Cyclin-dependent kinase inhibitor roscovitine attenuates liver inflammation and fibrosis by influencing initiating steps of liver injury

Yanjun Liu1#, Jiacheng Li2,3#, Liping Liao2,3#, Heming Huang1, Shijie Fan2,3, Rong Fu1,2, Jing Huang2,3, Cuicui Shi1, Liang Yu2, Kai-xian Chen2,3, Yuan-yuan Zhang*, Cheng Luo*, Guang-ming Li*

1Department of Gastroenterology, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, 1665 Kongjiang Road, Shanghai 200092, China

2Drug Discovery and Design Center, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China

3University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, China

#These authors contributed equally to this work.

*Correspondence:
Guangming Li, Department of Gastroenterology, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, 1665 Kongjiang Road, Shanghai 200092, China. Email: liguangming@xinhuamed.com.cn; Phone: +86-21-25077445
Cheng Luo, Drug Discovery and Design Center, State Key Laboratory of Drug
Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China. Email: cluo@simm.ac.cn, Phone: +86-21-50806918

Yuanyuan Zhang, Drug Discovery and Design Center, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China. Email: zhangyy@simm.ac.cn, Phone: +86-21-50806600

Abbreviations:

| Abbreviation | Description |
|--------------|-------------|
| ALT          | Alanine aminotransferase |
| AST          | Aspartate aminotransferase |
| CDK          | Cyclin-dependent kinase |
| DMEM         | Dulbecco’s modified eagle medium |
| ECM          | Extracellular matrix |
| ERK          | Extracellular regulated protein kinase |
| FBS          | Fetal bovine serum |
| GSEA         | Gene set enrichment analysis |
| HCC          | Hepatocellular carcinoma |
| H&E          | Hematoxylin-eosin |
| HSC          | Hepatic stellate cell |
| IHC          | Immunohistochemistry |
| JNK          | c-Jun N-terminal kinase |
| LPS          | Lipopolysaccharide |
| MAPK         | Mitogen-activated protein kinase |
| NF-kB        | Nuclear factor kappa B |
| PBS          | Phosphate buffered saline |
| RIN          | RNA Integrity Number |
| RNA-seq      | RNA sequencing |
| RT-PCR       | Real-time polymerase chain reaction |
| Ser2         | Serine 2 |
| Ser5         | Serine 5 |
| TUNEL        | Terminal deoxynucleotidyl transferase dUTP nick-end labeling |

Abstract
Liver diseases present a significant public health burden worldwide. Although the mechanisms of liver diseases are complex, it is generally accepted that inflammation is commonly involved in the pathogenesis. Ongoing inflammatory responses exacerbate liver injury, or even result in fibrosis and cirrhosis. Here we report that roscovitine, a cyclin-dependent kinase inhibitor, exerts beneficial effects on acute and chronic liver inflammation as well as fibrosis. Animal models of lipopolysaccharide/D-galactosamine- and acute or chronic CCl₄-induced liver injury showed that roscovitine administration markedly attenuated liver injury, inflammation and histological damage in lipopolysaccharide/D-galactosamine- and CCl₄-induced acute liver injury models, which is consistent with the results in vitro. RNA-seq analysis showed that roscovitine treatment repressed the transcription of a broad set of pro-inflammatory genes involved in many aspects of inflammation, including cytokine production and immune cell proliferation and migration, and inhibited the TGF-β signaling pathway and the biological process of tissue remodeling. For further validation, the beneficial effect of roscovitine against inflammation was evaluated in chronic CCl₄-challenged mice. The anti-inflammation effect of roscovitine was observed in this model, accompanied with reduced liver fibrosis. The anti-fibrotic mechanism involved inhibition of profibrotic genes and blocking of hepatic stellate cell activation. Our data show that roscovitine administration protects against liver diseases through inhibition of macrophage inflammatory actions and hepatic stellate cell activation at the onset of liver injury.

**Key words:** CDK inhibitor, roscovitine, liver inflammation, fibrosis

Clinical perspectives: 1) The incidence of liver diseases has been increasing in recent years. Inflammation serves as an essential driving factor of the pathogenesis of liver diseases progression. 2) In this study, roscovitine alleviated liver inflammation and hepatocyte injury in LPS/GalN- and CCl₄-induced acute liver injury models, accompanied with anti-fibrotic
potency presented in the chronic liver injury animal model. The underlying mechanism involved NF-κB and MAPK pathway inhibition, repression of pro-inflammatory gene transcription and suppression of profibrotic genes and hepatic stellate cell activation. 3) These data suggest that roscovitine alleviates liver injury via inhibition of macrophage inflammatory actions and hepatic stellate cell activation, and provides evidence that evidence that therapeutic targeting of these driving factors by pharmacological inhibition of cyclin dependent kinases could be used as effective strategies for the treatment of inflammation- or fibrosis-involved hepatic diseases.

