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The Promising Role of Anti-Fibrotic Agent Halofuginone in Liver Fibrosis/Cirrhosis

Berna Karakoyun

Abstract

Liver fibrosis is a complex inflammatory and fibrogenic process that results from chronic liver injury and represents an early step in the progression of cirrhosis. Several cell types [hepatic stellate cells (HSCs), hepatocytes, liver sinusoidal endothelial cells (LSECs), and Kupffer cells (KCs)], cytokines [platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, interferons (IFNs), interleukins (ILs)], oxidative stress, and microRNAs (miRNAs) are involved in the initiation and progression of liver fibrosis/cirrhosis. Generally, liver fibrosis begins with the stimulation of inflammatory immune cells to secrete cytokines, growth factors, and other activator molecules. These chemical mediators direct HSCs to activate and synthesize large amounts of extracellular matrix (ECM) components. Therefore, HSC activation is a pivotal event in the development of fibrosis and a major contributor to collagen (specifically type I) accumulation. The inhibitory effect of halofuginone on collagen type α1(I) synthesis and ECM deposition has been shown in several experimental models of fibrotic diseases. Halofuginone inhibits TGF-β–induced phosphorylation of Smad3, which is a key phenomenon in the fibrogenesis. It also regulates cell growth and differentiation, apoptosis, cell migration, and immune cell function in liver fibrosis/cirrhosis. This review discusses the etiology and mechanisms of liver fibrosis/cirrhosis and the promising role of anti-fibrotic agent halofuginone.

Keywords: liver fibrosis, liver cirrhosis, hepatic stellate cells, pathogenesis, anti-fibrotic, halofuginone

1. Introduction

Liver cirrhosis is the end-stage condition of several chronic liver diseases, and fibrosis is the critical pre-stage of cirrhosis. On a worldwide perspective, liver cirrhosis can be induced by
a number of well-defined etiological causes/factors or conditions such as chronic infection by hepatitis B, C viruses, chronic alcoholism and/or chronic exposure to toxins or drugs, infections, chronic exposure to altered metabolic conditions, inherited metabolic diseases such as hemochromatosis and Wilson’s disease, auto-immune diseases such as primary biliary cirrhosis, and auto-immune hepatitis [1–3]. These etiologies may work separately or in combination with each other to produce cumulative effects. While the causes of liver cirrhosis are multifactorial, there are some pathological characteristics that are common to all cases of cirrhosis, including degeneration and necrosis of hepatocytes, replacement of healthy liver parenchyma by fibrotic scar tissues and regenerative nodules, and loss of liver function [4–7].

Fibrosis is characterized by high levels of extracellular matrix (ECM, non-functional connective tissue) components extremely rich in collagen type I. The matrix metalloproteinases (MMPs, matrix degradation enzymes), and the tissue inhibitor of metalloproteinases (TIMPs) play a crucial role in the fine regulation of ECM turnover, which is altered in most pathological states associated with liver fibrosis [8]. The key cellular mediator of fibrosis comprises the activated hepatic stellate cells (HSCs), which serve as the primary ECM-producing cells. HSCs, which play a key role in the development of liver fibrosis [9, 10], are activated by several inflammatory cytokines and growth factors in a paracrine and autocrine manner [11, 12].

Liver fibrosis and cirrhosis are dynamic and highly integrated molecular, tissue and cellular processes that can progress and regress over time [13] and that require cellular cross-talk between various liver cell types [14]. At early stages of fibrosis, initiating signals [such as DNA, reactive oxygen species (ROS)], responding cells [Kupffer cells (KCs), platelets, liver sinusoidal endothelial cells (LSECs)], and soluble mediators [such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β] induce accompanying wound-healing responses to liver injury. With time, cells, cytokine responses, and ECM components become more specialized but continue to have strong interactions with each other [15].

Halofuginone is a non-toxic plant alkaloid [7-bromo-6-chloro-3-(3-hydroxy-2-piperidine)-2-oxopropyl-4(3H)-quinazoline] isolated from the roots of Dichroa febrifuga, and is used worldwide as an anti-parasitic drug [16]. Independent of this effect, halofuginone was found to be a potent inhibitor of collagen type α1 (I) gene expression [17], which was demonstrated in a broad range of cell types both in vitro and in vivo [16–20]. Due to its inhibitory effects on collagen synthesis (collagen type α1) and ECM deposition, halofuginone treatment was used in several experimental disease models characterized by excessive collagen accumulation, such as pulmonary, pancreatic and renal fibrosis [21–23], scleroderma and chronic graft-versus-host disease [24], post-operative peritendinous and abdominal adhesions [25, 26], urethral and esophageal strictures [27, 28], wound repair [29], burn injury [30], renal injury [31, 32], injury-induced arterial intimal hyperplasia [33], colitis [34], and liver fibrosis and cirrhosis [35–39]. Although the exact anti-fibrotic mechanism of halofuginone is not well understood, it was found that halofuginone affects collagen synthesis probably by inhibiting TGF-β-mediated Smad3 (intracellular protein) activation [40]. Halofuginone also regulates cell growth and differentiation, apoptosis, cell migration, and immune cell function [41]. It prevents concanavalin A-induced liver fibrosis by affecting T helper 17 (Th17) cell differentiation, which suggests a direct connection between the myofibroblasts/fibrosis pathway and
the Th17 pro-inflammatory pathway [38]. In addition, halofuginone treatment effectively inhibits the delayed-type hypersensitivity response, indicating suppression of T cell–mediated inflammation in vivo [42]. Moreover, it is a potent inhibitor of nuclear factor (NF)-κB, pro-inflammatory cytokines and p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation in activated T cells in vitro [42]. Also, it inhibits HSC proliferation and migration and up-regulates their expressions of fibrolytic MMP-3 and -13 via activation of p38 MAPK and NF-κB [43].

Although there are no highly effective anti-fibrogenic agents currently available, the potential candidates that can specifically inhibit ECM components in general and specifically inhibit collagen type I in particular, are considered to be promising for the prevention and treatment of liver fibrosis/cirrhosis. The present review aims to clarify the etiology and mechanisms of liver fibrosis/cirrhosis and focus on the anti-fibrotic potential of a novel and promising agent, halofuginone.

