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18α-Glycyrrhetinic acid monoglucuronide as anti-inflammatory agent through suppression of NF-κB and MAPK signaling pathway†

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† Electronic supplementary information (ESI) is available: Experimental procedures for compound synthesis, characteristic data, ^{1}H and ^{13}C NMR spectra, HRMS assays for Glycyrrhizin analogs. Details for the biological evaluation. Table of cytotoxicity analysis data. For ESI and other electronic format see DOI:

Bo Li and Yongan Yang contributed equally to this work.

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ABSTRACT: Based on SAR analysis of Glycyrrhizin, 18α-glycyrrhetinic acid monoglucuronide (18α-GAMG) with strong inhibition against LPS-induced NO and IL-6 production in RAW264.7 cells was discovered. Western blotting and immunofluorescence showed that 18α-GAMG reduced the expression of iNOS, COX-2, and MAPKs, as well as activation of NF-κB in LPS-stimulated RAW264.7 cells. The further *in vivo* results showed that 18α-GAMG could significantly improve pathological changes of CCl_4-induced hepatic fibrosis.

KEYWORDS: *Glycyrrhizin, anti-inflammatory activity*, *structure-activity relationship, signaling pathway, hepatic fibrosis*

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Introduction

Licorice, the roots and rhizomes of *Glycyrrhiza* species, is a natural sweetener and used as a traditional herbal medicine for the treatment of inflammation, gastric ulcers, liver disease, adrenal insufficiency, and tumours.[1–3] Glycyrrhizin (Glycyrrhizic acid, 18β-GA) is the major bioactive component in licorice with diverse pharmacological activities.[4–7] Researches showed that 18β-GA can promote maturation of murine dendritic cells (DCs), regulate interleukin (IL)-2, IL-10, IL-12, tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2),[8-11] down-regulate the production of IL-8 and eotaxin-1 in human lung fibroblast cells.[12] Glycyrrhizin also could prevent enteritis by reducing nuclear factor-κB (NF-κB) p65 and p38 mitogen-activated protein kinase (p38MAPK) expression in rat, attenuated neuroinflammation and oxidative stress in rotenone model of Parkinson's disease, and inflammatory response of isoflurane-induced cognitive deficits in neonatal rats.[13-15] The anti-inflammatory effect of 18β-GA may be due to directly binding to high-mobility group box 1 protein and thus inhibiting its chemoattractant and mitogenic activities.[16,17] These results indicated that 18β-GA could be used as immune modulators which precisely regulate the cellular immunity.

As a natural triterpene glycoside, 18β-GA contains one molecule of 18β-H-oleanane-type aglycone and two molecules of glucuronic acid (Fig. 1). 18β-Glycyrrhetinic acid (18β-GCCS), the aglycone of 18β-GA, is the active metabolite by intestinal bacteria after oral administration.[18] 18β-GA and 18β-GCCS exhibited similar anti-inflammatory effects by inhibiting the production of LPS-induced nitric oxide (NO), prostaglandin E_2 (PGE_2), TNF-α, IL-6, IL-1β and intracellular reactive oxygen species (ROS), reducing the expression of pro-inflammatory genes (iNOS and COX-2), and significantly blocking activation of transcription factors such as NF-κB and PI3K.[19,20] 18β-GA could be metabolized in the liver or be transformed *via* enzymolysis to 18β-glycyrrhetinic acid mono-glucuronide (18β-GAMG).[21–23] 18β-GAMG showed similar (or stronger) antitumor, antivirus, and anti-inflammatory activities to 18β-GA.[24–27] 18α-glycyrrhizin (18α-GA), a D/E-*trans*-stereoisomer, exhibited anti-inflammatory and anticancer activities by inhibiting the activation of key arachidonic acid (AA) metabolism

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enzymes including phospholipase A_2 (PLA_2), cyclooxygenase (COXs), and 5-lipoxygenase (LOX), and release of AA pathway-generated inflammatory lipid mediators.[28–30] Therefore, all glycyrrhizin analogues (Fig. 1, including metabolite and isomer) exhibited the anti-inflammatory activities, which prompted us to analyze structure-activity relationships (SAR) of 18β-GA.

