**Hesperetin derivative-14 alleviates inflammation by activating PPAR- γ in mice
with CCl₄-induced acute liver injury and LPS-treated RAW264.7 cells**

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Abstract

Hesperetin is a flavanone glycoside compound naturally occurring in the fruit peel
of *Citrus aurantium* L. (*Rutaceae*). Previous studies revealed that hesperetin possesses

various pharmacological effects, including anti-inflammation, anti-tumor, anti-oxidant and neuroprotective properties. Hesperetin derivative-14 (HD-14) is a derivative of hesperetin improved in water solubility and bioavailability. In this study, we indicated that HD-14 (2 μ M) significantly attenuated inflammation in LPS-treated RAW264.7 cells and had hepato-protective effects and anti-inflammatory effects (100 mg/kg) on C57BL/6J mice with CCl₄-induced acute liver injury. In addition, it was demonstrated that HD-14 dramatically up-regulated the expression of PPAR- γ in vivo and in vitro. Interestingly, over-expression of PPAR- γ had anti-inflammatory effects on the expressions of TNF- α , IL-6, and IL-1 β , whereas, knockdown of PPAR- γ with small interfering RNA had pro-inflammatory effects in LPS-treated RAW264.7 cells. Thus, our findings demonstrated that HD-14 alleviated inflammation by activating PPAR- γ expression at least in part. Further studies founded that HD-14 remarkably inhibited the expression of p-JAK1 and p-STAT1 through up-regulating PPAR- γ . Together, these results suggested that HD-14 served as an activator of PPAR- γ and the JAK1/STAT1 signaling pathway may be involved in the progress of inflammation. Collectively, HD-14 may be utilized as a potential anti-inflammation monomeric compound in the treatment of acute liver injury.

Keywords: Acute liver injury; Hesperetin derivative-14; Inflammation; PPAR- γ

Abbreviations: Acute liver injury, ALI; Kupffer cells, KCs; Hesperidin derivative-14, HD-14; Peroxisome Proliferator-activated Receptor- γ , PPAR- γ ; Tumor necrosis factor- α , TNF- α ; Interleukin-6, IL-6; Interleukin-1 β , IL-1 β ; Lipopolysaccharide, LPS; Methyl thiazolyl tetrazolium, MTT; Alanine aminotransferase, ALT; Aspartate

aminotransferase, AST; Aspartate aminotransferase, AST.

1. Introduction

Acute liver injury (ALI) threatens human health. During liver injury, resident macrophages, known as Kupffer cells (KCs), are critical in the initial stress response (Malhi et al., 2010). Activated Kupffer cells (KCs) are widely recognized as one of the main cell types responsible for ALI, releasing various metabolites that cause cellular damage including superoxide radicals, nitric oxide, eicosanoids, proteases, and pro-inflammatory cytokines (Akahori et al., 2007). Therefore, regulating the activity of macrophages provides an excellent therapeutic strategy to alleviate severe hepatic inflammation (Han et al., 2016). Furthermore, lipopolysaccharide (LPS) has also been shown to induce liver injury through the activation of inflammatory cytokines (Enomoto et al., 1998). It is now well accepted that pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF- α), interleukin1 β (IL-1 β) and interleukin6 (IL-6) play a major role in ALI. Thus, inhibition of KCs activity and pro-inflammatory cytokine production would be beneficial to alleviate liver injury (Levert et al., 2002).

In the past decades, treatment of ALI with Traditional Chinese Medicine has gained increasing attention. Hesperetin is a flavanone glycoside compound naturally occurring in the fruit peel of *Citrus aurantium* L. (*Rutaceae*). Previous study has illustrated that treatment of hesperetin reduced TNF- α , IL-6, and IL- β secretions in LPS-induced RAW264.7 cells (Ren et al., 2016). CCl₄-induced acute liver injury (ALI)

in mice is widely used as an experimental animal model for screening hepato-protective drugs (Niranjana Murthy et al., 2014; Zhang et al., 2014), whereas, the potential molecular mechanism of hesperetin suppressing inflammatory response in mice with CCl₄-induced ALI still unrevealed. More importantly, hesperetin has a lower bioavailability in the gastrointestinal tract and conjugated during absorption (Fisher, 1978). Based on the observation mentioned above, our laboratory synthesized a series of hesperetin derivatives to improve its water solubility, bioavailability and their anti-inflammatory activities with Mannich bases (Fig. 1A).