2. Role of different cell types in liver fibrosis/cirrhosis

The liver is composed of parenchymal cells (hepatocytes) and non-parenchymal cells (HSCs, LSECs, and KCs). Both parenchymal and non-parenchymal cells are involved in the initiation and progression of liver fibrosis/cirrhosis (Table 1).

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<td>KC-derived TGF-β1 stimulates proliferation and collagen formation of HSCs</td>
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Abbreviations: TGF-β1, transforming growth factor-β1; IL, interleukin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Table 1. Role of different cell types in liver fibrosis/cirrhosis.
2.1. Hepatic stellate cells (HSCs)

HSCs are one of the non-parenchymal cells of the liver located in the perisinusoidal space (space of Disse) between hepatocytes and sinusoidal endothelial cells. HSCs are also known as fat-storing cells, perisinusoidal cells, lipocytes, or vitamin A-rich cells, and their main function is storage of vitamin A and other retinoids \[7, 44\]. HSCs show two different phenotypes: quiescent type in the healthy liver and activated type in the diseased one. Quiescent HSCs mostly function as vitamin A reserves \[45\]. However, in response to liver injury, inflammatory cytokines such as tumor necrosis factor (TNF)-α, TGF-β, interleukin (IL)-1, and PDGF promote HSCs to undergo a phenotypic switch from a quiescent, vitamin A storing cell into proliferative, α-smooth muscle actin (α-SMA)-positive, myofibroblast-like cells which contribute to fibrosis by producing the abnormal ECM components \[46\]. Therefore, HSC activation is a pivotal phenomenon in initiation and progression of liver fibrosis and a major contributor to collagen accumulation \[47, 48\].

2.2. Hepatocytes

Hepatocytes are the primary parenchymal component of the liver and play an important role in fibrosis/cirrhosis. They are the main targets of several hepatotoxic agents including hepatitis viruses, alcohol metabolites, and bile acids \[11\]. Liver injury either promotes apoptosis or triggers compensatory regeneration of hepatocytes \[49\]. Hepatocyte-derived apoptotic bodies stimulate secretion of fibrogenic cytokines from KCs and promote HSC activation via interaction of toll-like receptor (TLR)-9 with DNA, which is released from apoptotic hepatocytes \[50–53\]. On the other hand, activated HSCs also act as phagocytes and phagocytize hepatocyte apoptotic bodies, which promote myofibroblasts survival and fibrogenesis \[54\]. Therefore, apoptosis of hepatocytes is a crucial event in liver injury and contributes to tissue inflammation, fibrogenesis, and development of cirrhosis. Also, in the cirrhotic stage, hypoxic hepatocytes become a primary source of TGF-β, which may augment liver fibrosis \[55\].

2.3. Liver sinusoidal endothelial cells (LSECs)

LSECs constitute the sinusoidal wall, also known as endothelium, or endothelial lining. The main characteristic of LSECs is having the fenestrae on the surface of the endothelium \[56, 57\]. The endothelial fenestrae control exchange of fluids, solutes, and particles between sinusoidal blood and hepatocytes \[58\]. In the healthy liver, the fenestrated endothelial cells prevent HSC activation through vascular endothelial growth factor-stimulated nitric oxide production \[59\]. However, LSECs have high endocytotic capacity \[56, 60\]. Upon liver injury, defenestration and capillarization of LSECs lead to impaired substrate exchange which is the major cause of hepatic dysfunction \[57, 58\] and HSC activation \[61, 62\]. It has been also revealed that LSECs can secrete the cytokine IL-33 to activate HSCs and promote liver fibrosis \[63\].

2.4. Kupffer cells (KCs)

KCs, also called stellate macrophages, are interspersed throughout the liver, situated within the sinusoids. KCs are responsible for the removal of circulating microorganisms, immune
complexes, and debris from the blood stream. They are usually activated by many injuri-
os factors such as viral infection and alcohol [64]. Activation of KCs is a key phenomenon
in initiation and preservation of liver fibrosis. Activated KCs express chemokine receptors,
secret inflammatory cytokines (such as TNF-α, IL-1, IL-6) and serve as antigen-presenting
cells, which lead to progression of fibrosis [65–68]. KCs are also involved in the activation
of HSCs and formation of liver fibrosis. For example, KC-conditioned medium promotes activa-
tion of cultured rat HSCs with enhanced ECM production and stimulates cell proliferation via
induction of PDGF receptors on the membrane of HSCs [69]. KC-derived TGF-β1 stimulates
proliferation and collagen formation of HSCs in a rat model of alcoholic liver fibrogenesis
[66]. Moreover, macrophage ablation has been shown to attenuate liver fibrosis. For example,
gadolinium chloride-mediated depletion of KCs has been shown to result in attenuation of
carbon tetrachloride (CCl₄)-induced fibrosis in rats with prevention of the increased TGF-β
expression [70]. Conversely, KCs produce interstitial collagenase MMP-13 when treated with
gadolinium chloride, which reduces ECM deposition during experimental liver fibrosis [71].
In addition, activated KCs can effectively kill HSCs by a caspase 9-dependent mechanism via
possible involvement of TNF-related apoptosis-inducing ligand (TRAIL) [72, 73].

3. Role of cytokines in liver fibrosis/cirrhosis

Cytokines, which mediate several immune and inflammatory reactions, are small signal-
ing proteins that facilitate intercellular communication between various cells. They func-
tion through cell-surface receptors, and down-stream signaling induces an alteration of cell
functions. Liver fibrosis/cirrhosis is a result of interaction of a complex network of cytokines,
which modify activities of circulating immune cells, HSCs, KCs, LSECs, and hepatocytes. The
role of cytokines in liver fibrosis/cirrhosis is summarized in Table 2.

3.1. Platelet-derived growth factor (PDGF)

PDGF is one of the most potent mitogen for HSCs isolated from mouse, rat, or human liver
[74]. PDGF and its receptors are significantly overexpressed in fibrotic tissues, and its activity
increases with the degree of liver fibrosis [75, 76]. Hepatocyte damage resulting from factors,
such as viruses, chemicals, or hepatotoxins, can induce KCs to synthesize and release PDGF
[77]. When PDGF binds to its specific receptor on the membrane of HSCs, it activates cor-
responding signal molecules and transcription factors, leading to the activation of its down-
stream target genes and activation of HSCs [74]. PDGF has been shown to up-regulate the
expression of MMP-2, MMP-9, and TIMP-1, and inhibit collagenase activity, thereby decreas-
ing ECM degradation [78].

