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# Dihydroartemisinin alleviates bile duct ligation-induced liver fibrosis and hepatic stellate cell activation by interfering with the PDGF-βR/ERK signaling pathway

Qin Chen [a], Lianyun Chen [a], Desong Kong [a,d], Jiangjuan Shao [a], Li Wu [a], Shizhong Zheng [a,b,c,*]

[a] Department of Pharmacology, School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, Jiangsu Province, China
[b] National First-Class Key Discipline for Traditional Chinese Medicine of Nanjing University of Chinese Medicine, Nanjing, China
[c] Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Material Medical, Nanjing University of Chinese Medicine, Nanjing, China
[d] Department of Science, Technology and Education, the Third Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210001, China

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ABSTRACT

Liver fibrosis represents a frequent event following chronic insult to trigger wound healing responses in the liver. Activation of hepatic stellate cells (HSCs), which is a pivotal event during liver fibrogenesis, is accompanied by enhanced expressions of a series of marker proteins and pro-fibrogenic signaling molecules. Artemisinin, a powerful antimalarial medicine, is extracted from the Chinese herb *Artemisia annua L.*, and can inhibit the proliferation of cancer cells. Dihydroartemisinin (DHA), the major active metabolite of artemisinin, is able to attenuate lung injury and fibrosis. However, the effect of DHA on liver fibrosis remains unclear. The aim of this study was to investigate the effect of DHA on bile duct ligation-induced injury and fibrosis in rats. DHA improved the liver histological architecture and attenuated collagen deposition in the fibrotic rat liver. Experiments in vitro showed that DHA inhibited the proliferation of HSCs and arrested the cell cycle at the S checkpoint by altering several cell-cycle regulatory proteins. Moreover, DHA reduced the protein expressions of a-SMA, α1 (I) collagen and fibronectin, being associated with interference of the platelet-derived growth factor β receptor (PDGF-βR)-mediated ERK pathway. These data collectively revealed that DHA relieved liver fibrosis possibly by targeting HSCs via the PDGF-βR/ERK pathway. DHA may be a therapeutic antifibrotic agent for the treatment of hepatic fibrosis.



## 1. Introduction

Hepatic fibrosis is a wound healing response to a variety of chronic stimuli, resulting in excessive production and deposition of extracellular matrix (ECM) in the liver [1,2]. Activation of hepatic stellate cell (HSC) is a well-accepted critical event in hepatic fibrosis and an attractive target for treatment [3]. HSC is activated upon liver injury, with changes in gene expression and major phenotypic transformation to α-smooth muscle actin (α-SMA)-positive myofibroblast that increases cell proliferation and produces large amounts of ECM including collagen I [4,5]. Some receptors for growth factors and their signal transductions are central to the pathogenesis of fibrosis and HSC activation. For example, accumulating evidence has indicated that platelet-derived growth factor-β receptor (PDGF-βR)-mediated ERK mitogen-activated protein kinase (MAPK) signaling stimulates collagen I gene expression in activated HSCs [6,7]; and many studies have also linked MAPK pathways to cell proliferation in the pathogenesis of liver fibrosis [8,9]. Fibrotic diseases can be effectively treated by inhibiting these signal transductions.

Although advances in understanding HSC activation have been impressive during the last decade, there are few breakthroughs in the therapeutic intervention of liver fibrosis. Therefore, it is urgent to identify antifibrotic agents that are innocuous. Most evolving antifibrogenic therapies are aimed at inhibiting HSC activation. Derived from a traditional Chinese herb, artemisinin is used for the treatment of fever and malaria [10]. Due to very low solubility of the natural compound, a number of derivatives have been synthesized, including artesunate, artemether, and dihydroartemisinin (DHA). DHA (Fig. 1), the major active metabolite of artemisinin, is a safe and effective antimalarial drug that has good absorption, wide distribution, and rapid metabolism [11,12]. Besides resisting malaria, DHA is also capable of anti-inflammation, immune regulation, and scar restraint [13–15]. Furthermore, DHA is able to alleviate lung injury and fibrosis [16]. However, its role in liver fibrosis remains unclear. This study evaluates the in vivo protective effects of DHA on liver injury and fibrogenesis caused by bile duct ligation (BDL) in a rat model and further explored the

* Corresponding author at: Department of Pharmacology, College of Pharmacy, Nanjing University of Chinese Medicine, 138 Xianlin Avenue, Nanjing 210023, Jiangsu, China.
*E-mail address:* nytws@163.com (S. Zheng).

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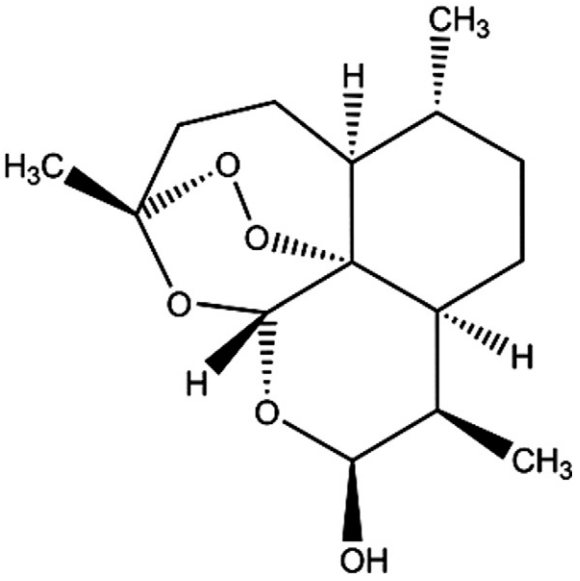


**Fig. 1.** Chemical structure of DHA.

underlying mechanisms. DHA protected the liver from BDL-induced injury and fibrogenesis, and inhibited the proliferation and fibrotic protein synthesis of HSCs. Disruption of the PDGF-βR/ERK pathway mediated DHA-inhibited HSC activation. The results provide meaningful indicators for clinical application of DHA in resisting hepatic fibrosis.