Peroxisome proliferator-activated receptors- γ (PPAR- γ) is expressed in various immune cells, such as primary peritoneal macrophages, dendritic cells, and T cells (Chinetti et al., 1998; Jiang et al., 1998; Ricote et al., 1998; Welch et al., 2003). Growing evidence supports an anti-inflammatory role for PPAR- γ . Activation of PPAR- γ by various ligands down-regulates the synthesis and release of proinflammatory cytokines (Zhao et al., 2011). The Janus kinase (JAK) signal transducer and activator of transcription (STAT) cascade is an essential inflammatory signaling pathway that mediates the immune responses (Darnell, 1997). JAK-1 activates STAT-1 in adipocytes (Waite et al., 2001). In addition, available evidence suggested that expression of STAT-1 was regulated by PPAR- γ (Stewart et al., 1999).

In the current study, we further explored the potential anti-inflammatory of HD-14 in mice with CCl₄-induced ALI and LPS-treated RAW264.7 cells. On the basis of our reports, HD-14 remarkably attenuated acute liver injury by reducing pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 release in vivo and in vitro. The

mechanism may attributable to modulating PPAR- γ expression and the JAK1 / STAT1 signaling pathway was involved.

2. Materials and methods

2.1 Animal and treatment

C57BL/6J mice (weighting about 20~22g each) were provided by the Experimental Animal Center, Anhui Medical University. All the animal experimental and procedures were approved by the Ethic Committee and the Animal Experimental Committee of Anhui Medical University. Mice were reviewed and performed under the permission of the Animal Experiments Guidelines and Animal Care of Chinese Academy of Sciences. For all experiments, C57BL/6J mice were divided randomly into six groups (n = 10 per group): Control group, CCl₄ group, HD-14 treatment group (50 mg/kg, 100 mg/kg, 200 mg/kg) (Lin et al., 2015), and Silymarin group (100 mg/kg) (Domitrovic et al., 2015). HD-14 treatment group, CCl₄ group and Silymarin group were injected intraperitoneally with a single dose of 1% CCl₄ (10 ml/kg in oil) to induce acute liver injury model (Huang et al., 2015), besides, mice in HD-14 treatment group and Silymarin group were given via gavage different concentrations of HD-14 and Silymarin for 7 days before. Control groups were injected intraperitoneally the same volume of vehicle (oil).

2.2 Materials and reagents

Hesperidin derivative-14 (HD-14) was synthesized by School of Pharmacy (Anhui,

China). Silymarin, Lipopolysaccharide (LPS), Dimethyl sulfoxide (DMSO), Collagenase (type IV), OptiPrep, Percol and Methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal antibody β -actin was purchased from Santa Cruz (California, USA). TNF- α polyclonal antibody were purchased from Abcam (Cambridge, UK). Rabbit IL-1 β , IL-6 polyclonal antibody were purchased from Bioworld (Minnesota, America). Rabbit PPAR- γ , JAK1, p-JAK1 polyclonal antibody were purchased from Cell Signaling Technology (MA, USA). Rabbit STAT1, p-STAT1 polyclonal antibody were purchased from Bioss (Beijin, China). ALT and AST activity assay kit were purchased from Jiancheng (Wuhan, China). ELISA kit was purchased from Elabscience. (Wuhan, China).

2.3 Cell culture and cell treatment

RAW264.7 (No. TCM13) cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% (v/v) heat-inactivated FBS (Every Green, China), 100U/mL penicillin, 100 mg/mL streptomycin and cultured at 37 °C with 5% CO₂. RAW264.7 cells were pre-treated with 1000 ng/ml LPS for 12h before treated different concentrations of HD-14 for 48 h.

2.4 Isolation of KCs

Isolated KCs were collected by in situ collagenase perfusion and differential centrifugation on OptiPrep density gradient as described previously (Hansen et al., 2002; Smedsrod and Pertoft, 1985). C57BL/6J mice were put through the portal vein then the inferior vena cava cut. PB, a kind of buffer solution diluted 40 ml PBC (NaCl, KCl, NaOH and Hepes) to 1000ml with double distilled water, perfused to purge blood of the liver followed by a digestion buffer ($1 \times$ PBC, supplemented with collagenase IV and Pronase E, 4.76 mM CaCl_2). The liver was disrupted after digestion in 1% BSA solution. Then cell in liver were fractionated using 25% Percol and 50% Percol. The intercushion fraction was washed and then adhered to plastic in medium. Furthermore, KCs adhered to the plastic, whereas the nonadherent fraction was washed off in culture medium.

2.5 Histopathology

The biggest lobe of the liver was excised and sectioned from each C57BL/6J mice and fixed with 4% paraformaldehyde for at least 24 h then embedded in paraffin. The pathological changes of liver was observed and photographed by Olympus BX-51 microscope after 4 μm thick sections stained with hematoxylin and eosin (H&E) with a standard procedure.