3.2. Transforming growth factor (TGF)-β

Among fibrotic mediators, TGF-β is one of the most important pro-fibrotic cytokine. The direct
targets in TGF-β pathway, Smads (especially Smad3) are critical mediators in fibrogenesis [79, 80].
The intracellular effectors of TGF-β signaling, the Smad proteins, are activated by receptors and
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<td><strong>Platelet-derived growth factor (PDGF)</strong></td>
<td>Activates HSCs</td>
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<td>Up-regulates expression of MMP-2, MMP-9, and TIMP-1 and inhibits collagenase activity</td>
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<td><strong>Transforming growth factor (TGF)-β</strong></td>
<td>Stimulates HSC activation</td>
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<td>Induces expression of matrix-producing genes, inhibits ECM degradation, and promotes TIMPs</td>
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<td>Inhibits DNA synthesis and induces apoptosis of hepatocytes</td>
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<td><strong>Tumor necrosis factor (TNF)-α</strong></td>
<td>Induces hepatocyte death by apoptosis</td>
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<td></td>
<td>Activates HSCs and stimulates ECM synthesis</td>
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<td>Induces/reduces apoptosis of activated HSCs</td>
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<td>Reduces glutathione and inhibits pro-collagen α1 mRNA expression</td>
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<td><strong>Interferons (IFNs)</strong></td>
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<td><strong>IL-1</strong></td>
<td>Activates HSCs and stimulates them to produce MMP-9, MMP-13 and TIMP-1</td>
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<td>Increases MCP-1 in hepatocytes and augments TLR-4-dependent up-regulation of inflammatory signaling in macrophages</td>
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<td><strong>IL-17</strong></td>
<td>Regulates production of TGF-β1 by KCs, induces activation of HSCs and induces production of collagen and α-SMA in HSCs via STAT3 pathway</td>
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<td><strong>IL-6</strong></td>
<td>Attenuates hepatocyte apoptosis and induces regeneration of hepatocytes through NF-κB pathway</td>
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<td><strong>IL-10</strong></td>
<td>Inhibits expression of TGF-β1, MMP-2 and TIMP-1</td>
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<td></td>
<td>Inhibits HSC activity</td>
<td>[117]</td>
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<td></td>
<td>Reduces TGF-β1, TNF-α, collagen α1, and TIMP mRNA up-regulation</td>
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<td><strong>IL-22</strong></td>
<td>Inhibits hepatocyte apoptosis via STAT3</td>
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<td>Induces HSC senescence</td>
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**Abbreviations:** HSC, hepatic stellate cell; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ECM, extracellular matrix; SMA, smooth muscle actin; MCP, monocyte chemoattractant protein; TLR, toll-like receptor; KC, Kupffer cell; STAT, signal transducer and activator of transcription; NF-κB, nuclear factor-κB.

Table 2. Role of cytokines in liver fibrosis/cirrhosis.
translocate into the nucleus, where they regulate transcription [79]. The main effect of TGF-β is to stimulate HSC activation, and the TGF-β autocrine cycle in activated HSCs is an important positive feedback to the progression of liver fibrosis [81, 82]. Though the main source of TGF-β in fibrotic liver is activated HSCs, LSECs, KCs, and hepatocytes also contribute to synthesis of this growth factor [83]. The level of TGF-β1 expression is increased during liver fibrosis and reaches a maximum at cirrhosis [55]. TGF-β1 induces expression of the matrix-producing genes, inhibits ECM degradation, and promotes TIMPs, leading to excessive collagen accumulation and promoting the development of liver fibrosis [84, 85]. Furthermore, TGF-β1 has been shown to inhibit DNA synthesis and induces apoptosis of hepatocytes. In particular, TGF-β1-induced apoptosis is thought to be responsible for tissue loss and decrease in liver size seen in cirrhosis [86–88].

3.3. Tumor necrosis factor (TNF)-α

TNF-α is a pro-inflammatory cytokine produced by different cell types. However, it is mainly produced by activated KCs in the liver. TNF-α is an important mediator in several processes such as cell proliferation, inflammation, and apoptosis [89]. TNF-α can induce cell death by apoptosis, and KCs can be stimulated by apoptotic hepatocytes to produce more TNF-α [90]. Furthermore, TNF-α plays an essential role in the HSC activation and ECM synthesis in liver fibrosis [91, 92]. TNF-α may act as a surviving factor for activated rat HSCs by up-regulating the anti-apoptotic factors (NF-κB, bcl-xL, and p21WAF1) and by down-regulating the pro-apoptotic factor (p53) [93]. On the other hand, TNF-α can induce apoptosis in HSCs [73]. It has been also demonstrated that TNF-α shows anti-fibrogenic effect in rat HSCs by reducing glutathione and inhibiting pro-collagen α1 mRNA expression [94].

3.4. Interferons (IFNs)

IFNs are potent pleiotropic cytokines that broadly alter cellular functions in response to viral and other infections. Leukocytes synthesize IFN-α and IFN-β in response to viruses, and T cells secrete IFN-γ upon stimulation with various antigens and mitogens. Although the primary action of IFN-α is to eradicate viruses, patients with hepatitis C treated with IFN-α exhibit a regression of liver fibrosis even if viral eradication is not achieved [95], indicating that IFN-α itself has anti-fibrotic activity via triggering the apoptosis of HSCs [96]. IFN-β treatment decreases α-SMA and collagen expression and inhibits HSC activation through inhibiting of TGF-β and PDGF pathways [97]. Similarly, IFN-γ reduces ECM deposition in vivo by inhibiting HSC activation [98] via TGFβ1/Smad3 signaling pathways [99]. Interestingly, IFN-α and IFN-γ may exert opposite effects on apoptosis in HSCs. IFN-α was shown to elicit an anti-apoptotic effect on activated HSCs, whereas IFN-γ was found to exert pro-apoptotic effect on HSCs by down-regulating heat-shock protein 70 [100].