1. Introduction

Liver, as the central metabolic organ, is constantly exposed to pathogen-derived molecules, as a result of which it must remain in an immune tolerant condition to sustain homeostasis [1, 2]. Injury to the liver, regardless of etiology, results in the disturbance of immune homeostasis and involves multiple cell types including hepatocyte, macrophages (Kupffer cells) and hepatic stellate cells (HSCs). Subsequent activation of signaling cascades initiates hepatic inflammation and tissue repair mechanisms. In pathological conditions, abnormal inflammatory responses can be fulminant or perpetual and result in various liver diseases including acute liver failure, steatohepatitis and liver fibrosis. Inflammatory cell infiltration is commonly involved in acute liver injury. Repetitive and/or sustained inflammation is an essential initiation factor leading to liver fibrosis, in which HSC activation and extracellular matrix (ECM) deposition cause hepatic tissue remodeling and fibrosis, or even promoting the development of cirrhosis and hepatocellular carcinoma (HCC) [3-5]. Therefore, the inhibition and treatment of uncontrolled liver inflammation is a crucial step in the treatment of liver diseases.

Cyclin-dependent kinases (CDKs) are serine/threonine protein kinases and are key regulators of the cell cycle and proliferation via regulation of phosphorylation state of various substrates involved in DNA replication and cell division [6]. Abnormal CDK activity and regulation are
commonly observed in proliferative diseases, infections and neurodegenerative disorders. Therefore, CDKs have been of great interest as essential therapeutic targets for various diseases, including breast cancer, non-small cell lung cancer, acute kidney injury, and Alzheimer’s disease [7-10]. Over the past decade, the broad-range purine analog CDK inhibitor roscovitine, also known as seliciclib or CY202, has been shown to exert promising inhibitory effect on various human cancers and has entered phase I and II clinical trials against multiple indications [11]. Apart from its potency against cancer, roscovitine also has anti-inflammatory properties. Roscovitine was proven effective in several in vivo models of inflammation including acute pleurisy, lung inflammation, and arthritis through the promotion of inflammation resolution and inhibition of proinflammatory cytokines transcription in macrophages [12-14]. The anti-inflammatory actions of roscovitine are thought to be due to its influence on immune cells: roscovitine promotes neutrophils apoptosis, blocks leukocyte extravasation and can downregulate the expression of pro-survival protein myeloid cell leukemia-1 at the transcription level [15]. Moreover, roscovitine has potential therapeutic effects in the treatment of cystic fibrosis, systemic sclerosis, and tubulointerstitial fibrosis [16-18]. However, the effect of roscovitine on liver inflammatory diseases and fibrosis remains to be elucidated.

In this study, we identified that the CDK inhibitor roscovitine alleviates hepatic inflammation and fibrosis by inhibiting macrophage proinflammatory cytokine production, downregulating the TGF-β pathway and genes involved in inflammatory signaling pathways and leukocyte proliferation and infiltration, and suppressing profibrotic genes and HSC activation. These results indicate that roscovitine exerts protective effects in the onset of liver injury via suppression of macrophage inflammatory activities and HSC activation, and provides evidence that pharmacological inhibition of CDKs could be used as anti-inflammatory or anti-fibrotic therapies and may benefit patients with related liver diseases.

2. Materials and methods

2.1 Animal experiments
Male C57BL/6J mice, aged 8-10 weeks and weighing 21-23g, were obtained from the Shanghai Institute of Materia Medica Animal Center (Shanghai, China). All mice were housed in a pathogen-free facility at 21±2°C with 12-h light-dark cycles and free access to regular chow and tap water. They were acclimated to the laboratory environment for 1 week prior to use. All animal experiments were approved by the Institute Animal Care and Use Committee and were performed at the Shanghai Institute of Materia Medica Animal Center at Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China).

2.1.1 LPS/GalN-induced liver injury model

After 1 week of acclimation, 24 male mice were randomly divided into 3 groups (n=8 per group): control group, mice only received the vehicle (0.9% saline); Lipopolysaccharide (LPS)/GalN group, mice received intraperitoneal injection (i.p.) of LPS (Escherichia coli, 0111:B4, Sigma, 2 mg/kg) and GalN (Sigma, 250 mg/kg) dissolved in phosphate buffered saline (PBS)[19]; and roscovitine treated group, mice were treated with roscovitine (10mg/kg, i.p.) 24 h and 2 h prior to LPS/GalN administration. Mice were sacrificed 4 h after the LPS/GalN challenge. To investigate the effect of roscovitine on LPS/GalN-induced lethality, a separate set of mice were allocated and treated identically (n=10 in normal control group, n=12 in model group, n=10 in roscovitine treatment group) to assess the survival rate. Observation of lethality was assessed over 48 h. The experiments were repeated for elongated observation of survival rate using a third set of mice (n=12 per group).

2.1.2 CCl₄-induced acute liver injury model

The second acute liver injury model was established with CCl₄. 24 mice were randomly divided into 3 groups (n=8 in each group): control group, mice only received the vehicle
(2ml/kg olive oil); CCl₄ group, mice received a single dose of CCl₄ (10% diluted in olive oil, 2ml/kg, i.p.) as reported previously [20]; and roscovitine treated group, mice were treated with roscovitine (10mg/kg, i.p.) 24 h and 2 h prior to CCl₄ challenge. All mice were sacrificed 48 h after CCl₄ administration.

2.1.3 CCl₄-induced chronic liver injury model

Liver fibrosis was induced by repeated CCl₄ challenge (10% diluted in olive oil, 1ml/kg, twice per week, i.p.) for 8 weeks [21]. 24 mice were randomly divided into 3 groups (n=8 per group): control group, mice received the vehicle (2ml/kg olive oil and 0.9% saline); CCl₄ group, mice received repeated CCl₄ challenge; roscovitine treated group, mice were injected with roscovitine (10mg/kg, once daily) from the beginning and for the following 8 weeks in parallel with ongoing CCl₄ challenge. Mice were sacrificed at the end of 8 weeks.