2.6 ALT/AST activity assay

Serum extracted from C57BL/6J mice was stored at -80°C . The serum levels of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were assayed

by using ALT and AST activity assay kits. The absorbance at 510 nm was obtained with a micro-plate reader model 680 (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 Immunohistochemistry

Liver tissues were embedded in paraffin after fixed in 10% formalin solution, slides were xylene dewaxed and dehydrated in alcohol, antigen retrieval was performed with 0.01 M citrate buffer immersed and microwave (98°C-100°C) heated to boiling for 15 min. The sections were treated with 5% BSA (37°C, 20 min) to block endogenous peroxidase activity, incubated with primary antibody against PPAR- γ (1:500) for 18 h at 4°C and secondary antibody for 1h at room temperature. The expression of PPAR- γ was visualized by 3,3'-diaminobenzidine tetrahydrochloride staining. The sections were counter stained with hematoxylin-redyed for 30 s and dehydrated with gradient ethanol and xylene. PPAR- γ positive areas were mounted by gums and observed by microscopic.

2.8 Cytokine assay by ELISA

The levels of TNF- α , IL-1 β , IL-6 concentrations in the supernatants of macrophage and mouse serum were measured using ELISA Kits (Elabscience, Wuhan, China) according to the manufacturer's instruction. The absorbance at 450 nm determined by Thermomax microplate reader immediately in each well.

2.9 Western blot analysis

Mouse KCs and RAW264.7 cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China) containing PMSF. Protein concentration of the extract was detected using BCA protein assay kit (Beyotime, Shanghai, China). Equal amounts of total protein were loaded and separated by SDS-PAGE and blotted onto PVDF membranes (Millipore Corp, Billerica, MA, USA). Nitrocellulose blots were incubated for 12h at 4°C with primary antibodies diluted in primary antibody dilution buffer (Beyotime, China) after incubated in TBST containing 5 % skim milk at room temperature for 1 h to blockade of nonspecific protein binding. The primary antibodies recognizing β -actin, PPAR- γ , TNF- α , IL-1 β , IL-6, JAK1, p-JAK1, STAT1 and p-STAT1 were used 1:3000, 1:1000, 1:100, 1:500, 1:500, 1:500, 1:500, 1:500, 1:300 and 1:300, respectively. Horseradish peroxidase conjugated anti-rabbit were used as secondary antibody (1:10000) treated the membranes at room temperature for 1 h after washed 3 times with TBS / Tween20 (0.075%). Proteins were visualized with ECL-chemiluminescent kit (ECL-plus, Thermo Scientific) after washed 3 times with TBS/Tween20 (0.075%).

2.10 Total RNA extraction and Real-time q-PCR

Total RNA was extracted from Mouse KCs and RAW264.7 cells using TRIzol reagents (Invitrogen, USA), according to the manufacture's instruction, and the first-strand cDNA was synthesized using a Thermoscript RT-PCR synthesis kit (Fermentas, Canada) according to the manufacturer's instructions. Real-time q-PCR analyses for mRNA of PPAR- γ , TNF- α , IL-1 β , IL-6 and β -actin were performed by

using ThermoScript RT-qPCR kits (Fermentas, USA) in an ABI Prizm step-one plus real-time PCR System (Applied Biosystems, USA). The mRNA level of GAPDH was used as an internal control. Relative mRNA expression levels calculated normalized to GAPDH. All experiments were performed in triplicate. Real-time q-PCR was performed 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and at 60°C about 1 minute by using Thermo Step One. The primers sequences were listed in Table1.

2.11 Immunofluorescence staining

Cultured RAW264.7 cells were induced by LPS (1000 ng/ml) and treated with different concentrations of HD-14 to determine the expression of PPAR- γ in vitro. Immunofluorescence staining was performed with FITC-conjugated anti-PPAR- γ (1:300). Alexa Fluor 594-conjugated anti-rabbit used as secondary antibody. Counterstaining of nuclei was performed with 4,6-diamidino-2-phenylindole (DAPI; Biyuntian biological technology, Shanghai, China). Images were determined using fluorescence microscopy.

2.12 RNA interference analysis

Small interfering RNA (PPAR- γ -RNAi) oligonucleotides against PPAR- γ and negative scrambled (Scrambled-RNAi) were purchased from GenaPharma Corporation (Shanghai, China). PPAR- γ -RNAi and Scrambled-RNAi were performed by using Lipofectamine™ 2000 (Invitrogen, California, USA) according to the

manufacturer's manuals. RAW264.7 cells were cultured in serum-free DMEM for 12 h, then performed reverse transfection with PPAR- γ -RNAi or the Scrambled-RNAi in Opti-MEM (Gibco, USA) culture medium. The culture medium was changed by DMEM 6 h after transfection, and LPS (1000 ng/ml) was added. Transfection was allowed to proceed for various times and cells were processed for different assays.