3.5. Interleukins (ILs)

ILs are immunomodulatory cytokines that are critically involved in the regulation of immune responses. They are produced by a variety of cell types such as CD4+ T lymphocytes, monocytes, macrophages, and endothelial cells. KCs and LSECs can rapidly produce ILs in response to liver injury. ILs can have pro- and anti-inflammatory functions in chronic liver diseases, dependent on the inflammatory stimulus and, the producing and the responding cell type.
The main function of pro-inflammatory ILs is to stimulate immune responses that result in the elimination of invading pathogens or damaged cells. On the other hand, anti-inflammatory ILs are produced to protect the host’s body from exaggerated immune responses and to limit organ damage. As soon as the pathogenic stimuli are removed, ILs production is no longer needed, and inflammation diminishes. If the stimulus continues, inflammation can become chronic and induce a variety of inflammatory diseases [101].

IL-1 is a pro-inflammatory and pro-fibrotic cytokine that directly activates HSCs and stimulates them to produce MMP-9, MMP-13, and TIMP-1, resulting in liver fibrogenesis [102]. IL-1 receptor-deficient mice exhibit ameliorated liver damage and reduced fibrogenesis [102]. Similarly, IL-1 receptor antagonist protects rats from developing fibrosis in dimethylnitrosamine-induced liver fibrosis [103]. Lack of IL-1α or IL-1β also makes the mice less susceptible to develop liver fibrosis in experimental model of steatohepatitis [104]. It has been also shown that IL-1β at physiological doses increases the inflammatory and prosteatotic chemokine monocyte chemoattractant protein (MCP)-1 in hepatocytes, and augments TLR-4-dependent up-regulation of inflammatory signaling in macrophages [105]. Thus, IL-1 is an important participant, along with other cytokines, and controls the progression from liver injury to fibrogenesis.

Another pro-inflammatory and pro-fibrotic cytokine IL-17 has been reported to be involved in many immune processes, most notably in inducing and mediating pro-inflammatory responses. Its expression increases with increasing degree of liver fibrosis [106, 107], suggesting that IL-17 may not only induce inflammation but also contribute to disease progression and chronicity [106]. IL-17 regulates production of TGF-β1 by KCs, which in turn, induces activation of HSCs into myofibroblasts, and further facilitates differentiation of IL-17 expressing cells [108]. Also, IL-17 directly induces production of collagen and α-SMA in HSCs via the signal transducer and activator of transcription (STAT)3 signaling pathway [108]. Furthermore, abrogation of IL-17 signaling by deletion of IL-17RA protects mice from fibrogenesis [108]. Similarly, blockade of endogenous IL-17 with neutralizing IL-17-specific antibody reduces liver fibrosis, whereas treatment with recombinant IL-17 increases fibrosis development [109].

IL-6 is a pleiotropic cytokine, which may affect differentiation of fibroblast to myofibroblast, and it plays an important role in fibrotic diseases [110, 111]. On the other hand, IL-6 has beneficial effects for the liver. For example, IL-6 reduces CCl₄-induced acute and chronic liver injury and fibrosis [112]. Also, it attenuates hepatocyte apoptosis and induces regeneration of hepatocytes through NF-κB signaling pathway [112]. In an experimental model of concavaline A-induced hepatitis, IL-6 pretreatment protects mice from liver injury. This protection requires gp130 signaling in hepatocytes and is mediated via the gp130/STAT3 signaling cascade [113]. Furthermore, systemic injection of IL-6 followed by intrahepatic transplantation of mesenchymal stem cells is also able to reduce hepatocyte apoptosis and liver fibrogenesis after CCl₄ treatment [114].

IL-10 is one of the major anti-inflammatory cytokines, with tissue protective functions during fibrogenesis. It down-regulates the pro-inflammatory response and has a modulatory effect on liver fibrogenesis [115, 116]. IL-10 has been shown to exert anti-fibrotic effects through inhibiting HSC activity [117]. IL-10-deficient mice show higher liver fibrosis with larger
inflammatory infiltrates in CCl₄-induced liver fibrosis compared to wild-type mice [118, 119]. IL-10 gene therapy reverses CCl₄-induced murine liver fibrosis by inhibiting the expression of TGF-β1, MMP-2, and TIMP-1 [115]. Additionally, IL-10 gene therapy reverses liver fibrosis and prevents cell apoptosis in a thioacetamide-treated murine liver, and reduces TGF-β1, TNF-α, collagen α1, and TIMP mRNA up-regulation, suggesting a therapeutic potential for treatment with IL-10 [120].

IL-22 is known to play important roles in the modulation of tissue immune responses to inflammation. It reduces inflammation-induced damage of hepatocytes both in vitro and in vivo by promoting their survival and inhibiting apoptosis [121]. This protective function is dependent on STAT3 signaling, as STAT3-deficient mice were not protected when treated with IL-22 [122]. Similarly, in CCl₄-induced liver fibrogenesis, IL-22 is protective through induction of senescence in HSCs via STAT3 signaling pathway [123]. Moreover, IL-22 is also involved in the restoration of functional liver mass after organ damage. Liver progenitor cells have been shown to express IL-22R, and IL-22 derived from inflammatory cells induces proliferation of liver progenitor cells [124].

### 4. Role of oxidative stress in liver fibrogenesis

Oxidative stress is caused by an imbalance between production of ROS and their elimination by anti-oxidant defenses [125]. As liver is an essential organ for detoxification and nutrients metabolism, it is more vulnerable to oxidative stress [125]. Oxidative stress-related molecules and pathways modulate tissue and cellular events involved in the liver fibrogenesis [126]. The generation of ROS plays a crucial role in producing liver damage and initiating liver fibrogenesis [126]. Oxidative stress disrupts lipids, proteins and DNA, induces necrosis and apoptosis of hepatocytes, resulting in the initiation of fibrosis [127]. ROS stimulate the production of pro-fibrogenic mediators from KCs and circulating inflammatory cells. Remarkably, ROS directly activate HSCs. The elevated oxidative stress contributes to fibrogenesis via stimulating collagen production from activated HSCs and release of other pro-fibrogenic cytokines and growth factors [126, 128].