In the above-mentioned animal experiments, roscovitine was initially dissolved in DMSO and diluted to the desired concentration with saline [22]. Mice were randomly divided using a random number table. At the end of each experiment, mice were killed by exsanguination under intraperitoneal injection of urethane anesthesia (1g/kg body weight). Serum and liver samples were collected.

2.2 Liver histopathology and immunohistochemistry

Liver tissues were fixed in 4% paraformaldehyde, dehydrated and paraffin embedded. The liver sections were stained with hematoxylin-eosin (H&E; Wuhan Servicebio G1005), TUNEL (Roche 11684817910) or Sirius Red (Wuhan Servicebio G1018) according to the manufacturer’s protocol. Immunohistochemistry (IHC) staining was performed using antibodies against F4/80 (1:100, MAB5580, RD), Ly-6G (1:100, MAB1037-100, RD), α-SMA (GB13044, 1:100), and Col1a1 (GB11022-1, 1:100) using standard methods. Photographs of random fields were blindly taken. Representative views are displayed.
2.3 Biochemical Analysis and cytokine measurement

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) serum levels were assessed using a Hitachi 7020 automatic Analyzer (Hitachi, Tokyo, Japan) following the manufacturer’s recommendations. The concentration of IL-6, TNF-α, and IL-1β in liver tissues or culture supernatants were assessed using ELISA kits (No. EMIL6RA, 88-7324-22, BMS6002; Thermo Scientific) according to the manufacturer’s instructions. All samples were analyzed in duplicate.

2.4 Cell culture and treatment

RAW264.7 and LX2 cell lines were cultured using Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Australia) supplemented with 10% fetal bovine serum (FBS, Gibco, Australia) and 1% antibiotics (Penicillin-Streptomycin), and incubated at 37°C with 5% CO₂. Primary hepatocytes and Kupffer cells were isolated from C57BL/6 mice by two-step in situ liver perfusion protocols as previously reported [23]. Primary hepatocytes were grown in Hepatocyte Medium (Sciencell, America). Kupffer cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% antibiotics (Penicillin-Streptomycin).

After 24 h of culture, RAW264.7 cells were pretreated with or without roscovitine for 24 h or 1 h and were then subsequently co-incubated with LPS (Escherichia coli, 055:B5, Sigma, 1 μg/mL) for 4 h; LX2 cells were starved in serum-free DMEM for 24h before being treated with recombinant human TGF-β1 (Peprotech,100-21C,10ng/ml) with/without roscovitine for 24h; primary hepatocytes and Kupffer cells were pretreated with roscovitine for 1 h, followed by co-incubation with LPS (1 μg/mL) for 4 h.

2.5 RNA-Seq analysis

Flash frozen mouse liver tissues from the LPS/GalN-induced liver injury model and TGF-β stimulated LX2 cells were used for total RNA extraction. The isolated RNA was used to
prepare cDNA libraries and subsequently sequenced on the Illumina HiSeq2000 platform. RNA Integrity Number (RIN) value was used to assess the quality of the isolated RNAs. Only RNA sequences with RIN$\geq 7.0$ were used for sequencing. The sequencing reads were mapped to mm10 by STAR 2.5 and gene counting was quantified using feature counting software [24, 25]. The DEseq2 R package was used for differential gene expression analysis [26]. The p value was adjusted by the Benjamini and Hochberg methods, and a 5% FDR cutoff value and fold change greater than 2 were set as the thresholds of the significant genes in the LPS/GalN-induced liver injury in vivo model, while the fold change threshold was 1.5 in the LX2 cells study. Gene set overlays were determined using BioVenn web tool [27]. Differentially expressed genes were further analyzed by gene-annotation enrichment analysis using the DAVID 6.8 bioinformatics platform and ClusterProfiler R package [28]. Gene Set Enrichment Analysis (GSEA) and leading edge analysis were performed using GSEA 4.0.3 software [29]. STRING 11.0 was used for network analysis, and enrichment analysis for GO was mapped onto the network [30].

2.6 RNA extraction and quantitative RT-PCR

Total RNA was extracted from cells and liver tissues and reverse transcribed according to the manufacture’s protocol (Vazyme, China). Relative transcript levels were measured by RT-qPCR using SYBR green (Vazyme, China) on a Quant Studio 6 Flex Real-Time PCR system (ABI). Target gene expression was normalized to GAPDH expression. The $\Delta\Delta$Ct method was employed for calculation. Primer sequences are shown in Supplemental Table 1. These experiments were performed in triplicate.

2.7 Western blotting
Total protein was isolated from frozen liver tissues and cultured cells. Proteins were separated by electrophoresis, blotted and incubated with primary antibodies and corresponding secondary antibodies (anti-rabbit/mouse HRP conjugated antibody; PeproTech, China) for 1h. Finally, protein bands were visualized using a chemiluminescence detection kit (Super signal, Thermo Scientific). These experiments were repeated for 2 or 3 times. Primary antibodies used in this study are listed Supplement Table 2.