In this paper, we have synthesized Glycyrrhizin analogs with different glucuronide unit and/or 18-α/β-stereoisomer to investigate the SAR and pharmacological mechanism of Glycyrrhizin as anti-inflammatory agent. Then their anti-inflammatory activities *in vitro* and *in vivo* were further evaluated.

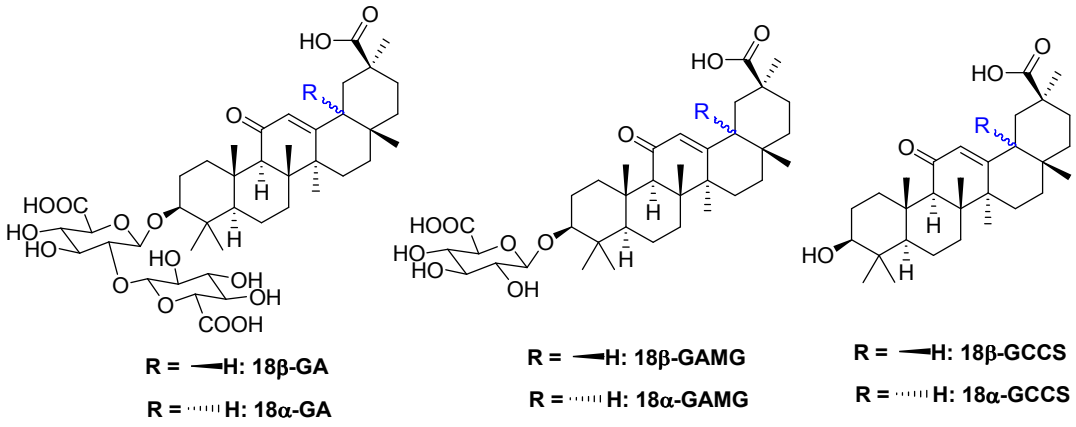

Fig. 1 Chemical structures of Glycyrrhizin analogs.

Results and discussion

Glycyrrhizin analogs were synthesized and characterized by NMR and ESI-MS, their details synthetic and structural information are located in the Supporting Information (Scheme 1).

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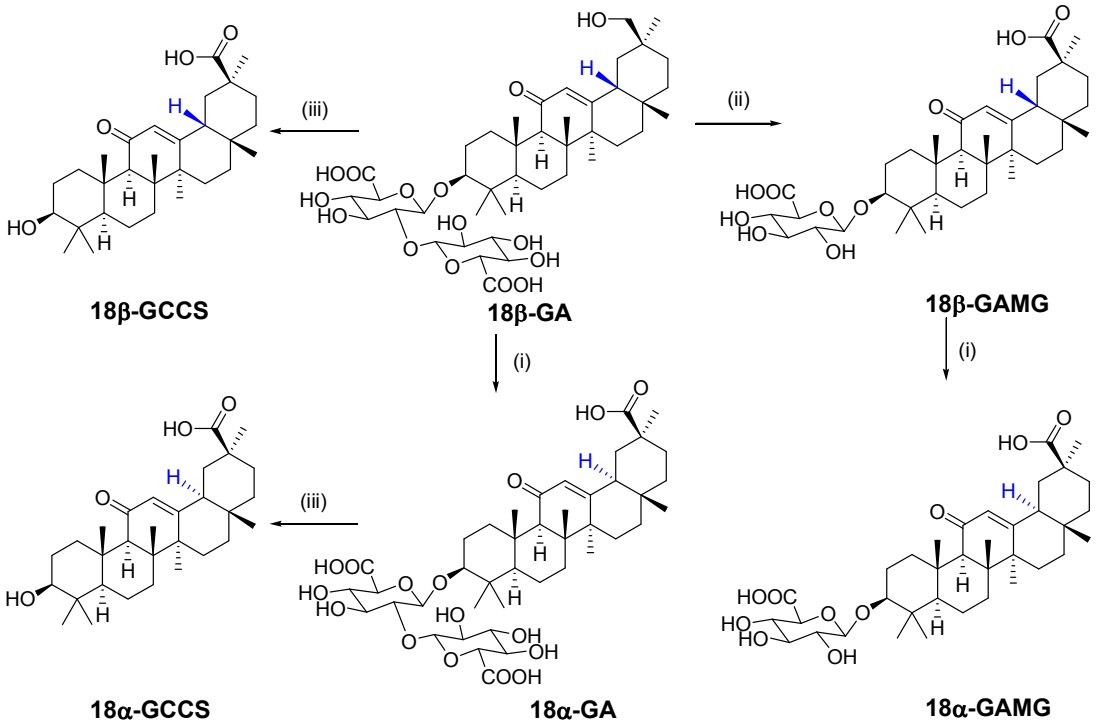

