


 Cite this: *Med. Chem. Commun.*,
2017, 8, 1498

18 α -Glycyrrhetic acid monoglucuronide as an anti-inflammatory agent through suppression of the NF- κ B and MAPK signaling pathway^{†‡}

 Bo Li,^a Yongan Yang,^b Liuzeng Chen,^a Shichao Chen,^a
Jing Zhang^{*c} and Wenjian Tang ^{*a}

 Received 26th April 2017,
Accepted 28th May 2017

DOI: 10.1039/c7md00210f

rsc.li/medchemcomm

Based on the SAR analysis of glycyrrhizin, 18 α -glycyrrhetic acid monoglucuronide (18 α -GAMG) with strong inhibition against LPS-induced NO and IL-6 production in RAW264.7 cells was discovered. Western blotting and immunofluorescence results showed that 18 α -GAMG reduced the expression of iNOS, COX-2, and MAPKs, as well as activation of NF- κ B in the LPS-stimulated RAW264.7 cells. Further *in vivo* results showed that 18 α -GAMG could significantly improve the pathological changes of CCl₄-induced hepatic fibrosis.

Introduction

Licorice, the roots and rhizomes of the *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, 18 β -GA) is the major bioactive component in licorice with diverse pharmacological activities.^{4–7} Researches showed that 18 β -GA can promote the maturation of murine dendritic cells (DCs) and can 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} additionally, it can down-regulate the production of IL-8 and eotaxin-1 in human lung fibroblast cells.¹² Moreover, glycyrrhizin could prevent enteritis by reducing the nuclear factor- κ B (NF- κ B) p65 and p38 mitogen-activated protein kinase (p38MAPK) expression in rat, attenuated neuroinflammation and oxidative stress in the rotenone model of Parkinson's disease, and inflammatory response of isoflurane-induced cognitive deficits in neonatal rats.^{13–15} The anti-inflammatory effect of 18 β -GA may be due to direct bind-

ing to high-mobility group box 1 protein, thus inhibiting its chemoattractant and mitogenic activities.^{16,17} These results indicated that 18 β -GA could be used as immune modulators, which precisely regulate cellular immunity.

As a natural triterpene glycoside, 18 β -GA contains one molecule of 18 β -H-oleanane-type aglycone and two molecules of glucuronic acid (Fig. 1). 18 β -Glycyrrhetic acid (18 β -GCCS), the aglycone of 18 β -GA, is the active metabolite produced by the intestinal bacteria after the oral administration of 18 β -GA.¹⁸ 18 β -GA and 18 β -GCCS exhibited similar anti-inflammatory effects by inhibiting the production of LPS-induced nitric oxide (NO), prostaglandin E₂ (PGE₂), TNF- α , IL-6, IL-1 β , 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- κ B and PI3K.^{19,20} 18 β -GA could be metabolized in the liver or be transformed *via* enzymolysis to 18 β -glycyrrhetic acid mono-glucuronide (18 β -GAMG).^{21–23} 18 β -GAMG showed (or stronger) antitumor, antiviral, and anti-inflammatory activities similar to those of 18 β -GA.^{24–27} 18 α -Glycyrrhizin (18 α -GA), a *D/E-trans*-stereoisomer, exhibited anti-inflammatory and anticancer activities by inhibiting the activation of key arachidonic acid (AA) metabolism enzymes including phospholipase A₂ (PLA₂), 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 anti-inflammatory activities, which prompted us to analyze the structure–activity relationships (SAR) of 18 β -GA.

In this study, we synthesized glycyrrhizin analogs with different glucuronide units and/or 18- α/β -stereoisomer to investigate the SAR and pharmacological mechanism of glycyrrhizin

^a School of Pharmacy, Anhui Medical University, Hefei 230032, China.

E-mail: ahmupharm@126.com; Fax: (+86) 551 65161115;

Tel: (+86) 551 65161115

^b Elion Nature Biological Technology Co., Ltd, Nanjing 210038, China

^c Anhui Prevention and Treatment Center for Occupational Disease, Anhui No. 2 Province People's Hospital, Hefei 230022, China. E-mail: hfzj2552@163.com

[†] Bo Li and Yongan Yang contributed equally to this work.