### 5. Role of microRNAs (miRNAs) in liver pathophysiology

miRNAs are a family of small non-coding RNAs (20–25 nucleotides in length) that control gene expression by binding to mRNAs to repress translation or induce mRNA cleavage [129]. Many researchers have reported that the unusual expression of miRNAs in liver tissue was related to the pathogenesis of liver disease of any etiology [130, 131]. Recently, miRNAs have been found to play fundamental roles in liver fibrosis, including those in HSC activation and ECM production [132]. For example, miRNA-21 exhibits an important role in the pathogenesis and progression of liver fibrosis. A natural product 3,3’-Diindolylmethane (DIM) inhibits TGF-β signaling pathway by down-regulating the miRNA-21 expression in thioacetamide-induced experimental liver fibrosis. Furthermore, DIM can suppress HSC activation via down-regulating
miRNA-21 levels in HSCs by inhibiting activity of the transcription factor AP-1 [133]. Inhibition of miRNA-21 also reduces liver fibrosis through concomitant reduction of CD24+ liver progenitor cells [134]. In mouse and human studies, the expression levels of miRNA-199a, antisense miRNA-199a*, miRNA-200a, and miRNA-200b are found to be positively and significantly correlated with progression of liver fibrosis. Overexpression of these miRNAs dramatically increases the expression of fibrosis-related genes in HSCs [135]. Also, miRNA-221 and miRNA-222 are up-regulated in human liver in a fibrosis progression-dependent manner [136]. Similarly, in isolated primary human liver cells, miRNA-571 is up-regulated in hepatocytes and HSCs in response to the pro-fibrogenic cytokine TGF-β [137]. miRNA-214 appears to participate in the development of liver fibrosis by modulating the epidermal growth factor (EGF) receptor and TGF-β signaling pathways. Also, inhibition of miRNA-214 by locked nucleic acid-antimiRNA-214 ameliorates liver fibrosis in PDGF c transgenic mice [138]. In addition, miRNA-214-5p may play crucial roles in HSC activation and progression of liver fibrosis. The overexpression of miRNA-214-5p in human stellate cells increases the expression of fibrosis-related genes such as MMP-2, MMP-9, α-SMA, and TGF-β1 [139].

miRNAs may also play anti-fibrogenic roles. It has been demonstrated that both miRNA-150 and miRNA-194 inhibit HSC activation and ECM production in rats with liver fibrosis by decreasing the expression of c-myb (target for miRNA-150) and rac 1 (target for miRNA-194) [140]. Interestingly, miRNAs such as miRNA-19b, miRNA-29, miRNA-133a, and miRNA-146a are significantly down-regulated in HSCs isolated from experimental animals with liver fibrosis, and restoration of these miRNAs alleviates fibrogenesis [47, 141, 142]. Moreover, miRNA-133a overexpression inhibits both human and murine primary HSCs proliferation and prevents the progression of liver fibrosis [142].

Multiple studies have proposed that miRNAs may serve as biomarkers for HSC activation and liver fibrosis progression, and can be possible candidates for future therapies targeting liver fibrosis/cirrhosis.

6. Pathogenesis of liver fibrosis/cirrhosis

Liver fibrosis and its end-stage consequence, cirrhosis, represent the final common pathway of almost all chronic liver diseases. Fibrosis and cirrhosis of the liver remain major medical problems with significant morbidity and mortality worldwide. Liver fibrosis is in fact a wound-healing response to liver injury and is characterized by accumulation of fibrotic scar tissue. Although the scar tissue formation is beneficial at first because it encapsulates the injury, the chronic activation of this healing process eventually progresses to advanced fibrosis/cirrhosis. This leads to altered vascular architecture and microcirculation, ischemia, and widespread hepatocyte cell death [143]. Also, in cirrhosis, collagen strands become so prevalent and divide the liver parenchyma into distinct structurally abnormal regenerative nodules, resulting in organ dysfunction [143].

In fact, liver damage leading to cirrhosis is the result of a complex mechanism involving, from direct toxic effects to a sustained inflammatory process, driving to the death of hepatocytes...
via apoptosis and liver fibrosis, mediated by secretion of several cytokines [144]. The inflammatory reaction is the coordinated process by which the liver responds to local insults, trying to restore the hepatic architecture and function after acute liver injury [128]. However, if the liver is faced to a sustained local damage, the chronic inflammatory response gives rise to a progressive replacement of healthy liver tissue by non-functional fibrotic scar tissue. The imbalance between tissue regeneration and fibrosis determines the outcome toward health recovery or liver cirrhosis [144].

6.1. Imbalance between extracellular matrix synthesis and degradation

Liver fibrosis can be defined as a dynamic and highly integrated molecular, tissue and cellular process regarded as the result of an imbalance between ECM synthesis and degradation. In the healthy liver, ECM is composed of several components such as collagens (mainly the interstitial types I, III, V, VI, and the basement membrane types IV, XV, XVIII, and XIX), glycoproteins (such as laminin isoforms and fibronectin), proteoglycans and elastin [145–147]. Normally, ECM components comprise less than 3% of the relative area of a liver tissue section and approximately 0.5% of the wet weight. During the development of liver fibrosis, there is a 5- to 10-fold increase in the content of collagenous and non-collagenous components, particularly of fibrillar collagen type I and III [146], and an increase of elastin, laminins, and proteoglycans [148]. The total amount of ECM is not only dependent on the rate of synthesis but also largely on the balance between the matrix MMPs, and the TIMPs, especially TIMP-1 [15].

The MMPs are a family of zinc-dependent endopeptidases that can degrade both collagenous and non-collagenous components of ECM in the extracellular space [149]. MMP activity is regulated by TIMPs, which bind to MMPs, blocking their proteolytic activity. The MMPs and TIMPs play a crucial role in the fine regulation of the ECM turnover and the resulting increase in the TIMPs/MMPs ratio in liver promotes fibrosis by protecting accumulated matrix from degradation by MMPs (Figure 1) [8].

6.2. Mechanisms and mediators of liver fibrogenesis

Liver fibrosis, which is characterized by the excessive deposition of ECM (non-functional connective tissue) components [150], involves both parenchymal and non-parenchymal cells, as well as infiltrating immune cells [151, 152]. Furthermore, several critical signaling pathways have important roles in liver fibrosis. The complex interactions between these signaling pathways and different cells contribute to the progression of liver fibrosis [153].