Scheme 1 Synthesis of Glycyrrhizin analogs. *Reagents and conditions:* (i) NaOH solution (5.0 M), 90 °C, 12 h. (ii) β-glucuroidase; (iii) AcOH, 5 N HCl, 100 °C.

To evaluate the anti-inflammatory effect of Glycyrrhizin analogs, Griess reagent was used to detect the level of LPS-induced NO release in RAW264.7 cells. Excessive release of NO is regarded as an important factor in inflammatory responses.[31] As shown in Fig. 2, after Glycyrrhizin analogs treatment, the increase of LPS-induced NO release was significantly alleviated in RAW264.7 cells. The SAR analysis showed: (i) the anti-inflammatory activity of 18α-epimer of the oleanane-type aglycone was superior to that of 18β-epimer (18α-GA > 18β-GA, 18α-GAMG > 18β-GAMG, 18α-GCCS > 18β-GCCS); (ii) the number of glucuronic acid at the C-3 position had effect on the anti-inflammatory activity (mono-glucuronide > aglycone > bis-glucuronide, such as 18β-GAMG > 18β-GCCS > 18β-GA, 18α-GAMG > 18α-GCCS > 18α-GA). Glycyrrhizin analogs displayed preferable anti-inflammatory activity, among them, 18α-GAMG exhibited the strongest activity, and the NO inhibition rate exceeded 70% at the concentration of 40 μM.

The cytotoxicity of Glycyrrhizin analogs was evaluated by MTT assay in RAW264.7

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cells. As shown in Table S1, Glycyrrhizin analogs and LPS showed a low toxicity, and the relative cell viabilities treated with them were all more than 96%. These results indicated that Glycyrrhizin analogs did not possess significant cytotoxic effects against the cells at the used concentrations.

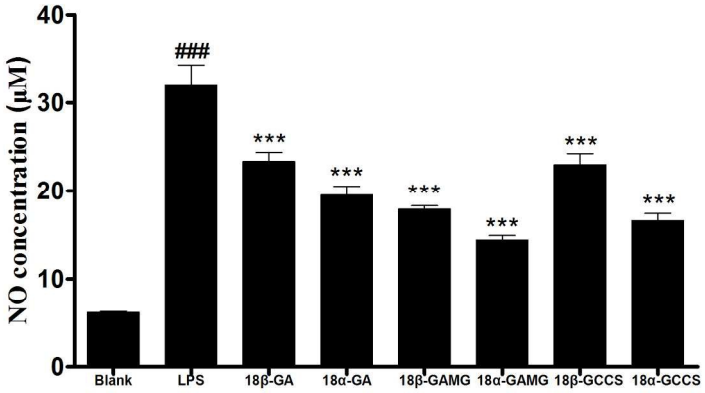

Fig. 2 The inhibitory effects of Glycyrrhizin analogs on NO production in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with Glycyrrhizin analogs (40 μM) for 2 h, and then in the presence or absence of LPS (1 μg/mL) for 20 h. The results were showed as means ± SD (n=3) of at least three independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the blank group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with LPS-stimulated group.