2.13 Plasmid construction

Plasmid construction of pEX-2-PPAR- γ and empty pEX-2-control were purchased from Genechem (Shanghai, China) and extracted by using GoldHi EndoFree Plasmid Maxi Kit (Kangwei, Beijing, China), empty pEX-2-control serve as a control in parallel. RAW264.7 cells were transfected with pEX-2-PPAR- γ and empty pEX-2-control by using Lipofectamine™ 2000 according to the manufacturer's manuals.

2.14 Statistical analysis

Data are presented as the mean $\pm$ S.D and analyzed using Kruskal-Wallis one-way analysis of variance (ANOVA), followed by post-hoc tests using GraphPad Prism 5. In all cases, $P < 0.05$ was considered statistically significant.

Result

3.1. Hepato-protective effects of HD-14 on mice with CCl₄-induced ALI

In order to determine the effects of HD-14 on mice with CCl₄-induced ALI,

histopathological study, serological test, Western blot, Real-time q-PCR and ELISA assay were performed. Fig. 1A showed the molecular structural formula of hesperetin and hesperetin derivative-14 respectively; In Fig. 1C, histopathological study was performed to investigate the effect of HD-14 on mice with CCl₄-induced ALI. Mice liver tissues sections stained with hematoxylin and eosin (H&E) showed that CCl₄ (1%) injection caused remarkable ALI exhibited hepatocyte necrosis, irregularly arranged, inflammatory cells infiltration compared with the control group. Interestingly, HD-14 of different concentrations (50 mg/kg, 100 mg/kg, 200 mg/kg) and Silymarin (100 mg/kg) treatment showed hepato-protective effects of mice with ALI compared to control group. Besides, mice treated with HD-14 (50 mg/kg, 100 mg/kg, 200 mg/kg) and Silymarin (100 mg/kg) markedly attenuated the serum levels of ALT and AST as shown in Fig. 1D. Moreover, in primary KCs, Results of Western blot, quantitative data and ELISA assay demonstrated that HD-14 pretreatment significantly attenuated pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 protein expression and production levels in a concentration-dependent manner, which was further confirmed by Real-time q-PCR in mRNA levels in CCl₄-induced ALI, additionally, the HD-14 treatment group (200 mg/kg) showed similar Hepato-protective and anti-inflammatory effects compared with Silymarin (100 mg/kg) group (Shown in Fig. 1E, F and G). All the features suggested that HD-14 have significantly effects on Hepato-protective and suppressed inflammatory response in CCl₄-induced ALI.

3.2. Anti-inflammatory effects of HD-14 in LPS-induced RAW264.7 cells

To further investigate the anti-inflammatory effects of HD-14 on LPS-induced RAW264.7 cells, Western blot, Real-time q-PCR and ELISA assay were performed to detect the pro-inflammatory cytokine production. In Fig. 2A, results of MTT assay indicated that HD-14 in the concentration of 25 μ M had limited suppressive effect on the cell viability of RAW264.7 cells. In addition, results of Western blot and ELISA assay demonstrated that HD-14 at the concentrations of 1, 2, 4, 8, 16 μ M markedly inhibited pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 protein expression and production levels in a concentration-dependent manner (Shown in Fig. 2B and C). Additionally, results of Real-time q-PCR indicated that HD-14 substantially inhibited the mRNA levels of pro-inflammatory cytokines with concentration higher than 2 μ M in a concentration-dependent manner (Shown in Fig. 2D). In conclusion, these results suggest that higher dose concentrations (2 μ M and above) of HD-14 exhibited a markedly anti-inflammatory feature in LPS-induced RAW264.7 cells.

3.3. HD-14 increased expression of PPAR- γ in mice with ALI and LPS-induced RAW264.7 cells.

Emerging evidences supports the anti-inflammatory effect of PPAR- γ in inflammation related diseases. Our findings suggested that HD-14 substantially elevated the expression of PPAR- γ in vivo and in vitro. In Fig. 3A, results of immunohistochemical (IHC) staining analysis showed that the level of PPAR- γ was dropped in mice with CCl₄-induced ALI compared to control group. More importantly,

HD-14 pretreatment was significantly up-regulated the expression of PPAR- γ . Consistently, results of Western blot and Real-time q-PCR indicated that the expression of PPAR- γ markedly reduced both in primary KCs and LPS-induced RAW264.7 cells compared to control group, whereas, the expression of PPAR- γ dramatically promoted with HD-14 treated at concentration higher than 8 μ M (Shown in Fig. 3B and C). Moreover, as shown in Fig. 3D, results of immunofluorescence (IF) assay indicated that HD-14 in the concentration of 8 μ M largely increased the expression of PPAR- γ (green) in LPS-treated RAW264.7 cells. All the results indicated that HD-14 could activate the expression of PPAR- γ . Besides, 8 μ M HD-14 was employed in the following experiments.