[‡] Electronic supplementary information (ESI) available: Experimental procedures for compound synthesis, characteristic data, ¹H and ¹³C NMR spectra, HRMS assays for glycyrrhizin analogs. Details for the biological evaluation. Table of cytotoxicity analysis data. See DOI: 10.1039/c7md00210f

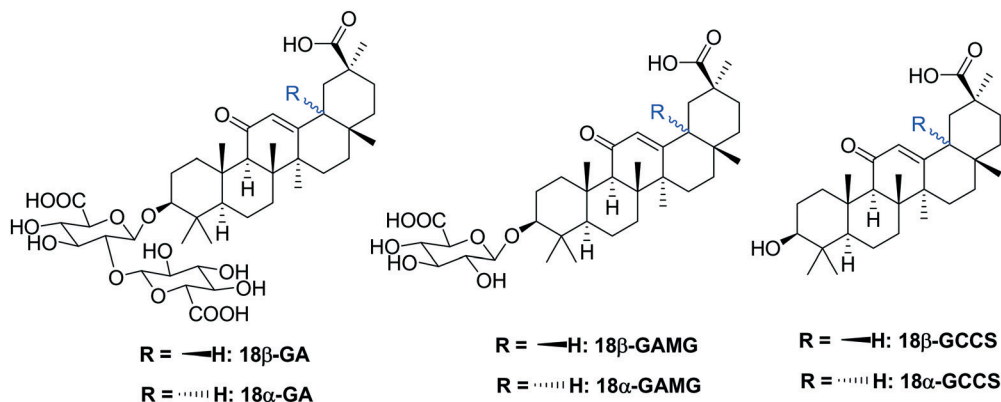
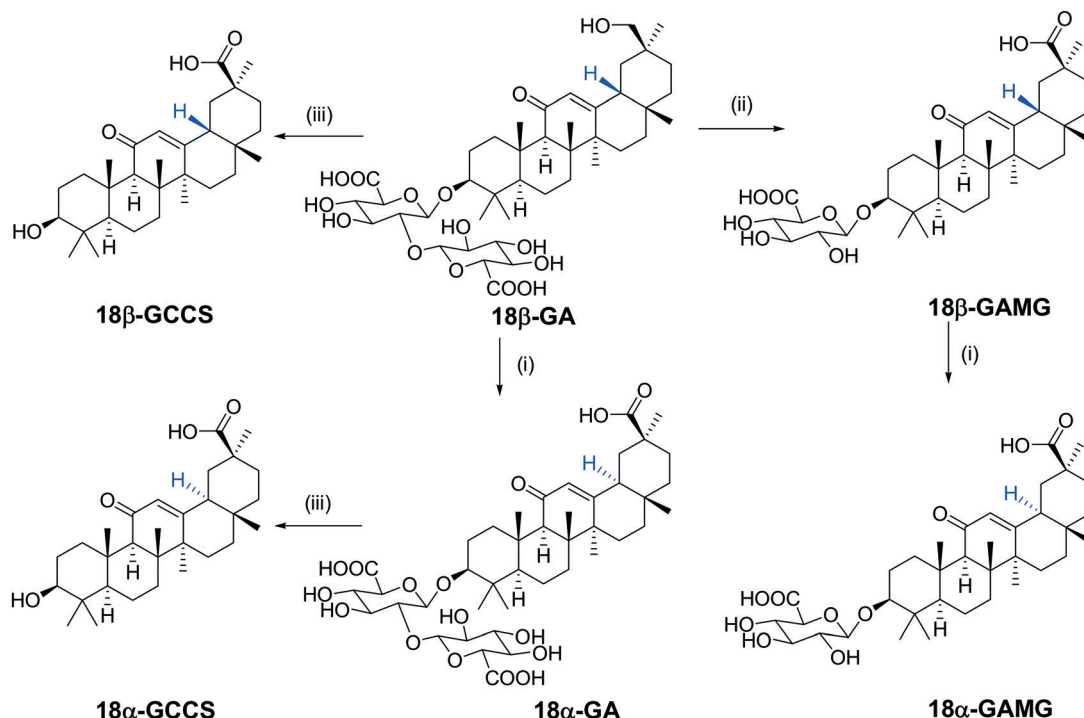


Fig. 1 Chemical structures of the glycyrrhizin analogs.