2.8 Statistical analysis

All results are expressed as mean ± SD using GraphPad Prism 8.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Analysis of differences between different groups was performed via a two-tailed unpaired t-test. P < 0.05 was considered statistically significant.

3. Results

3.1 Roscovitine reduces expression of inflammatory mediators in a dose-dependent manner in vitro

Roscovitine has shown a great anti-inflammatory potency in vitro [13, 31]. To further validate the effect of roscovitine on inflammation, LPS-challenged RAW264.7 macrophages were used. RAW cells were pretreated with or without roscovitine at different concentrations for 24 h followed by 1 ug/ml LPS stimulation for 4 h. As indicators of macrophage inflammatory activation, expression levels of proinflammatory cytokines including Il-6, Il-1β and Tnf-α were determined. RT-PCR results showed the mRNA expression levels of these cytokines were significantly increased in LPS-stimulated cells. These changes were reversed by roscovitine co-treatment in a dose-dependent manner (Figure 1A). Similar results were observed when the roscovitine pretreatment time was shortened to 1 h (Figure 1B). ELISA was used to detect IL-6, IL-1β and TNF-α protein secretion levels in cell culture supernatants and concordant results were obtained (Figure 1C & 1D). The anti-inflammatory effect of roscovitine was further validated in primary Kupffer cells and hepatocytes and parallel results
were observed (Figure 1E). Collectively, roscovitine exerted anti-inflammatory and hepatoprotective effects against LPS-induced cell injury in vitro.

3.2 Roscovitine pretreatment protects against LPS/GalN-induced liver injury and inflammation in mice

Given the anti-inflammatory properties roscovitine observed in vitro, we further examined whether roscovitine has any protective effect on hepatic injury and inflammation in vivo. Acute liver injury was induced in mice using LPS/GalN. Examination of LPS/GalN-induced lethality revealed marked increase in lethality in mice in the model group, while roscovitine treatment significantly reduced overall mortality (Figure 2A). Elongated observation time of another set of LPS/GalN challenged mice produced similar results (Figure S1A). Additionally, roscovitine significantly ameliorated liver injury and cell death. Mice from roscovitine treated group displayed significantly attenuated ALT and AST levels when compared with mice in the model group (Figure 2B). The gross liver morphology also appeared to be improved by roscovitine treatment (Figure 2C). Histologically, H&E staining of liver tissues after LPS/GalN exposure revealed a significant degree of liver injury, characterized by extensive tissue structure destruction, congestion, inflammatory cell infiltration, and cell necrosis. This injury was remarkably ameliorated by roscovitine (Figure 2D). TUNEL staining and its quantification consistently revealed that the increased hepatic cell death induced by LPS/GalN was reversed by roscovitine intervention (Figure 2E). Collectively, these data indicate that roscovitine treatment significantly mitigates the degree of liver injury.

We next evaluated whether roscovitine alleviates hepatic inflammation. The extent of inflammatory cell infiltration was examined by IHC staining in liver sections. Macrophage (marked by F4/80) and neutrophil (marked by LY-6G) infiltration was markedly inhibited in roscovitine-treated mice compared with vehicle-treated mice (Figure 3A). Abundant studies have proven that the extent of liver injury is closely related to the cytokine response; inhibition of inflammatory cytokines production is associated with alleviation of liver injury [32-35]. ELISA detection of TNF-α, IL-6 and IL-1β pro-inflammatory mediators in serum (Figure 3B) and liver tissues (Figure 3C) was diminished by roscovitine. Consistent with this,
mRNA expression of IL-6, IL-1β, and Tnf-α were markedly attenuated via roscovitine treatment (Figure 3D). These data are consistent with the previous study that showed that roscovitine promoted clearance of inflammatory cells and resolution of inflammation [12]. These results indicate that in mice with acute liver injury treatment with roscovitine regulates inflammatory cell infiltration and inflammatory cytokine expression in the liver, contributing to suppression of inflammatory responses in vivo.

3.3 Roscovitine regulates the expression of genes related to inflammatory and TGF-β signaling pathways

To gain a deep insight into the underlying mechanism of the protective and anti-inflammatory effects of roscovitine on liver injury, RNA-seq analysis was performed using liver tissues from control mice and LPS/GalN-challenged mice treated with or without roscovitine. A total of 2505 LPS/GalN-induced genes were identified as significantly up-regulated upon LPS/GalN stimulation. Most LPS/GalN-induced genes were involved in the inflammation response, and excessive and unchecked expression of pro-inflammatory genes leads to tissue damage. Of the 2505 genes, 1114 were down-regulated after roscovitine treatment (Figure 4A and B), suggesting that roscovitine treatment represses the expression of genes involved in the inflammation response. Gene-annotation enrichment analysis revealed that genes affected by roscovitine were predominantly enriched in the inflammation response, including in the activation, proliferation and adhesion function of immune cells, indicating that the roscovitine regulated inflammation response is comprehensive (Figure 4C, Supplement Table 3). In addition, the majority of differentially expressed genes annotated to these GO terms were pro-inflammatory cytokines, chemokines, and adhesion molecules and their receptors, indicating that roscovitine hinders transcriptional activation of a broad set of pro-inflammatory genes induced by LPS/GalN stimulation (Figure S1B). These observations are consistent with the role of roscovitine as a pan-CDK inhibitor.