3.4. HD-14 reduced TNF- α , IL-1 β and IL-6 expression by targeting PPAR- γ

To determine the potential molecular mechanism of HD-14 exhibited anti-inflammatory features through modulating PPAR- γ . In Fig. 4 A, B, results of immunofluorescence and Western blot showed that the expression of PPAR- γ was effectively decreased by PPAR- γ -RNAi. Moreover, results of Western blot, quantitative data and ELISA assay revealed that observably increased of protein, mRNA levels and production of TNF- α , IL-1 β and IL-6 with PPAR- γ -RNAi treatment, additionally, effects of HD-14 attenuated inflammatory response was substantially weakened in LPS-induced RAW264.7 cells (Shown in Fig. 4C, D and E). On the contrary, In Fig. 4 F, G, immunofluorescence and Western blot suggest that the expression of PPAR- γ was significantly increased with pEX-2-PPAR- γ treatment,

results of Western blot, quantitative data and ELISA assay investigated that dramatically decreased of protein, mRNA levels and production of TNF- α , IL-1 β and IL-6, anti-inflammatory effects significantly promoted with pEX-2-PPAR- γ . Moreover, effects of HD-14 attenuated inflammatory response remarkably pronounced with PPAR- γ over-expression in LPS-induced RAW264.7 cells (Shown in Fig. 4H, I and J). Herein, these results suggest that HD-14 inhibited inflammatory response may by targeting PPAR- γ .

3.5. HD-14 suppressed p-JAK1 and p-STAT1 expression through activating PPAR- γ expression

Previous studies indicated that a potential role of JAK1/STAT1 signaling pathway in inflammatory response by contributes to inhibit inflammatory mediators. Our studies founded that HD-14 significantly decreased the phosphorylation and activation of JAK1 and STAT1 through up-regulating PPAR- γ . In Fig. 5A, Results of Western blot indicated that p-JAK1 and p-STAT1 were significantly increased in primary KCs isolated from mice with ALI. However, HD-14 inhibited the protein expression of p-JAK1 and p-STAT1 with a concentration-dependent manner. Furthermore, the protein levels of p-JAK1 and p-STAT1 were significantly up-regulated with PPAR- γ -RNAi treatment in LPS-induced RAW264.7 cells. However, the phosphorylation and activation of JAK1 and STAT1 obvious dropped with pEX-2-PPAR- γ treated in LPS-induced RAW264.7 cells. These findings suggested that the JAK1 and STAT1 induced inflammatory signaling pathway may

involve in PPAR- γ modulate at least in part.

4. Discussion

Inflammation is a major component of the initiation and exacerbation of ALI (Srungaram et al., 2015). Macrophage is one of the key mediators which regulate the progress of hepatic inflammation (Han et al., 2016). Furthermore, Increasing reports indicate that liver macrophages play an important role in modulating inflammation through phagocytosis cytokine production, and antigen presentation (Chen et al., 2014; Wijesundera et al., 2014). In response to microenvironmental signals, It has been reported that long-term pro-inflammatory cytokines, such as TNF- α , IL-6, and IL-1 β , are strongly associated with the development of acute and chronic inflammation (Murakami and Ohigashi, 2007). It is now well accepted that mediators, attenuating inflammatory response, has therapeutic potential in treatment of ALI (Ambade et al., 2012).

The present study demonstrated treatment of hesperetin not only effectively reduced the secretion of TNF- α , IL-6, and IL- β , but also decreased the expression of iNOS and COX-2 in LPS-induced RAW264.7 cells (Ren et al., 2016). However, no detailed analysis supports the anti-inflammatory feature of hesperetin in mice with CCl₄-induced ALI. Herein, the molecular mechanism of action remains to be elucidated. More importantly, hesperetin has a lower bioavailability in the gastrointestinal tract and conjugated during absorption (Fisher, 1978). On the basis of these strategies, our laboratory synthesized a series of hesperetin derivatives to active

the anti-inflammatory features of hesperetin by improve its water solubility, bioavailability. In the current study, we showed that HD-14 has a direct inhibitory effect on inflammation in mice with CCl₄-induced ALI and LPS-treated RAW264.7 cells.

