Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

Association for Academic Surgery

Antifibrosis effect of novel oridonin analog CYD0618 via suppression of the NF-κB pathway

Claire B. Cummins, MD,[a] Xiaofu Wang, BS,[a] Jimin Xu, PhD,[b] Byron D. Hughes, MD, MPH,[a] Ye Ding, PhD,[b] Haiying Chen, BS,[b] Jia Zhou, PhD,[b,1] and Ravi S. Radhakrishnan, MD, MBA[a,*,1]

[a] Department of Surgery, University of Texas Medical Branch, Galveston, Texas
[b] Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas

ARTICLE INFO

Article history:
Received 28 February 2018
Received in revised form
7 May 2018
Accepted 14 June 2018
Available online xxx

Keywords:
Liver fibrosis
Therapeutics
Oridonin
NF-κB pathway
Hepatic stellate cells

ABSTRACT

Background: Liver fibrosis is characterized as excessive deposition of the extracellular matrix proteins, primarily by activated hepatic stellate cells (HSCs). NF-κB has been reported as one of the major mediators of HSC activation. Previously, our team reported that oridonin exhibited antihepatic fibrogenetic activity *in vitro*. In this study, we examined the effects of its novel derivative CYD0618 on HSC viability, apoptosis, and NF-κB signaling.
Methods: Cell proliferation of activated human and rat HSC lines LX-2 and HSC-T6 was measured using Alamar Blue Assay. Apoptosis was measured by a Cell Death Detection ELISA kit. Cellular proteins were determined by Western blots and immunofluorescence.
Results: CYD0618 significantly inhibited LX-2 and HSC-T6 cell proliferation in a dose-dependent manner. CYD0618 induced cell apoptosis in both cell lines. CYD0618 treatment increased cell cycle inhibitory protein p21, p27, and induced apoptosis marker cleaved poly (ADP-ribose) polymerase, while suppressing the expression of Collagen type 1. CYD0618 blocked lipopolysaccharide (LPS)-induced NF-κB p65 nuclear translocation and DNA binding activity and prevented LPS-induced NF-κB inhibitory protein IκBα phosphorylation and degradation. LPS-stimulated NF-κB downstream target cytokines IL-6 and MCP-1 were attenuated by CYD0618. Endogenous and LPS-stimulated NF-κB p65 S^{536} phosphorylation was inhibited by CYD0618 treatment.
Conclusions: The potent antihepatic fibrogenetic effect of CYD0618 may be mediated via suppression of the NF-κB pathway.

Introduction

Hepatic fibrosis is a wound-healing response that is associated with the sustained inflammatory signals of chronic liver disease.[1,2] Hepatic fibrosis typically evolves over decades and can ultimately lead to its most advanced form, cirrhosis.[1] Hepatic cirrhosis is a significant global disease, estimated to have caused over a million deaths in 2010, and is caused by a

Presented at the Academic Surgical Congress Meeting, Jacksonville, Florida, January 30-February 1, 2018.

* *Corresponding author*. Department of Surgery, The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0353. Tel.: +1 409 772 5666; fax: +1 409 772 4253.
E-mail address: rsradhak@utmb.edu (R.S. Radhakrishnan).
[1] Co-Corresponding authors.

0022-4804/$ – see front matter © 2018 Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.jss.2018.06.040

wide range of etiologies.[3] Although traditional treatments have focused on reducing the exposure to etiological agents, more recent research has been focused on finding agents that prevent the progression of fibrosis to cirrhosis or induce regression of advanced fibrosis.[4]

NF-κB has been proposed to be the central link between hepatic injury, fibrosis, and hepatocellular carcinoma, and it represents a target for the prevention or treatment of liver fibrosis.[5] NF-κB plays an important role in the suppression of apoptosis and is necessary for normal hepatic development and homeostasis.[6] Blockage of the NF-κB pathway is sufficient to increase the rate of apoptosis in hepatic stellate cells (HSCs).[7] Initiation of the classic NF-κB pathway leads to activation of the IκB kinase (IKK) complex, which results in phosphorylation and proteasome-dependent degradation of NF-κB inhibitor IκBα, nuclear translocation of NF-κB dimers (e.g., p50/p65), and ultimately synthesis of antiapoptotic factors.[6,8] In an alternative pathway for NF-κB activation, phosphorylation of NF-κB subunit p65 plays an important role in modulating transcriptional activity of NF-κB independent of IκB proteins and phosphorylation specific to the serine 536 (S^{536}) site has been shown to alter the association with basal components of the transcriptional machinery.[9,10]