To further evaluate the effects of Glycyrrhizin analogs on LPS-induced IL-6 production, RAW264.7 cells were cultured with LPS (1 μg/mL) in the presence of Glycyrrhizin analogs for 20 h, and the levels of IL-6 in the supernatant were determined by ELISA. As shown in Fig. 3, the LPS-induced IL-6 production was decreased after Glycyrrhizin analogs treatment, and inhibitory effects with the above conclusions were observed: (i) for inhibition of IL-6 production, 18α-epimer was better than 18β-epimer (18α-GA > 18β-GA, 18α-GAMG > 18β-GAMG, 18α-GCCS ≈ 18β-GCCS); (ii) the glucuronic acid' number had effect on the anti-inflammatory activity (mono-glucuronide > aglycone > bis-glucuronide, such as 18β-GAMG > 18β-GCCS > 18β-GA, 18α-GAMG > 18α-GCCS > 18α-GA). Thus, combining with the anti-inflammatory activity and cytotoxicity of Glycyrrhizin analogs, 18α-GAMG was selected to further explore the mechanisms of the anti-inflammatory effect.

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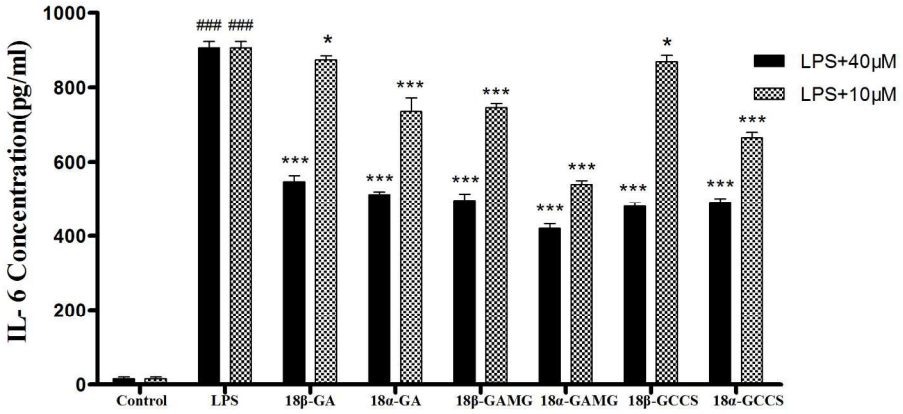

Fig. 3 The inhibitory effects of Glycyrrhizin analogs on LPS-induced IL-6 production in RAW264.7 cells. RAW264.7 cells were incubated with Glycyrrhizin analogs (40 μM and 10 μM) for 2 h, and then in the presence or absence of LPS (1 μg/mL) for 20 h. The results were showed as means ± SD (n = 3) of at least three independent experiments. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with the control group; $^{*}p < 0.05$, $^{***}p < 0.001$ compared with LPS-stimulated group.

18α-GAMG was the most effective in inhibiting NO and IL-6 production. Thus, it was used to investigate the expression of inflammation-related proteins.[32,33] The expression of nitric oxide synthase (iNOS) and COX-2 was examined in LPS-stimulated RAW264.7 cells. Western blotting showed that 18α-GAMG strongly attenuated the expression of iNOS and COX-2 in LPS-stimulated RAW264.7 cells in a dose-dependent manner (Fig. 4). These preliminary results demonstrated that 18α-GAMG may participate in signaling pathways activated by LPS in macrophages.