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

as an anti-inflammatory agent. Then, their anti-inflammatory activities *in vitro* and *in vivo* were further evaluated.

Results and discussion

Glycyrrhizin analogs were synthesized and characterized *via* NMR and ESI-MS, and their detailed synthetic and structural information are provided in the ESI† (Scheme 1).

To evaluate the anti-inflammatory effect of the glycyrrhizin analogs, Griess reagent was used to detect the level of the LPS-induced NO release in the RAW264.7 cells. Excessive release of NO is regarded as an important factor in inflammatory responses.³¹ As shown in Fig. 2, after treatment with glycyrrhizin analogs, the increase in the LPS-induced NO re-

lease was significantly alleviated in the RAW264.7 cells. SAR analysis showed that (i) the anti-inflammatory activity of 18 α -epimer of the oleanane-type aglycone was superior to that of 18 β -epimer (18 α -GA > 18 β -GA, 18 α -GAMG > 18 β -GAMG, 18 α -GCCS > 18 β -GCCS) and (ii) the number of glucuronic acids at the C-3 position had an effect on the anti-inflammatory activity (mono-glucuronide > aglycone > bis-glucuronide, such as 18 β -GAMG > 18 β -GCCS > 18 β -GA, 18 α -GAMG > 18 α -GCCS > 18 α -GA). Glycyrrhizin analogs displayed preferable anti-inflammatory activity; among them, 18 α -GAMG exhibited the strongest activity, and the NO inhibition rate exceeded 70% at a concentration of 40 μ M.

The cytotoxicity of the glycyrrhizin analogs was evaluated by the MTT assay in the RAW264.7 cells. As shown in Table

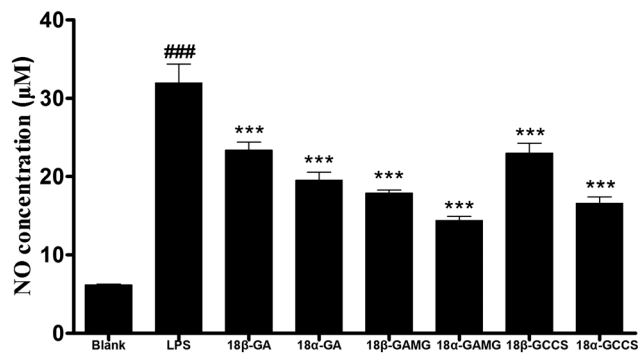


Fig. 2 The inhibitory effects of the glycyrrhizin analogs on the NO production in the LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with glycyrrhizin analogs (40 μM) for 2 h and then in the presence or absence of LPS (1 $\mu\text{g mL}^{-1}$) for 20 h. The results have been shown as means \pm 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.

S1,[†] glycyrrhizin analogs and LPS showed low toxicity, and the relative viabilities of the cells treated with them were more than 96%. These results indicated that glycyrrhizin analogs did not possess significant cytotoxic effects against the cells at the concentrations used herein.

To further evaluate the effects of glycyrrhizin analogs on the LPS-induced IL-6 production, RAW264.7 cells were cultured with LPS (1 $\mu\text{g mL}^{-1}$) in the presence of glycyrrhizin analogs for 20 h, and the levels of IL-6 in the supernatant were determined *via* ELISA. As shown in Fig. 3, the LPS-induced IL-6 production decreased after treatment with the glycyrrhizin analogs, and inhibitory effects were observed with the following conclusions: (i) for the inhibition of the IL-6 production, 18 α -epimer was better than 18 β -epimer (18 α -GA > 18 β -GA, 18 α -GAMG > 18 β -GAMG, 18 α -GCCS \approx 18 β -GCCS); (ii) the number of glucuronic acids had an effect on the anti-inflammatory activity (mono-glucuronide > aglycone > bis-glucuronide, such as 18 β -GAMG > 18 β -GCCS >