HSCs are central effectors of fibrogenesis although other cells and processes can make significant contributions. In the healthy liver, HSCs are in a quiescent state with low proliferation rates, store dietary vitamin A, control the ECM synthesis, regulate the local vascular contractility, and serve as the pericytes for the sinusoidal endothelial cells. Damage to hepatocytes activates HSCs transformation into myofibroblast-like cells that play a fundamental role in the development of fibrotic liver response [14]. Myofibroblast-like cells with high proliferative capacity, without vitamin A, exhibit increased expression of α-SMA fibers [3]. These cells contribute to fibrosis by producing large amounts of ECM components and collagens (specifically type I) to encapsulate
the injury [152]. Although HSCs are classically considered to be a major source of myofibroblasts [154, 155], other cell types like portal myofibroblasts and cells recruited from the bone marrow also contribute to the expansion of the myofibroblast population observed during the liver injury [154]. Activated HSCs also secrete an increased amount of MMPs and their inhibitors, TIMPs, which are necessary for the ECM remodeling [154, 156]. HSC activation leads to the up-regulation of TIMPs and TGF-β1 with the inhibition of MMP activity. The TIMP activation thus stimulates collagen type I synthesis and ECM deposition in the extracellular space [157]. Besides injured hepatocytes, hepatic macrophages (KCs), endothelial cells, and lymphocytes also drive HSC activation [158].

HSC activation is still the primary pathway leading to the liver fibrosis and it consists of two main stages: initiation and perpetuation (Figure 2) [126]. The initiation stage is related with the early changes in gene expression and phenotype that render the cells responsive to several cytokines and stimuli. Initiation of HSC activation is stimulated by several soluble factors such as oxidant stress signals (ROS), apoptotic bodies, and paracrine stimuli from neighboring cell types including hepatocytes, KCs, sinusoidal endothelium, and platelets [8, 72]. Hepatocytes
are believed to represent a major source of ROS as well as of other oxidative stress-related reactive mediators or intermediates [1]. Hepatocyte apoptosis leads to the release of cellular contents such as DNA and ROS that activate KCs to release pro-inflammatory (such as TNF-α, IL-1β, IL-6, MCP-1, TGF-β, etc.) and pro-fibrogenic (especially TGF-β) factors [158]. Hepatocyte apoptosis following injury also promotes initiation of HSC activation through a process mediated by Fas, and this process may involve the TRAIL [159]. After stimulation by cytokines or engulfment of apoptotic bodies, KCs stimulate matrix synthesis and cell proliferation through the actions of cytokines including TGF-β1 and ROS/lipid peroxides [64]. Endothelial cells are also likely to participate by conversion of TGF-β from the latent to the active, pro-fibrogenic form [126]. Platelets are another important source of paracrine stimuli, including PDGF, TGF-β1, and EGF [126]. On the other hand, perpetuation stage results from the effects of these stimuli on maintaining the activated phenotype and generating liver fibrosis. This stage involves...
autocrine as well as paracrine cycles. It includes HSC activation and related cellular changes such as proliferation, chemotaxis, fibrogenesis, contractility, and matrix degradation [126]. Activated HSCs proliferate in response to various kinds of cytokines, chemokines, and growth factors such as TGF-β, EGF, and PDGF [2, 8]. TGF-β, which has been identified as the most pro-fibrotic cytokine, promotes expression of collagen type I by activated HSCs and inhibits ECM degradation through the expression of TIMPs [160]. In parallel, PDGF has emerged as the most potent proliferative cytokine for HSCs [8]. Also, activated HSCs show chemotactic response, migrate toward damaged area and start to accumulate [3]. They express the cytoskeleton protein (α-SMA), equipping the cells with a contractile apparatus and collagens (especially type I) [12, 161, 162]. Thus, HSCs are able to constrict individual sinusoids as well as the entire fibrotic liver [3]. The net effect of these changes is to increase ECM deposition. In addition, cytokine release by HSCs can expand the inflammatory and fibrogenic tissue responses, and matrix proteases may hasten the replacement of normal matrix with fibrotic scar [126]. Briefly, activated HSCs are major effectors of liver fibrogenesis by integrating all incoming paracrine or autocrine signals released from both parenchymal and non-parenchymal cells (pro-inflammatory cytokines, growth factors, chemokines, ROS, and others).

Chronic inflammation and fibrosis are inseparably linked and the interactions between immune cells, local fibroblasts and especially subsets of macrophages determine the outcome of liver injury [8]. Macrophage phenotype and function are critical determinants of fibrotic scarring or resolution of injury. Macrophages, which are typically categorized into classically activated (M1) or alternatively activated (M2) phenotypes, play dual roles in the progression and resolution of liver fibrosis [163]. Typically, M1 macrophages play a pro-inflammatory role in liver injury and produce inflammatory cytokines, while M2 macrophages exert an anti-inflammatory role during tissue repair and fibrosis. The imbalance of M1 and M2 macrophages mediates the progression and resolution of liver fibrosis [164]. During the early stages of liver injury, bone marrow-derived monocytes are extensively recruited to the liver and then differentiate into inflammatory macrophages (mostly M1 macrophages) to produce pro-inflammatory and profibrotic cytokines, thereby promoting inflammatory responses and HSC activation. Afterwards, recruited macrophages switch their phenotypes (mostly M2 macrophages) to secrete MMPs for the successful resolution in hepatic scar [153, 165, 166]. Therefore, a complicated interplay between M1 and M2 types of macrophages plays a critical role in fibrogenesis [128].

**6.3. Liver fibrosis is potentially reversible**

Liver fibrosis is thought to be a potentially reversible condition if the cause of liver injury is removed (such as virus suppression or alcohol absence) (Figure 2). Regression of liver fibrosis is associated either with elimination of activated HSCs via apoptosis or senescence or with reversal of activated HSCs to a more quiescent phenotype. It has been shown that HSCs are sensitive to Fas and TRAIL-mediated apoptosis, and natural killer cells can induce apoptosis of HSCs by a TRAIL-mediated mechanism [167]. Similarly, TRAIL expressed by KCs is also thought to mediate HSC apoptosis [168]. In addition, apoptosis of activated HSCs is for sure followed by a decrease in collagen production as well as a reduction in TIMP synthesis with an increase in the hepatic MMP expression [1]. Therefore, activated HSCs, the primary source of ECM, are the most attractive target for reversing liver fibrosis [169].
7. Halofuginone

Halofuginone, a non-toxic and low molecular weight plant alkaloid [7-bromo-6-chloro-3-(3-hydroxy-2-piperidine)-2-oxopropyl-4(3H)-quinazoline] (Figure 3) isolated from the roots of Dichroa febrifuga (Chinese medicinal plant), is used worldwide as an anti-parasitic drug in commercial poultry production [16]. At first, halofuginone was identified as a potent inhibitor of collagen type α1 gene expression and ECM deposition. At present, it is being evaluated in clinical trial for Duchenne muscular dystrophy, in which fibrosis is the main complication.