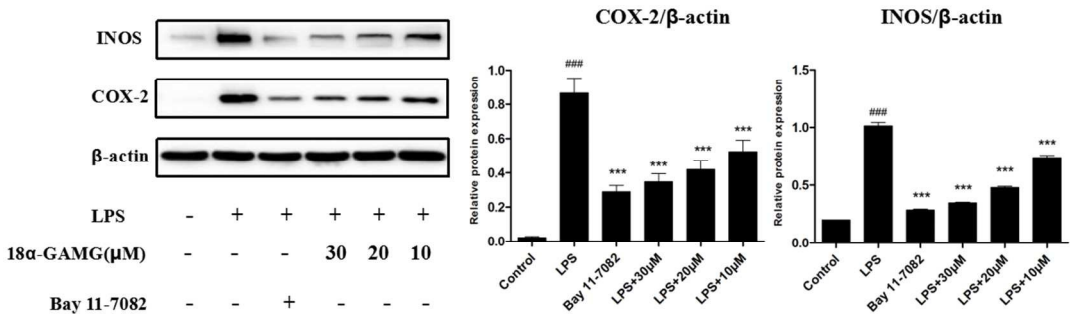

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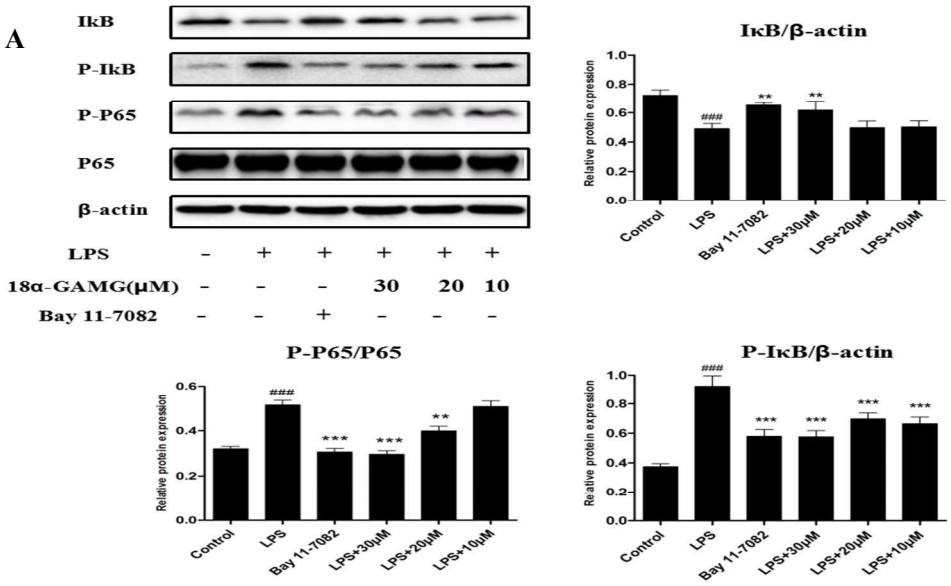
Fig. 4 Effects of 18α-GAMG on LPS-induced iNOS and COX-2 gene expression in RAW264.7 cells. The cells were treated with different concentrations of 18α-GAMG, and then in the presence or absence of LPS (1 μg/mL) for 20 h. Bay 11-7082 is the NF-κB inhibitors (20 μM). The results were showed as means ± SD (n = 3) of at least three independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with LPS-stimulated group.

NF-κB is a well-known transcription factor that positively regulates inflammatory genes such as iNOS, COX-2, and IL-6 in response to inflammatory stimuli.[34] NF-κB activation is controlled by phosphorylation and degradation of IκB-α, a cognate regulatory subunit of NF-κB.[35] Therefore, western blotting was used to examine the effects of 18α-GAMG on NF-κB pathways in LPS-stimulated RAW 264.7 cells. As shown in Fig. 5A, LPS significantly increased the levels of phosphorylated NF-κB p65, IκBα and 18α-GAMG treatment could attenuate activation of these proteins in varying degrees.Furthermore, the nuclear translocation of NF-κB was examined in LPS-stimulated RAW264.7 cells. Immunofluorescence analysis showed that 18α-GAMG clearly inhibited NF-κB p65 nuclear translocation from the cytosol to the nucleus (Fig. 5B).These results further confirmed that 18α-GAMG might regulate the expression of pro-inflammatory proteins through inhibition of NF-κB signaling pathways.