Peroxisome proliferator-activated receptors (PPARs) are members of a nuclear receptor superfamily containing PPAR- α , PPAR- β/δ , and PPAR- γ (Straus and Glass, 2007). With respect to immunoregulation, PPAR- γ activation has been shown to repress LPS-, phorbol ester-, and cytokine-induced activation of a number of inflammatory genes, such as iNOS, gelatinase B, scavenger receptor A, cyclooxygenase-2, TNF- α , IL-1, IL-6, and chemokines (Harris et al., 2002; Janabi, 2002; Jiang et al., 1998; Marx et al., 2000; Ricote et al., 1998; Simonin et al., 2002). Moreover, Emerging evidence has demonstrated that activation of PPAR- γ attenuates production of various inflammatory mediators in macrophages (Ricote and Glass, 2007). The PPAR- γ agonist inhibited inflammatory responses through mediating reduction in phosphorylation leads to the suppression of JAK-STAT-dependent (Park et al., 2003). JAK-1, the kinase that activates STAT-1 in adipocytes (Waite et al., 2001). The activation of JAK family kinases leads to the phosphorylation of the receptor subunits, which can recruit STAT1 leads to phosphorylation, nuclear translocation, and regulate gene expression (Zvonic et al., 2004). In this study, our results suggested that HD-14 has direct active effect on the expression of PPAR- γ , in which JAK1/STAT1 signaling pathway may be involved.

In the present study, our findings suggested that level of PPAR- γ was decreased

in mice with CCl₄-induced ALI and LPS-treated RAW264.7 cells compared to control group. Interestingly, HD-14 exhibited anti-inflammatory effects by acting as an agonist of PPAR- γ . Furthermore, HD-14 dramatically inhibited the phosphorylation of JAK1 and STAT1 in primary kupffer cells isolated from mice with ALI and LPS-treated RAW264.7 cells. Moreover, the phosphorylation and activation of JAK1 and STAT1 obvious reduced when treated with pEX-2-PPAR- γ , conversely, phosphorylation of JAK1 and STAT1 were increased by blocking the expression of PPAR- γ .

5. Conclusion

Taken together, HD-14 has direct inhibitory effect on acute liver injury via attenuating macrophages derived pro-inflammatory cytokines production. At least in part, HD-14 acts as an effectively anti-inflammatory modulator via a mechanism involving the PPAR- γ activation pathway. The mechanism may attributable to its effect on the JAK1/STAT1 pathway.

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Fig 1

A

Chemical structure of Hesperetin ($C_{16}H_{14}O_6$)

Chemical structure of Hesperetin derivative-14 ($C_{34}H_{32}Cl_2N_2O_6$)

B

C

D

Western blot analysis showing the expression of TNF- α (26 KDa), IL-6 (23 KDa), and IL-1 β (31 KDa) in H9c2 cells treated with CCl₄ and Silymarin. β -actin (42 KDa) is used as a loading control. The lanes are labeled: Control, CCl₄, Low, Middle, High, and Silymarin. The blots show that CCl₄ treatment increases the expression of all three cytokines, and Silymarin treatment reduces this increase in a dose-dependent manner.

Fig 2

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Fig 5

Figure legends

Fig.1. Hepato-protective effects of HD-14 on mice with CCl₄-induced ALI, n=6 mice per group. **A.** Molecular structural formula of hesperetin and hesperetin derivative-14. **B.** Representative images from C57/BL-6 mice fresh livers in Control, CCl₄, HD-14 treatment group and Silymarin treatment group. **C.** Pathology observation of mice liver tissues sections stained with hematoxylin and eosin (H&E), Representative views from each group are presented (×100). **D.** Effect of different concentrations of HD-14 and Silymarin on serum ALT and AST levels of each group. **E.** Effect of different concentrations of HD-14 and Silymarin on CCl₄-induced protein levels of TNF- α , IL-1 β and IL-6 by Western blot in primary KCs. **F.** Effect of different concentrations of HD-14 and Silymarin on CCl₄-induced mRNA levels of TNF- α , IL-1 β and IL-6 by Real-time q-PCR in primary KCs. **G.** Effect of different concentrations of HD-14 and Silymarin on CCl₄-induced production levels of TNF- α , IL-1 β and IL-6 by ELISA assay in primary KCs. Results of Western blot, quantitative data and ELISA assay showed that HD-14 (100 mg/kg, 200 mg/kg) pretreatment significantly attenuated the expression of pro-inflammatory cytokines. Additionally, HD-14 treatment group (200 mg/kg) showed similar Hepato-protective and anti-inflammatory effects compared with Silymarin (100 mg/kg) group in mice with CCl₄-induced ALI. Data represent the mean $\pm$ S.D. for at least three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to the DMSO control. #P<0.05, ##P<0.01, ###P<0.001 compared to the DMSO + CCl₄ treatment. HD-14: hesperetin derivative-14; Low: HD-14(50 mg/kg) + CCl₄; Middle: HD-14(100 mg/kg)

+ CCl₄; High: HD-14(200 mg/kg) + CCl₄.