Roscovitine can inhibit both transcription-associated CDKs (CDK7, CDK9) and cell cycle-associated CDKs (CDK1, CDK2). Transcription-associated CDKs, especially CDK7 and CDK9, play important roles in the inflammation response by regulating the
transcriptional process of pro-inflammatory cytokines and chemokines. Phosphorylation of serine 5 (Ser5) and serine 2 (Ser2) on the extended C-terminal domain of RNA polymerase II induced by CDK7 and CDK9 mediates initiation and elongation of transcription, and subsequent synthesis of inflammatory mediators involved in the inflammation response. Our data show that roscovitine inhibits Ser2 and Ser5 phosphorylation \textit{in vivo} and \textit{in vitro}, suggesting phosphorylation modifications affected by roscovitine might repress transcriptional activation of pro-inflammatory genes (Figure 4D and Figure S2A). GSEA was performed to identify the pathways and biological processes affected by roscovitine treatment. Roscovitine repressed some biological process driving the inflammation response and subsequent tissue damage, including the positive regulation of cytokine production, positive regulation of cytokine secretion, regulation of leukocyte migration, and regulation of leukocyte proliferation, which indicate that roscovitine-mediated impaired transcription activation of pro-inflammatory genes affects multiple aspects of the inflammation response to ameliorate liver injury (Figure 4E). Representative genes in leading edge subsets of enriched pathways and biological processes were validated by RT-PCR (Figure 4F). Network analysis results also revealed significant enrichment in the biological processes of cytokine production, immune cell proliferation and migration (Figure 4G).

Nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways serve as essential components in inflammation and can be activated by various cytokines including TNF, IL-6, and IL-1β [36, 37]. Suppression of proinflammatory genes and cytokines was clearly observed in roscovitine treated mice and gave rise to inhibition of the related pathways. Western blot analysis revealed significant activation of the NF-κB and MAPK pathways following LPS/GalN administration, and that roscovitine intervention dramatically impaired NF-κB and MAPK pathway activation (Figure 4H, top). Concordant dose-dependent results were observed \textit{in vitro} (Figure 4H, bottom). Quantification of the western blot results is shown in Figure S2B and S2C. These data indicate that roscovitine suppressed the activation of inflammatory signaling pathways and may explain its beneficial effect in alleviating liver inflammation and injury.
Interestingly, the TGF-beta signaling pathway and biological process of tissue remodeling also showed significant down-regulation after roscovitine treatment (Figure 4D). Members of these two gene sets are involved in regulation of liver disease progression, from initial liver injury caused by inflammation to liver fibrosis and cirrhosis. Downregulation of TGF-beta and other representative genes regulating tissue remodeling was validated by RT-PCR *in vitro* and *in vivo* (Figure 4F). Taken together, these results show that roscovitine inhibits the transcription of a broad set of pro-inflammatory genes involved in many aspects in inflammation, and represses the TGF-beta signaling pathway and biological process of tissue remodeling, further ameliorating inflammation and tissue damage. Moreover, inhibition of the expression of genes involved in immune cell migration and proliferation might promote resolution of inflammation and reduce liver injury caused by immune cell infiltration during the course of chronic liver injury and inflammation.

3.4 Roscovitine attenuates liver injury and inflammation in the CCl₄-induced acute liver injury model

The limitation of the LPS/GalN-induced acute liver injury model is that it largely depends on the early production of detrimental inflammatory mediators, leading to massive hepatocyte apoptosis and lethal outcomes at the late stage [38]. Therefore, the anti-inflammatory and hepatoprotective effects of roscovitine were further confirmed in the CCl₄-induced acute liver injury model. H&E staining revealed massive centrilobular hepatic necrosis and inflammation in liver sections from CCl₄-induced acute liver injury model mice (Figure 5A). Moreover, serum ALT and AST levels were significantly increased in these mice after CCl₄ injection (Figure 5B). These pathological changes were altered by roscovitine, suggesting alleviated liver damage. IHC staining showed that roscovitine treatment reduced F4/80⁺ macrophage and LY-6G⁺ neutrophil infiltration in the CCl₄-induced acute liver injury model (Figure 5C). Similarly, RT-PCR results demonstrated the suppression of *Il-6*, *Il-1β* and *Tnf-α* inflammatory genes expression in roscovitine-treated mice (Figure 5D). Together, these data suggest that roscovitine exerts protective effects on inflammation and injury of liver parenchyma, in addition to attenuating inflammation-dominant liver injury.
3.5 Roscovitine protects against chronic CCl₄-induced liver inflammation in mice

Based on the promising hepatoprotective efficacy of roscovitine in acute liver injury models, we hypothesized that roscovitine could exert similar protective effects against inflammation in a chronic liver injury model. We tested this hypothesis using a chronic CCl₄-challenged mice model. At the endpoint of the experiment, CCl₄-challenged mice demonstrated disturbed lobular architecture with inflammation and hepatocyte ballooning evidenced by H&E staining (Figure 6A) and elevated serum ALT levels (Figure 6B). The liver injury was accompanied by increased mRNA levels of proinflammatory cytokines including Il-6, Il-1β and Tnf-α in the CCl₄-treated group (Figure 6C). These pathological changes were moderately attenuated by roscovitine. Together, these data indicate that roscovitine demonstrates consistent anti-inflammatory properties in chronic CCl₄-induced liver injury.