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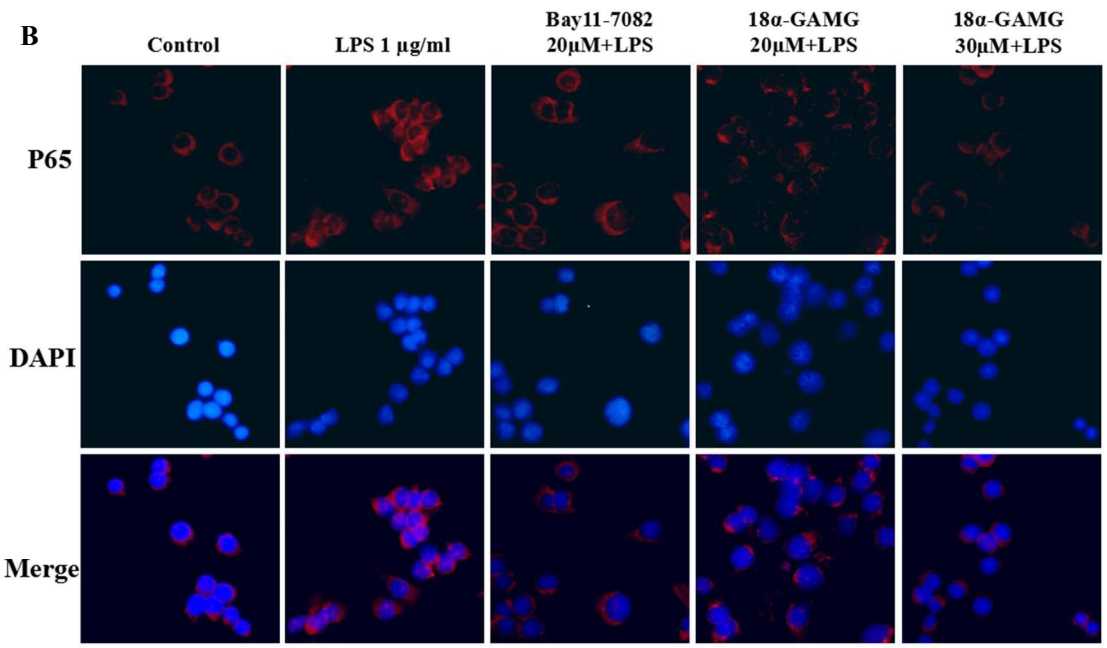

Fig. 5 **A:** 18α-GAMG suppressed LPS-induced activation of NF-κB signaling pathway in RAW 264.7 cells. RAW264.7 cells were treated with 18α-GAMG (10 μM – 30 μM) and LPS (1 μg/mL) for 30 min. The levels of NF-κB p65, IκB, and their phosphorylated forms were analyzed using western blotting. Bay 11-7082 is the NF-κB inhibitors (20 μM). The results were showed as means ± SD (n = 3) of at least three independent experiments. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with LPS-stimulated group.

B: 18α-GAMG clearly inhibited NF-κB p65 nuclear translocation. RAW264.7 were pretreated with 18α-GAMG for 1 h and then stimulated with LPS (1 μg/mL) for 3 h.

The mitogen-activated protein kinases (MAPK) transduction pathway is activated by NF-κB in mammalian cells.[36,37] Inhibiting the activation of MAPK down-regulates the expression of inflammatory mediators and thus improve the outcome of experimental inflammatory diseases.[38,39] To determine the role of 18α-GAMG in modulating MAPK activation in LPS-stimulated RAW264.7 cells, the expression of ERK, JNK, and p38 was examined. As expected (Fig. 6), levels of phosphorylation of p38, JNK, and ERK were increased after LPS-stimulated for 30 min. 18α-GAMG dose-dependently (10, 20, and 30 μM) inhibited LPS-induced phosphorylation of ERK, but had little effect on phosphorylation of JNK or p38 in RAW264.7 cells. These results suggested that the

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anti-inflammatory activity of 18α-GAMG might be associated with its negative effects on ERK activation.