Previous studies from our group have demonstrated that oridonin, an active compound isolated from *Rabdosia rubescens*, has potent antihepatic fibrogenetic effects by decreasing HSC viability and inducing HSC apoptosis.[11] *Rabdosia rubescens* has been used as a traditional medicine for the treatment of various cancers and inflammatory disorders.[12-15] *In vivo* studies have demonstrated that oridonin has protective effects against acute liver injury in mice.[16] In addition, several studies have shown that oridonin affects glutathione depletion, reactive oxygen species, and the NF-κB pathway.[17,18] However, oridonin has poor aqueous solubility and bioavailability, and its short biological half-life presents obstacles for its clinical use.[19] To this end, novel derivatives of oridonin have been developed and demonstrated enhanced antihepatic fibrogenetic effects.[20,21] CYD0618, a novel oridonin analog, was chosen for further study due to its enhanced potency and aqueous solubility. We hypothesized that CYD0618 will potently inhibit hepatic fibrogenetic activity through suppression of both the classic and alternative NF-κB signaling pathways.

Methods

Reagents

All cell culture mediums and trypsin were purchased from Life Technology Corp. (Carlsbad, CA). Oridonin and Bay-11-7082 were purchased from Sigma–Aldrich Co, LLC. (St. Louis, MO). CYD0618 is a newly designed nitrogen-enriched oridonin analog with a thiazole ring fused at C-1 and C-2 of the A-ring, resulting in significantly improved aqueous solubility (Fig. 1). CYD0618 was synthesized following our previously reported protocols.[22,23] All experiments were conducted within a biosafety level 2 laboratory in full compliance with the University of Texas Medical Branch Biological Safety Committee.

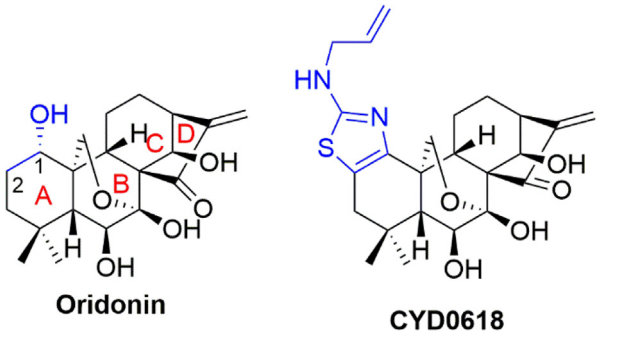

Fig. 1 – Chemical structures of oridonin and its new analog CYD0618. (Color version of figure is available online.)

Cell culture

The human immortalized HSC line LX-2 and rat immortalized HSC line HSC-T6 were a gift from Dr Scott Friedman (Mount Sinai Medical Center, New York) and cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium with a high glucose concentration (4.5 g/L) supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin. All experiments were performed on cells within 6 wk of culture from liquid nitrogen. Human and rat cell lines were used to ensure that these effects were not limited to a single cell line.

Cell viability assay

Cell viability was assessed using Alamar Blue assay (Cat#-Dal1025) purchased from Life Technologies (Grand Island, NY) by following the manufacturer's instructions. Fluorescence intensity was monitored using a SpectraMax M5 microplate reader from Molecular Devices, LLC (Sunnyvale, CA) with excitation and emission wavelengths set at 544 and 590 nm, respectively. Assay was performed in triplicate and repeated at least three times.

Cell death detection ELISA assay

A total of 8×10^3 cells/well were seeded into 96 well plates. The next day, after reaching 70%-80% confluence, cells were replaced with fresh complete medium and treated as indicated. Apoptosis was determined using a Cell Death Detection ELISA kit (product # 11 774 425 001) from Roche Diagnostic Corp. (Indianapolis, IN) by following the manufacturer's protocol. Assay was performed in duplicate and repeated twice.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described[24] with anti-NF-κB p65 (Cat#8242) from Cell Signaling Technology Inc, (Danvers, MA). After the indicated treatments and staining, the cells were visualized by the Nikon Eclipse Ti confocal microscope at 20× magnification (Nikon Instruments Inc, Melville, NY).