3.6 Roscovitine protects against liver fibrosis in vivo and in vitro

Repeated CCl₄ treatment induced the development of significant liver fibrosis, which was alleviated by roscovitine (Figure 6D). Perpetual hepatic inflammation and inflammatory cells infiltration serve as crucial driving factors of HSC activation and liver fibrosis [39]. Moreover, activated HSC transdifferentiates to fibrogenic myofibroblasts characterized by enhanced ECM production, which is a crucial step in the development of liver fibrosis. Therefore, the anti-fibrotic effect of roscovitine was investigated by assessing its effect on inflammation and HSC activation. The expression levels of α-SMA and Col1a1 were assessed as dominant components of ECM and well-established markers of fibrosis and HSC activation [40]. IHC and western blot assays revealed significantly increased α-SMA and COL1a1 expression in the model group which was reversed by roscovitine treatment (Figure 6E, 6F, and S2D). RT-PCR assessment of the expression levels of typical profibrotic markers was consistent with the IHC staining and western blot results (Figure 6G).

For further validation, the human HSC LX-2 cell line was used to directly investigate the effect of roscovitine on HSC activation. This analysis revealed that roscovitine inhibits COL1A1 and α-SMA mRNA expression in the presence of TGF-β, a profibrotic cytokine, in a
dose-dependent manner, indicating that roscovitine inhibits HSC activation (Figure S3A). To profile gene expression change during HSC activation and roscovitine treatment, transcriptome analysis was performed in LX2 cells treated with TGF-β with or without roscovitine. A specific subset of profibrotic genes induced by TGF-β was suppressed by roscovitine (Figure S3B). Further gene enrichment analysis revealed that genes effected by roscovitine are involved in many well-known biological processes that play critical roles in HSC activation and fibrosis including cell-matrix adhesion, focal adhesion assembly and the tumor necrosis factor-mediated signaling pathway (Figure S3D). Notably, the expression of important marker genes in these gene sets was inhibited by roscovitine (Figure S3C), suggesting that roscovitine is an effective inhibitor of HSC activation.

Taken together, our results show that liver inflammation and HSC activation were effectively inhibited by roscovitine.

4. Discussion

Liver disease can be caused by various etiologies, including alcohol, viral hepatitis and chemical insults and is an enormous global health burden [41, 42]. Inflammation is a physiological response to injury and leads to subsequent secretion of inflammatory mediators involved in cellular defense and tissue repair [43]. Chronic injury left unchecked leads to persistence of liver inflammation and might result in tissue damage and disease progression. Ongoing chronic inflammation induces the activation of macrophages to release proinflammatory and profibrotic mediators. TGF-β, predominantly produced by macrophages, activates HSCs and is generally considered the most potent fibrogenic cytokine [44]. However, there is a lack of efficient anti-inflammatory medications for liver diseases. The identification of effective pharmacotherapies for related hepatic diseases is urgently needed.

Roscovitine is a 2, 6, 9-substituted purine analogue that function as a potent inhibitor of CDK-1, -2, -5, -7 and -9 [6]. Recent studies have reported the protective role of roscovitine in lipoteichoic acid-induced lung inflammation and LPS-induced murine testis inflammation [45, 46]. Although roscovitine has shown effective anti-inflammatory capacity in multiple
preclinical models associated with inflammation, whether it exerts a protective effect on liver inflammation had not been examined. Given the roles of inflammation in initiating liver injury and in tissue remodeling and fibrogenesis, we explored roscovitine as a potential anti-inflammatory agent in the liver and assessed its effect in the treatment of mouse experimental liver injury. We found that mRNA and protein levels of proinflammatory cytokines were significantly reduced by roscovitine in both cells and cell culture supernatant evidenced by RT-PCR and ELISA respectively. Moreover, LPS-induced cytotoxicity against hepatocytes was also relieved by roscovitine treatment. These results are concordant with the existing data that show that roscovitine can alleviate inflammation-related responses in vitro through inhibition of proinflammatory cytokines and nitric oxide production [13]. Our in vivo study used a well-accepted mice model of LPS/GalN-induced acute liver injury, which involves excessive production of proinflammatory cytokines and hepatocyte necrosis. Our results show that roscovitine acts as a hepatoprotective agent by significantly suppressing proinflammatory cytokines and inflammatory signaling pathways, prolonging survival time and improving liver morphology and function. In consideration of the limitations of the LPS/GalN model, the effects of roscovitine in the CCl4-induced acute liver injury mice model were assessed and concordant hepatoprotective results were observed.

RNA-seq and transcriptomic analysis revealed that roscovitine regulates a broad set of genes related to inflammation. Gene-annotation enrichment analysis was used to identify the genes most predominantly affected by roscovitine. The majority of these genes were annotated as pro-inflammatory cytokines, chemokines, adhesion molecules and their receptors, and were enriched in multiple processes of the inflammatory response including activation, proliferation and adhesion function of immune cells. Representative genes in the enriched pathways were validated via RT-PCR. Expression of Il-6, Il-1β, Tnf-α, Ccl2, and Ccl5, proinflammatory cytokines and chemokines which aggravate inflammatory cell infiltration and the inflammation response, were significantly reduced by roscovitine. Mechanistically, the hepatoprotective and anti-inflammatory effects of roscovitine may occur through inhibition of inflammatory cell proliferation and inflammatory cytokine expression induced
by macrophage activation. Our data are consistent with those of previous studies that show that roscovitine promotes inflammation resolution by driving inflammatory cell apoptosis [12, 47, 48].