## 2. Materials and methods

### *2.1. Reagents and antibodies*

DHA was purchased from Nanjing Dizhao Biological Technology Co., Ltd. (Jiangsu, China). Acetaminophen (N-acetyl-p-aminophenol, APAP) was obtained from Xi'an Helin Biological Engineering Co., Ltd. (Xi'an, China). The MEK inhibitor U0126 was purchased from Cell Signaling Technology (Danvers, MA, USA). PDGF-βR inhibitor imatinib was obtained from Nanjing EnoGene Biotechnology (Nanjing, China). All these compounds were dissolved in dimethylsulfoxide (DMSO; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for experiments. Primary antibodies against α-SMA, α1 (I) procollagen, fibronectin, TGF-βRII, p53, PPARγ and TIMP-1 were purchased from Epitomics (San Francisco, CA, USA). Primary antibodies against CDK2, cyclin A, cyclin E, p-ERK, ERK, p-JNK, JNK, p-p38, and p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against p21, TGF-βRI, PDGF-βR and EGFR were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody against β-actin was purchased from Sigma (St. Louis, MO, USA).

### *2.2. Animal procedures and treatments*

All experimental procedures were approved by the institutional and local committee on the care and use of animals of Nanjing University of Chinese Medicine (Nanjing, China), and all animals received humane care according to the National Institutes of Health (USA) guidelines. Male Sprague-Dawley rats (180–220 g) were obtained from Nanjing Medical University (Nanjing, China). All animals were housed in cages (two rats per cage) for 7 days. The light cycle was controlled to provide 12 h light and 12 h darkness; temperature was 22 °C and humidity was 40–60%. Forty-eight rats were randomly divided into six groups (eight rats per group). Group 1 was the vehicle control in which rats did not receive BDL or DHA treatment. Group 2 was the sham group without DHA treatment. Group 3 was the BDL group without DHA treatment. Groups 4, 5, and 6 were treatment groups in which rats received BDL and intraperitoneal injection of DHA at 3.5, 7, and 14 mg/kg, respectively. Groups 3–6 were bile duct ligated for 6 weeks. After BDL for 14 days, groups 4–6 were intraperitoneally injected with DHA five times a week for 3–6 weeks. DHA was dissolved in DMSO. At the end of the experiment, the rats were sacrificed after being anesthetized by i.p. pentobarbital (50 mg/kg). Blood was collected, and livers were isolated. A small portion of the liver was removed for histopathological and immunohistochemical studies by being fixed with 10% formalin and subsequently embedded with paraffin. The remaining liver was cut in pieces and rapidly frozen with liquid nitrogen to extract hepatic proteins.

BDL was performed using a standard technique according to a described protocol [17]. Briefly, the rats were anesthetized with chloral hydrate [0.3 g, intraperitoneally (i.p.)]. After midline abdominal incision, the ventral side of the liver was lifted so that it could stick to the diaphragm and the hepatic hilum became clearly visible. The bile duct was separated carefully from the flanking portal vein and hepatic artery using a micro-serration forceps. Subsequently, a suture was placed around the bile duct and secured with a surgical knot. Thereafter, a second suture was placed in close proximity to the first suture and knotted around the bile duct. The suture was shortened and all organs replaced to their physiological position. The sham operation was performed similarly without BDL.

### *2.3. Liver histopathology*

Harvested liver tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Liver sections of 5 μm thickness were prepared and stained with hematoxylin and eosin and Masson's trichrome stain by using standard methods. For sirius red collagen staining, thin sections were deparaffinized and stained with picro-sirius red for 1 h at room temperature. After being washed, sections on the slides were dehydrated in 100% ethanol and in xylene, and then they were mounted in Permount. Photographs were taken in a blinded fashion at random fields. Representative views of the liver sections are shown.

### *2.4. Hydroxyproline examination*

The hydroxyproline levels in liver tissue and blood were determined by using a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocol. Briefly, three small pieces of liver tissues randomly excised from the liver of every rat were hydrolyzed in 6 N HCl at 110 °C for 24 h, and subsequently they were neutralized with NaOH. Isopropanol in citrate acetate-buffered chloramine T was added to aliquots of the hydrolysate, followed by the addition of Ehrlich reagent. The chemical reaction occurred in dark for 25 min at 60 °C. After centrifugation, absorbance of the supernatant of each sample was read at 558 nm by using a 96-well plate spectrometer. *Trans*-hydroxyproline was used as the standard for quantification. Values were normalized to control.

### *2.5. Cell isolation and culture conditions*

Primary HSCs were isolated from male Sprague–Dawley rats as we previously described in detail [18]. Primary rat HSCs (HSCs) were cultured in DMEM (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Sijiqing Biological Engineering Materials, Hang Zhou, China), 1% antibiotics, and maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. HSCs at passages 2 to 4 were used in experiments. Human HSCs cell line LX-2 was purchased from The Cell Bank of Chinese Academy of Sciences (Shanghai, China) and was maintained under the conditions described above. Cell morphology was assessed using an inverted microscope with a Leica Qwin System (Leica, Germany).