Fig.2. Anti-inflammatory effects of HD-14 on LPS-induced RAW264.7 cells. A.

Effect of different concentrations of HD-14 on viability of RAW264.7 cells determined by MTT assay. **B.** Effect of different concentrations of HD-14 on LPS-induced protein levels of TNF- α , IL-1 β and IL-6 by Western blot in RAW264.7 cells. **C.** Effect of different concentrations of HD-14 on LPS-induced mRNA levels of TNF- α , IL-1 β and IL-6 by Real-time q-PCR in RAW264.7 cells. **D.** Effect of different concentrations of HD-14 on LPS-induced production levels of TNF- α , IL-1 β and IL-6 by ELISA assay in RAW264.7 cells. Results of Western blot, quantitative data and ELISA assay showed that HD-14 in the concentration of 2 μ M was unable to significantly suppressed inflammatory response in RAW264.7 cells with a concentration-dependent manner. Data represent the mean $\pm$ S.D. for at least three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to the DMSO control. #P<0.05, ##P<0.01, ###P<0.001 compared to the DMSO + LPS treatment. HD-14: hesperetin derivative-14.

Fig.3 HD-14 increased expression of PPAR- γ in mice with ALI and LPS-induced

RAW264.7 cells. A. The expression of PPAR- γ was analyzed by immunohistochemical (IHC) staining analysis. Representative views from each group are presented ($\times 100$). **B.** Effect of different concentrations of HD-14 and Silymarin on CCl₄-induced protein and mRNA levels of PPAR- γ by Western blot and Real-time

q-PCR in primary KCs. Data represent the mean $\pm$ S.D. for at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the DMSO control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to the DMSO + CCl₄ treatment. HD-14: hesperetin derivative-14. Low: HD-14(50 mg/kg) + CCl₄; Middle: HD-14(100 mg/kg) + CCl₄; High: HD-14(200 mg/kg) + CCl₄. **C.** Effect of different concentrations of HD-14 on LPS-induced protein and mRNA levels of PPAR- γ by Western blot and Real-time q-PCR in RAW264.7 cells. **D.** Effect of HD-14 on the expression of PPAR- γ (green) in LPS-induced RAW264.7 cells detected by Immunofluorescence staining, 4,6-diamidino-2-phenylindole (DAPI) stained nuclei blue. Results of Western blot and quantitative data showed that the expression of PPAR- γ dramatically alleviated whereas HD-14 was unable to significantly activated PPAR- γ expression in mice with CCl₄-induced ALI and LPS-treated RAW264.7 cells with concentration of 8 μ M and 200 mg/kg. Results of immunofluorescence (IF) assay indicated that HD-14 in the concentration of 8 μ M largely increased the expression of PPAR- γ compared to the LPS treatment group. Representative views from each group are presented ($\times 100$). Data represent the mean $\pm$ S.D. for at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the DMSO control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to the DMSO + LPS treatment. HD-14: hesperetin derivative-14.

Fig.4 HD-14 reduced TNF- α , IL-1 β and IL-6 expression by targeting PPAR- γ .

Results of Western blot (A) showed that the expression of PPAR- γ was effectively

reduced in RAW264.7 cells by PPAR- γ -RNAi. **(B, C)**. Essential role of PPAR- γ blockade in the effects of HD-14 on protein levels of TNF- α , IL-1 β , IL-6 and PPAR- γ in LPS-induced RAW264.7 cells. **(D, E)**. Essential role of PPAR- γ blockade in the effects of HD-14 on mRNA levels of TNF- α , IL-1 β , IL-6 and PPAR- γ in LPS-induced RAW264.7 cells. **F**. Essential role of PPAR- γ blockade in the effects of HD-14 on production levels of TNF- α , IL-1 β and IL-6 in LPS-induced RAW264.7 cells. Results of Western blot, quantitative data and ELISA assay showed that blockade of PPAR- γ expression observably increased pro-inflammatory cytokines levels, moreover, anti-inflammatory feature of HD-14 was remarkably weakened when knockdown the expression of PPAR- γ . Data represent the mean $\pm$ S.D. for at least three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to the DMSO control. #P<0.05, ##P<0.01, ###P<0.001 compared to the DMSO + LPS treatment. ¢P<0.05, ¢¢P<0.01, ¢¢¢P<0.001 compared to the control. &P<0.05, &&P<0.01, &&&P<0.001 compared to the NC. NC: Scrambled-RNAi; siRNA: PPAR- γ -RNAi.