Inflammatory responses are thought to be regulated by multiple signaling pathways, and NF-κB and MAPK pathways play a predominant role in the innate immune response. NF-κB and MAPK contact with upstream Toll-like receptor 4 (TLR4), an LPS sensor, and become activated to initiate proinflammatory reactions [49, 50]. Activated NF-κB directs the production of proinflammatory cytokines, particularly TNF-α, which is also by itself an activator of NF-κB, resulting in a positive feedback loop. Roscovitine administration down-regulates p-p65, p-IκB, and TNF-α expression levels, suggesting blockade of the NF-κB signaling pathway and its consequential activation. The MAPK pathway, which can also be activated by TNF-α, plays an essential role in stress and inflammatory responses [51]. JNKs and ERKs, two extensively-studied MAPKs, have a close relationship with macrophage-triggered inflammatory responses [52]. Deletion of JNK1 and JNK2 causes decreased polarization to the M1 phenotype in macrophages, indicating a pro-inflammatory role for JNK signaling [53]. The ERK1 and ERK2 is closely related to cytokine production regulation in macrophages [52]. Western blot analysis of the activation state of the MAPK signaling pathway confirmed that roscovitine suppresses MAPK pathway signaling in vivo and in vitro. These findings suggest that roscovitine can suppress macrophage inflammatory reactions, a preliminary step in the biological process of liver injury, possibly via inhibiting proinflammatory genes and related signaling pathways, leading to alleviated hepatic inflammation in the LPS/GalN challenged mice model.

Cell death and inflammation are central elements in the progression and deterioration of liver fibrosis. In addition, excessive fibrosis is often detected in patients with chronic inflammation [54]. The encouraging results of roscovitine on the alleviation of hepatic inflammation and hepatocyte injury in the acute liver injury models led us to explore the pharmacological effects of roscovitine in a chronically injured murine model. As expected, liver injury and fibrosis were alleviated by roscovitine in the chronic CCl₄-induced liver fibrosis model. These
results are consistent with those of previous reports showing the anti-fibrotic abilities of roscovitine in various models. Roscovitine efficiently reduces fibrosis in the renal interstitial fibrosis model through TGF-β1/p38 MAPK pathway regulation [17]. The anti-fibrotic mechanism of roscovitine was explored by assessing its effect on inflammation and HSC activation. Examination of histological sections and proinflammatory cytokine expression revealed that roscovitine reduced hepatocyte injury and inflammatory response. HSC activation, measured by α-SMA and Col1a1 expression levels, was reduced in vivo and in vitro. Further transcriptome analysis of TGF-β stimulated LX2 cells incubated with or without roscovitine revealed that roscovitine suppresses the expression of various genes involved in HSC activation and fibrosis.

As a CDK inhibitor, the cell death-inducing actions of roscovitine, such as antitumor properties and neutrophil apoptosis-inducing abilities, have been well studied. The role of roscovitine in the influence on the onset of liver injury has been studied to a lesser extent. Our results indicate that roscovitine can effectively suppress macrophage inflammatory actions and subsequent HSC activation, which are two preliminary steps of significant importance in liver injury initiation and progression. These data provide evidence that therapeutic targeting of these driving factors can be effective strategies for the treatment of inflammation- or fibrosis-involved hepatic diseases. However, additional research is required to translate these findings into a therapeutic tool that can prevent progression and deterioration in liver diseases.

**Data availability statement:** All supporting data are available by contacting the corresponding author.

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Figure 1. Roscovitine inhibits LPS-induced inflammatory responses in vitro.

RAW264.7 cells were pretreated with roscovitine for 24 h (A) or 1 h (B) and then stimulated with LPS for 4 h, after which the Il-6, Il-1β, and Tnf-α mRNAs were quantified by RT-qPCR analysis. RAW264.7 cells were pretreated with roscovitine for 24 h (C) or 1 h (D) and then stimulated with LPS for 4 h, after which the IL-6, IL-1β and TNF-α concentrations in culture supernatants were determined by ELISA. (E) Primary isolated Kupffer cells and hepatocytes were pretreated with roscovitine for 1 h and then stimulated with LPS for 4 h, after which the Il-6, Il-1β, and Tnf-α mRNAs were quantified by RT-qPCR analysis. All data are presented as mean $\pm$ SD from three independent experiments. ####P < 0.0001 versus normal control. ns > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus LPS group.

Figure 2. Roscovitine exerted protective effects on the LPS/GalN-induced liver injury model in mice.

(A) Survival curves for mice from the control group (n=10), LPS/GalN group (n=12), and Roscovitine treated group (n=10). (B) Serum ALT, AST in mice from the control group, LPS/GalN group, and Roscovitine-treated group (n=6/group). (C) Morphological changes of livers in mice from the indicated groups. (D) Representative images of H&E-staining in liver sections (Scale bar: 50μm). (E) Representative images of TUNEL staining in liver sections from the indicated groups and its quantification (n=6/group) (Scale bar: 50μm). All data are presented as mean $\pm$ SD. ####P < 0.0001 versus normal control group. ****P < 0.0001 versus vehicle-treated LPS-stimulated group.
Figure 3. Roscovitine attenuates inflammatory cell infiltration and inflammation reactions in LPS/GalN-induced liver injury mice.