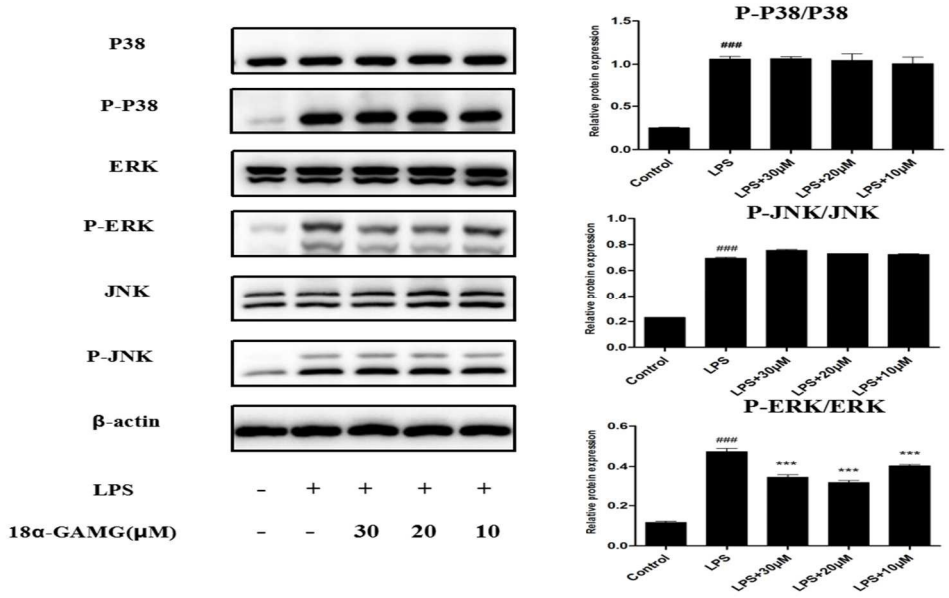

Fig. 6 18α-GAMG suppressed LPS-induced activation of MAPK signaling pathway in RAW 264.7 cells. RAW264.7 cells were treated with 18α-GAMG (10 μM – 30 μM) and LPS (1 μg/mL) for 30 min. The levels of JNK, ERK1/2, and p38 MAPK proteins, and their phosphorylated forms were analyzed using western blotting. The results were showed as means ± SD (n = 3) of at least three independent experiments. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with LPS-stimulated group.

In order to further verify the anti-inflammatory activity of 18α-GAMG *in vivo*, mice model of CCl_4-induced hepatic fibrosis was established in this study to investigate its effect on hepatic fibrosis.[40] Healthy C57BL6 mice (SPF, male, 20 ± 2 g) were purchased from the experimental animal center of Anhui Medical University. Animals were housed in a temperature (22 ± 2 °C) and relatively humidity (50%)-controlled room on a 12 h light/dark cycle, given free access to food and water, and acclimatized for at least one week prior to use. All the animal experiments were performed in accordance with the Regulations of the Experimental Animal Administration issued by the State Committee of Science and Technology of China. Efforts were made to minimize the number of animals used and their suffering. Animals were maintained in accordance with the Guides of

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Center for Developmental Biology, Anhui Medical University for the Care and Use of Laboratory Animals and all experiments used protocols approved by the institutions' subcommittees on animal care.

As shown in Fig. 7, in the control group, the structure of the liver was clear, and the size of hepatocytes was constant. The hepatic lobule was intact, without denaturation or necrosis (Fig. 7A, 7F). In the model group, the amount of blue collagen fibers was obviously increased. The fatty degeneration was apparent and ballooning degeneration of hepatocyte can be seen in model group (Fig. 7B, 7G). The extent of inflammatory cell infiltration, blue collagen fibers and fibrosis of liver in colchicine group (0.1 mg/kg), high-dose 18α-GAMG group and low-dose 18α-GAMG were significant decreased, and high-dose group was better than low-dose group (Fig. 7C, 7D, 7E, 7H, 7I, 7J). These results showed that 18α-GAMG could significantly improve pathological changes of CCl_4-induced hepatic fibrosis.

