection leads to fibrosis regression, this is a long process. Therefore, once HCV infection is cured, the therapeutic targets for reversing liver fibrosis focus on using antifibrotic agents.

Current antifibrotic approaches targeting HCV-associated liver fibrosis are mainly based on reducing inflammation, hepatocyte injury, and HSC activation; or inducing ECM degradation after HCV removal. To date, there is a wide variety of antifibrotic drugs that are being tested in clinical trials, but most of them are analyzed on NASH patients. These antifibrotic drugs should also be evaluated in patients cured of HCV infection, although it is expected that the results with NASH patients can be extrapolated to CHC patients. However, no compound is currently approved by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA). Moreover, the promising results obtained in preclinical steps do not accurately predict outcomes in human clinical trials. Furthermore, due to the diverse stages of liver fibrosis, the complexity of the process, and different patients' genetic backgrounds, more research is needed to explore the question of whether or not antifibrotic drugs are effective treatments.

Liver fibrosis regression requires new therapeutic strategies, such as the use of mitochondrial open reading frames of the 12S ribosomal RNA type-c (MOTS-c) agonists [346], cell-based therapy [347-349], or combined treatments. Nanoparticles can also be an attractive tool because they accumulate in the liver [350]. The combinations of two or more antifibrotic compounds addressing multiple pathways offer the most exciting approaches. The advantages of combination therapies rely on using lower doses of drugs, which reduces toxicity problems and side-effects derived from prolonged treatments, and has a higher efficacy due to additive or even synergistic effects compared to monotherapy.

Several promising antifibrotic drugs and targets are currently undergoing preclinical studies and will be evaluated in the clinic shortly. Moreover, the use of novel techniques, such as precision-cut liver slice cultures, human liver organoids, humanized mice, as well as omics technology for the analysis of signaling pathways triggered during chronic liver disease [2, 351-354], will provide valuable information for testing possible antifibrotic drugs.

Declarations

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Competing interests

The authors declare that they have no conflicts of interest.

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Not applicable.

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Authors' contributions

Conceptualization: SR and IM.

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