(A) Representative images and quantification of F4/80$^+$ and LY-6G$^+$ cells staining in liver sections (n=6-8/group) (Scale bar: 50μm). (B and C) Proinflammatory cytokines concentrations detected by ELISA in serum (n=6/group) (B) and liver tissues (n=8/group) (C). (D) mRNA expression of proinflammatory cytokines in liver tissues (n=5/group). All data are presented as mean ± SD. ####P < 0.0001 versus normal control group. ****P < 0.0001 versus vehicle-treated LPS-stimulated group.

Figure 4. Roscovitine suppressed gene expression of pro-inflammatory genes and attenuated inflammatory pathway activation.

Total RNA was extracted for RNA-seq analysis from liver tissues in control (n=4), LPS/GalN model (n=4) and roscovitine-treated group (n=4). (A) The heatmap showing differentially expressed genes between roscovitine-treated group and model group. Differentially expressed genes were filtered for absolute value of fold change ≥2 and adjusted P values (Padj) of <0.05. (B) The overlap between the gene set of LPS/GaIN induced gene and the gene set of roscovitine suppressed genes. (C)Top 10 enriched GO items for differentially expressed genes between roscovitine-treated group and model group. (D) Western blotting was performed to validate phosphorylation state of Ser2 and Ser5 by CDK7 and CDK9 on the extended C-terminal domain of RNA polymerase II both in vivo and in vitro. (E) GSEA results shows effected pathways and biological processes in the LPS/GaIN challenged mice after treatment of roscovitine. (F) Representative genes of enriched pathway and biological processes were evidenced by qPCR. (G) Network analysis on differentially expressed genes between roscovitine-treated group and LPS/GaIN model group. Protein-protein interaction network was constructed using STRING11.0, and enrichment analysis for GO was mapped onto the network. Nodes are colored in green and genes involved in cytokine production, leukocyte...
proliferation and leukocyte migration are highlighted in orange, red and purple, respectively.

(H) Regulation of NF-κB and MAPK signaling pathways by roscovitine was demonstrated by western blot in liver tissues and RAW264.7 cells. Samples of western blots derived from the same experiment. All data are presented as mean ± SD from three independent experiments.

Figure 5. Roscovitine attenuates the CCl4-induced acute liver injury and inflammation. For these experiments, liver tissues were collected 48 hours following a single dose of intraperitoneal CCl4 injection.

(A) Representative images of H&E stained liver sections from the control group, CCl4-induced acute liver injury model group, and Roscovitine-treated group (Scale bar: 50μm). (B) Serum ALT, AST levels in the respective groups (n=6/group). (C) Representative images and quantification of F4/80⁺ and LY-6G⁺ cells staining in liver sections (n=5-6/group) (Scale bar: 50μm). (D) mRNA expression of proinflammatory cytokines (Il-6, Il-1β and Tnf-α) detected by RT-qPCR (n=5/group). All data are presented as mean ± SD. ####P < 0.0001 versus normal control group. **P < 0.01, ***P < 0.001, ****P < 0.0001 versus vehicle-treated CCl4-challenged group.

Figure 6. Roscovitine attenuates CCl4-induced chronic liver injury, inflammation and fibrosis. Livers of mice exposed to CCl4 for 8 weeks were excised and processed for histological and biochemical investigations.

(A). Representative H&E stained images of liver sections (Scale bar: 50μm). (B) Serum ALT levels in the respective groups (n=6/group). (C) mRNA expression of proinflammatory cytokines (Il-6, Il-1β and Tnf-α) in liver tissues following 8 weeks of CCl4 administration (n=5/group). (D) Representative images and quantification of Sirius red staining in liver
sections (Scale bar: 50μm) (n=7-8/group). (E) Representative image and quantification of α-SMA and Col1α1 staining of liver sections from the indicated groups (n=7-8/group) (Scale bar: 50μm). (F) Western blot analysis and quantifications of α-SMA and COL1α1 protein levels in mouse liver tissues. Samples of western blots derived from the same experiment. (G) mRNA expression of fibrosis markers (a-Sma and Col1α1) in hepatic tissues from respective groups of mice exposed to CCl₄ for 8 weeks (n=5/group). All data are presented as mean ± SD from three independent experiments. ****P < 0.0001 versus normal control group. **P < 0.01, ***P < 0.001, ****P < 0.0001 versus vehicle-treated repeated CCl₄-challenged group.
A

Control

Acute CCl₄ model

Roscovitine treated

HE

100×

100×

100×

B

ALT (U/L)

AST (U/L)

NC

CCl₄

Roscovitine

NC

CCl₄

Roscovitine

Control

Acute CCl₄ model

Roscovitine treated

F4/80

200×

200×

200×

LY-6G

200×

200×

200×

Positive area (%)

F4/80

LY-6G

C

Control group

CCl₄ group

CCl₄ + Roscovitine group

D

IL-6 mRNA

NC

CCl₄

Roscovitine

NC

CCl₄

Roscovitine

TGF-β mRNA

NC

CCl₄

Roscovitine

NC

CCl₄

Roscovitine