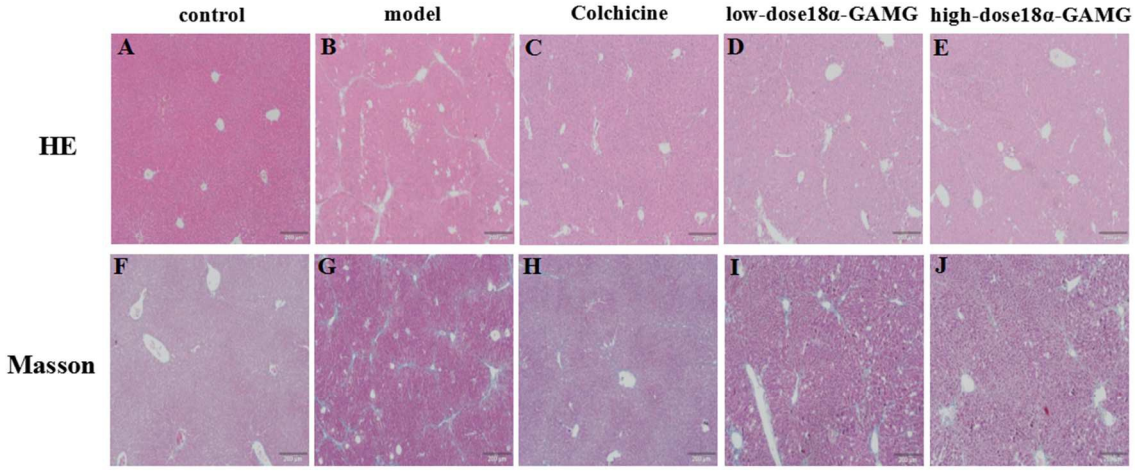

Fig. 7 Typical light photomicrographs of liver tissue showing influence of 18α-GAMG on histological changes (H&E and Masson staining). (**7A-7E**) represented H&E staining of the control group, model group, colchicine group (0.1 mg/kg), low-dose 18α-GAMG group (100 mg/kg), and high-dose 18α-GAMG group (200 mg/kg), respectively. (**7F-7J**) represented Masson staining of the control group, model group, colchicine group (0.1 mg/kg), low-dose 18α-GAMG group (100 mg/kg), and high-dose 18α-GAMG group (200 mg/kg), respectively.

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Conclusions

In conclusion, the clear structure-activity relationships of glycyrrhizin with anti-inflammatory activity was explained, among them, glucuronide unit and 18-α/β-stereoisomer are important factors. In these compounds, 18α-glycyrrhetinic acid monoglucuronide (18α-GAMG) with the strongest inhibition was found. Western blotting and immunofluorescence showed that 18α-GAMG decreased the expression of iNOS, COX-2, and MAPKs, as well as activation of NF-κB in LPS-stimulated RAW264.7 cells. Taken together, 18α-GAMG exerted its anti-inflammatory activity through inhibition of NO generation as a result of inhibiting NF-κB and MAPKs-related inflammatory signaling pathways. In addition, the *in vivo* results showed that 18α-GAMG could significantly improve pathological changes of CCl_4-induced hepatic fibrosis. Therefore, 18α-GAMG may be clinically useful for the reduction of inflammation in the future.

Acknowledgements

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Conflict of Interest

The authors declare no competing interests.

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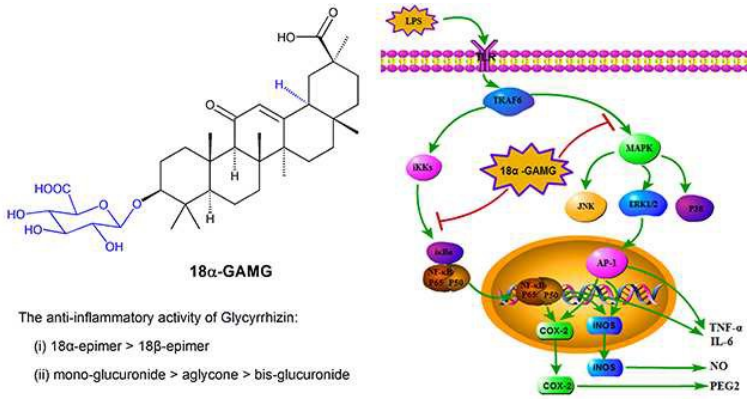

18α-GAMG exhibited strong anti-inflammatory activity through inhibiting the expression of iNOS, COX-2, and MAPKs, as well as activation of NF-κB.