7.1. Halofuginone and its effect on collagen synthesis

Halofuginone was found to be a potent inhibitor of collagen type α1 gene expression [17], which was demonstrated in a broad range of cell types including rat, mouse, chicken, and human, both in vitro and in vivo [16–20]. The discovery of the inhibitory effect of halofuginone on collagen synthesis and ECM deposition has led to intensive studies that were aimed to control many diseases associated with excessive collagen accumulation, such as pulmonary, pancreatic and renal fibrosis [21–23], scleroderma and chronic graft-versus-host disease [24], post-operative peritendinous and abdominal adhesions [25, 26], urethral and esophageal strictures [27, 28], wound repair [29], burn injury [30], renal injury [31, 32], injury-induced arterial intimal hyperplasia [33], colitis [34], and liver fibrosis and cirrhosis [35–39]. Inhibition is independent of the route of administration (intraperitoneally, administered locally, or given orally).

Halofuginone was found to inhibit collagen type I synthesis but not that of type II [17] or III [170] in vitro. The inhibitor effect of halofuginone on collagen α1 synthesis appears not to be a direct effect but rather dependent on new protein synthesis, because concurrent treatment of fibroblasts with protein synthesis inhibitors blocks the suppressive effect of halofuginone on collagen α1 mRNA gene expression [18].

Because of the significant impact of fibrosis on human health, there is an unmet need for safe and effective therapies that directly target fibrosis. In animal models of fibrosis, regardless of the tissue, halofuginone had a minimal effect on collagen levels in the control (non-fibrotic) animals; however, it displayed a strong inhibitory effect in the fibrotic organs. This suggests that the regulation of the low-level expression of collagen type I genes differs from that of the

Figure 3. Chemical structure of halofuginone.
overexpression induced by the fibrogenic stimulus, which is usually an aggressive and rapid process [171]. Halofuginone mainly affects the stimulated collagen synthesis, therefore, when it is administered systemically, it is actually targeted to the desired fibrotic location without affecting collagen synthesis in other regions.

7.2. Halofuginone and TGF-β pathway

TGF-β is a “master switch” in chronic liver disease, being involved in all stages of the disease progression, from initial liver injury, inflammation, fibrosis, to cirrhosis and hepatocellular carcinoma at the end [172]. TGF-β signals through transmembrane receptor serine/threonine kinases to activate novel signaling intermediates called Smad proteins, which then modulate transcription of target genes [173]. TGF-β, signaling via Smad3, is the most pro-fibrogenic cytokine present in the liver and the major promoter of ECM synthesis [173, 174]. It induces pro-fibrotic cellular and transcriptional responses such as induction of the synthesis of ECM components, especially collagen, as well as fibronectin and laminin, and it inhibits the matrix degradation enzymes [175]. In various experimental fibrotic models, no effect of halofuginone was observed on the expression of the TGF-β receptors gene or on TGF-β levels [176–178]. This finding supports the hypothesis that the halofuginone target is down-stream in the TGF-β pathway. Halofuginone is an inhibitor of Smad3 phosphorylation down-stream of the TGF-β signaling pathway [177, 179, 180]. In chemically induced liver fibrosis, halofuginone affects TGF-β regulated genes through inhibition of Smad3 phosphorylation of activated HSCs [181]. It inhibits TGF-β-induced phosphorylation of Smad3 and also increases the expression of the inhibitory Smad7 in several cell types (such as fibroblasts, hepatic and pancreatic stellate cells, tumor cells and myoblasts) [178, 181–183]. The inhibition of Smad3 phosphorylation is associated with the halofuginone-dependent activation of Akt MAPK/ERK and p38 MAPK phosphorylation [182]. Thus, drugs that selectively target individual signaling pathways down-stream of the TGF-β receptor are likely to be more successful.

7.3. Halofuginone affects pre-existing fibrosis

Halofuginone affects fibrosis as a preventive agent when it was administered before or together with the fibrotic stimulus [21, 26, 27, 35, 184]. It can elicit resolution of established fibrosis, a capability that sets it apart from all other preventive anti-fibrotic agents. For example, in rats with established thioacetamide-induced liver fibrosis, addition of halofuginone to the diet results in almost complete resolution of the fibrotic condition as measured by hydroxyproline levels in the liver [36]. This is probably due to up-regulation of the collagen degradation pathway by inhibition of the TIMP-1, and activation of MMPs [43]. In addition, halofuginone given orally before fibrosis induction prevents the activation of most of the stellate cells and the remaining cells expressed low levels of collagen α1 gene, resulting in low levels of collagen [36]. Furthermore, halofuginone administration in low concentrations prior to and following partial hepatectomy in cirrhotic rats does not inhibit normal liver regeneration, despite the reduced levels of collagen type I mRNA [37]. When given to rats with established fibrosis, halofuginone causes significant reductions in α-SMA, TIMP-2, collagen type I gene expression, and collagen accumulation [37]. These animals demonstrate improved capacity for regeneration, suggesting the possible beneficial use of halofuginone before and during fibrotic/cirrhotic liver regeneration.
7.4. Halofuginone as an anti-fibrotic agent

In recent years, much attention was focused on halofuginone against liver fibrosis (Table 3). Although the exact anti-fibrotic mechanism of halofuginone is not well understood, it is found to be associated with inhibition of TGF-β signaling [179], which is known to inhibit mesangial