G. Results of Western blot showed that the expression of PPAR- γ was effectively increased in RAW264.7 cells by pEX-2-PPAR- γ . **H**. Essential role of PPAR- γ up-regulated in the effects of HD-14 on protein levels of TNF- α , IL-1 β , IL-6 and PPAR- γ in LPS-induced RAW264.7 cells. **D**. Essential role of PPAR- γ up-regulated in the effects of HD-14 on mRNA levels of TNF- α , IL-1 β , IL-6 and PPAR- γ in LPS-induced RAW264.7 cells. **E**. Essential role of PPAR- γ up-regulated in the effects of HD-14 on production levels of TNF- α , IL-1 β and IL-6 in LPS-induced RAW264.7 cells. On the contrary, Over-expression of PPAR- γ remarkably increased

anti-inflammatory effects, and anti-inflammatory effects of HD-14 was largely pronounced in LPS-treated RAW264.7 cells. Data represent the mean $\pm$ S.D. for at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the DMSO control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to the DMSO + LPS treatment. $^{\text{a}}P < 0.05$, $^{\text{ab}}P < 0.01$, $^{\text{abc}}P < 0.001$ compared to the DMSO control. $^{\text{s}}P < 0.05$, $^{\text{ss}}P < 0.01$, $^{\text{sss}}P < 0.001$ compared to the pEX-2. HD-14: hesperetin derivative-14. pEX-2: pEX-2-control; PPAR- γ : pEX-2-PPAR- γ .

Fig.5 HD-14 suppressed p-JAK1 and p-STAT1 expression through activating

PPAR- γ expression. A. Effect of different concentrations of HD-14 and Silymarin on

CCl₄-induced protein levels of p-JAK1 and p-STAT1 by Western blot in primary KCs.

Results of Western blot showed that HD-14 (50 mg/kg, 100 mg/kg, 200 mg/kg) and

Silymarin(100 mg/kg) inhibited the protein expression of p-JAK1 and p-STAT1 in

primary KCs from ALI. Data represent the mean $\pm$ S.D. for at least three independent

experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the DMSO control.

$P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to the DMSO + CCl₄ treatment. HD-14:

hesperetin derivative-14. Low: HD-14(50 mg/kg) + CCl₄; Middle: HD-14(100 mg/kg)

+ CCl₄; High: HD-14(200 mg/kg) + CCl₄. **(B, C)** Essential role of PPAR- γ blockade

or up-regulated in the effects of HD-14 on protein levels of p-JAK1 and p-STAT1 in

LPS-induced RAW264.7 cells. Results of Western blot showed that HD-14 inhibited

phosphorylation and activation of JAK1 and STAT1 protein by activating PPAR- γ at

least in part in LPS-induced RAW264.7 cells. Data represent the mean $\pm$ S.D. for at

least three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to the DMSO control. #P<0.05, ##P<0.01, ###P<0.001 compared to the DMSO + LPS treatment. &P<0.05, &&P<0.01, &&&P<0.001 compared to the NC. \$P<0.05, \$\$P<0.01, \$\$\$P<0.001 compared to the pEX-2. HD-14: hesperetin derivative-14; NC: Scrambled-RNAi; siRNA: PPAR- γ -RNAi; pEX-2: pEX-2-control; PPAR- γ : pEX-2-PPAR- γ .

Gene	Forward	Reverse
TNF- α	5'-CACCACCATCAAGGACTCAA-3'	5'-AGGCAACCTGACCACTCTCC-3'
IL-1 β	5'-CTTTGAAGTTGACGGACCC-3'	5'-TGAGTGATACTGCCTGCCTG-3'
IL-6	5'-GAGGATACCACTCCCAACAGACC-3'	5'-AAGTGCATCATCGTTGTTTCATACA-3'
PPAR- γ	5'-CCCAATGGTTGCTGATTACA-3'	5'-GGACGCAGGCTCTACTTTGA-3'
GAPDH	5'-GGACCTCATGGCCTACATGG-3'	5'-TAGGGCCTCTCTTGCTCAGT-3'

Table 1. Primers sequences used for Real-time q-PCR (mouse)