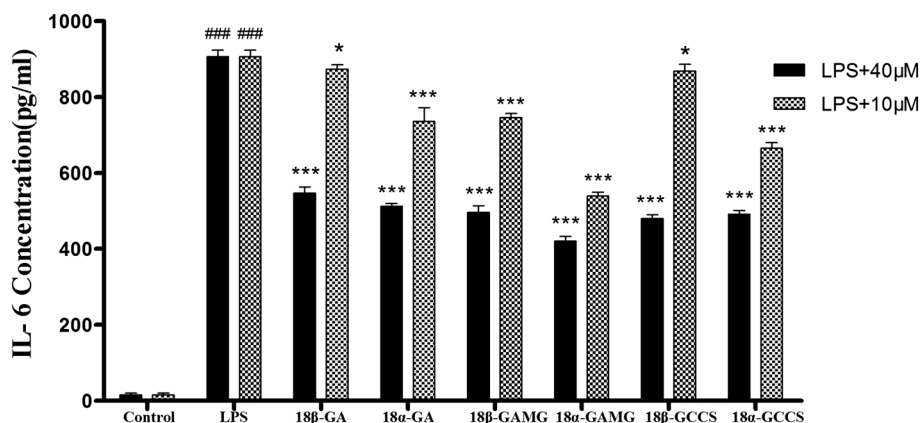


Fig. 3 The inhibitory effects of the glycyrrhizin analogs on the LPS-induced IL-6 production in the RAW264.7 cells. RAW264.7 cells were incubated with glycyrrhizin analogs (40 μM and 10 μM) for 2 h and then in the presence or absence of LPS (1 $\mu\text{g mL}^{-1}$) for 20 h. The results have been shown as means \pm 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.

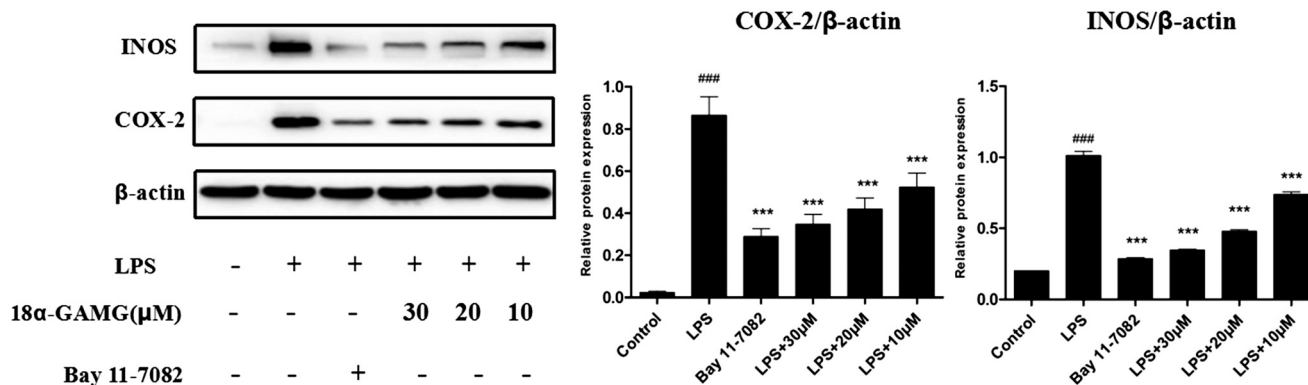


Fig. 4 Effects of 18 α -GAMG on LPS-induced iNOS and COX-2 gene expression in the RAW264.7 cells. The cells were treated with different concentrations of 18 α -GAMG and then in the presence or absence of LPS (1 $\mu\text{g mL}^{-1}$) for 20 h. Bay 11-7082 is the NF- κ B inhibitors (20 μM). The results have been shown as means \pm 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.