<table>
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<td>DMN-induced liver fibrosis/cirrhosis in rats</td>
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<td>Prevents increase in collagen type I gene expression</td>
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<td>TAA-induced liver fibrosis in rats</td>
<td>Causes almost complete resolution of fibrosis</td>
<td>Reduces collagen levels, collagen α1(I) gene expression, TIMP-2 content, and SMA-positive cells</td>
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<tr>
<td>TAA-induced liver cirrhosis in rats</td>
<td>Improves liver regeneration</td>
<td>Reduces α-SMA, TIMP-2, collagen type I gene expression, and collagen accumulation</td>
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<td>ConA-induced liver fibrosis in rats</td>
<td>Prevents liver fibrosis</td>
<td>Decreases Th17 cell differentiation and its related cytokines production</td>
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<td>Attenuates liver fibrosis</td>
<td>Suppresses synthesis of collagen 1, α-SMA and TIMP-2; down-regulates TGF-β1/Smad3 signaling pathway; decreases pro-inflammatory cytokines</td>
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<td>TAA-induced liver fibrosis in rats</td>
<td>Up-regulates MMP-3 and TIMP-1 (in vivo); inhibits HSC proliferation and migration (in vitro)</td>
<td>Activates p38 MAPK and NF-κB</td>
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<td>TAA-induced liver fibrosis in rats</td>
<td>Inhibits HSC activation and collagen synthesis; prevents activation of TGF-β-dependent genes</td>
<td>Inhibits Smad3 phosphorylation</td>
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Table 3. Effects of halofuginone in various experimental liver diseases.
cell proliferation and ECM deposition [185]. In several animal models of fibrosis, in which excess collagen is the characteristic of the disease, halofuginone prevents transition of the fibroblasts to myofibroblasts by inhibition of Smad3 phosphorylation down-stream of the TGF-β signaling pathway [186, 187], thereby inhibits collagen synthesis [186]. Halofuginone also regulates cell growth and differentiation, apoptosis, cell migration, and immune cell function [41]. It prevents concanavalin A-induced liver fibrosis by affecting Th17 cell differentiation, which suggests a direct link between the myofibroblasts/fibrosis pathway and the Th17 pro-inflammatory pathway [38]. Th17 cells, a distinct subset of CD4+ T cells with IL-17 as their major cytokine, orchestrate the pathogenesis of inflammation [171]. It has been suggested that halofuginone-dependent inhibition of fibrosis includes selective inhibition of the Th17 cell development by activating the amino acid starvation response [188, 189]. Halofuginone activates the amino acid starvation response by directly inhibiting the prolyl-tRNA synthetase activity of glutamyl-prolyl-tRNA synthetase [190]. Furthermore, addition of exogenous proline reverses a broad range of halofuginone-induced cellular effects, indicating that glutamyl-prolyl-tRNA synthetase-inhibition underlies the therapeutic activities of halofuginone [190].

TGF-β is required for facilitation of differentiation of the inflammatory Th17 cell subset [191], which suggests the presence of a connection between the TGF-β signaling inhibition and the amino acid starvation response [187]. Treatment with halofuginone also effectively inhibits the delayed-type hypersensitivity response, indicating suppression of T cell–mediated inflammation in vivo [42]. Moreover, it was shown that halofuginone is a potent inhibitor of NF-κB, pro-inflammatory cytokines, and p38 MAPK phosphorylation in activated T cells in vitro [42]. Also, submicromolar concentrations of halofuginone inhibit HSC proliferation and migration and up-regulate their expression of fibrolytic MMP-3 and -13 via activation of p38 MAPK and NF-κB. The remarkable induction of MMP-3 and -13 makes halofuginone a promising agent for anti-fibrotic combination therapies [43]. Halofuginone also affects the cross-talk between the hepatocytes and the HSCs by up-regulating the synthesis and secretion of insulin-like growth factor binding protein-1 (IGFBP-1), which inhibits HSC migration [192]. It also affects the expression of early genes of liver regeneration, IGFBP-1 whose synthesis and secretion is regulated in part by TGF-β [192] and tyrosine phosphatase (PRL-1) whose synthesis is regulated by transcription factor early growth response-1 (Egr-1) probably via TGF-β [193].

7.5. Anti-tumoral role of halofuginone

In many types of tumor, there is a strong relationship between tissue fibrosis and increased risk of tumor development. For example, the leading risk factor for hepatocellular carcinoma is liver cirrhosis, and its associated inflammation, regeneration, and fibrosis [194, 195]. Tumor cells develop and metastasize more effectively in fibrotic tissues; therefore, any reduction in tissue fibrosis reduces the risk of cancer [171]. Halofuginone reduces tumor growth and mortality in xenograft mice implanted with human hepatoma cells [196]. In diethylnitrosamine and N-nitosomorpholine-induced, spontaneously metastasizing hepatocellular carcinoma, halofuginone suppresses lung metastasis in rats through MMP inhibition [197]. Moreover, halofuginone treatment results in effective inhibitory effects on the cascade of events leading to angiogenesis (formation of new blood vessels), such as abrogation of endothelial cell MMP-2 expression, basement membrane invasion, capillary tube formation, vascular sprouting, and
deposition of sub-endothelial ECM in vitro [171]. Inhibition of angiogenesis is mostly accompanied by inhibition of the fibroblasts to myofibroblasts transition, reduction in tumor stroma ECM, and inhibition of tumor growth [171]. The high effectiveness of halofuginone in reducing fibrosis, which affects tumor growth and tissue regeneration in the liver, arises from its dual role in inhibiting the TGF-β signaling and Th17 cell development [187].

8. Conclusion

Fibrosis is a pathological process associated with excessive ECM deposition that leads to destruction of organ architecture and function. Fibrosis contributes enormously to deaths worldwide; thus, effective therapies are of a great need. Halofuginone has great potential as an anti-fibrotic therapeutic. Systemic administration of halofuginone in animal models and humans is well tolerated [24]. Additionally, in most animal models of fibrosis, halofuginone has a minimal effect on collagen levels in non-fibrotic animals, while exerting strong inhibitory effects in fibrotic organs. It mainly affects stimulated collagen synthesis without altering the usual low physiological level of collagen expression. Because halofuginone inhibits collagen type I synthesis on the transcriptional level and reduces ECM deposition, it is a promising candidate for treatment of diseases associated with excessive ECM, such as liver fibrosis/cirrhosis. Thus, halofuginone meets the criteria as a promising anti-fibrotic drug for further evaluation in the treatment of liver fibrosis/cirrhosis.

Conflicts of Interest

The author reports no conflicts of interest.

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