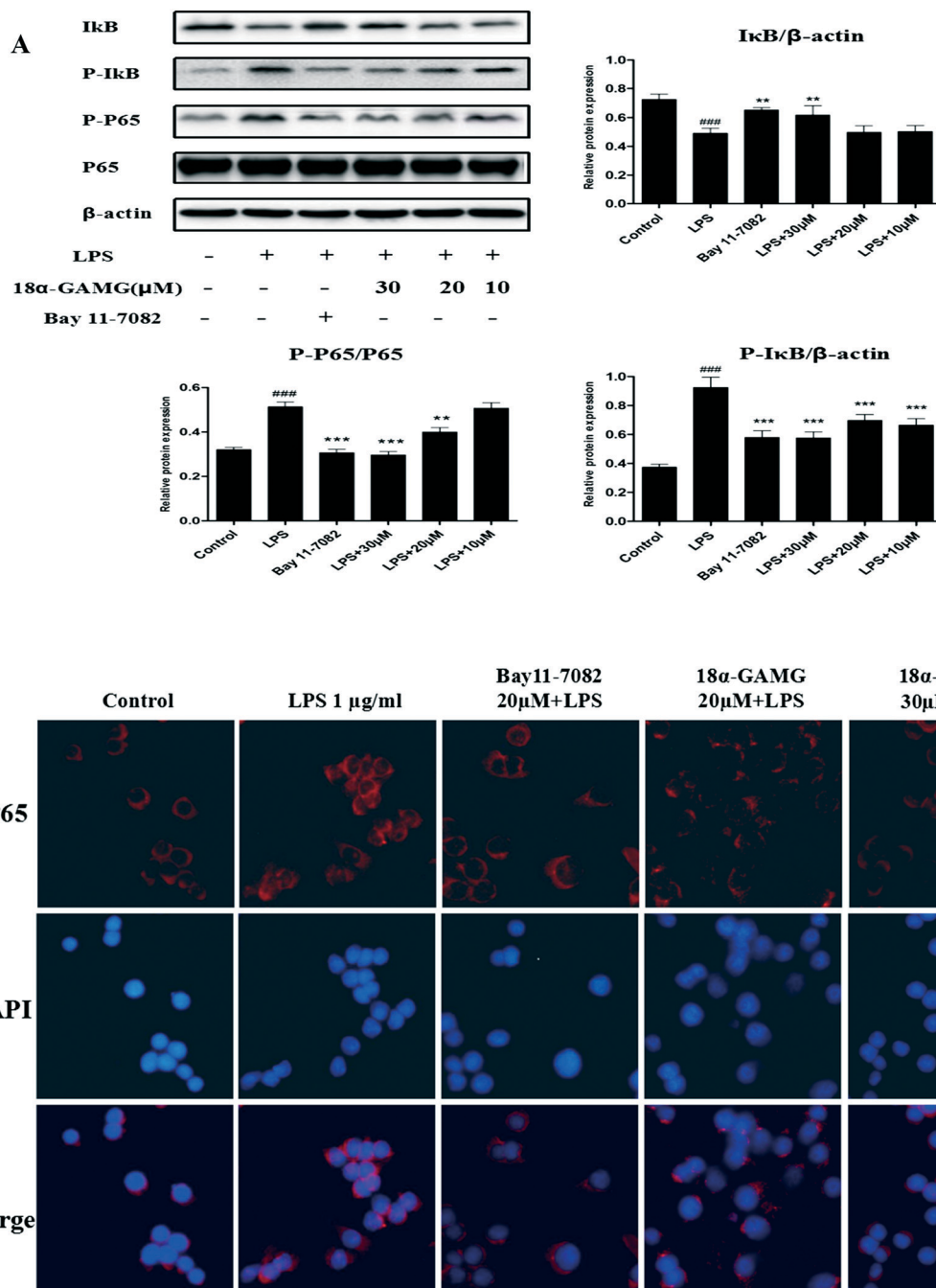


Fig. 5 A: 18α-GAMG suppressed LPS-induced activation of the NF-κB signaling pathway in the RAW264.7 cells. RAW264.7 cells were treated with 18α-GAMG (10–30 μM) and LPS (1 μg mL⁻¹) for 30 min. The levels of NF-κB p65 and IκB, and their phosphorylated forms were analyzed using western blotting. Bay 11-7082 is the NF-κB inhibitors (20 μM). The results have been shown 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⁻¹) for 3 h.

18β-GA, 18α-GAMG > 18α-GCCS > 18α-GA). Thus, combining the anti-inflammatory activity and cytotoxicity of the glycyrrhizin analogs, 18α-GAMG was selected to further explore the mechanisms of the anti-inflammatory effect.

18α-GAMG was most effective in inhibiting the NO and IL-6 production. Thus, it was used to investigate the expression of the inflammation-related proteins.^{32,33} The expression of

nitric oxide synthase (iNOS) and COX-2 was examined in the LPS-stimulated RAW264.7 cells. Western blotting results showed that 18α-GAMG strongly attenuated the expression of iNOS and COX-2 in the 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.

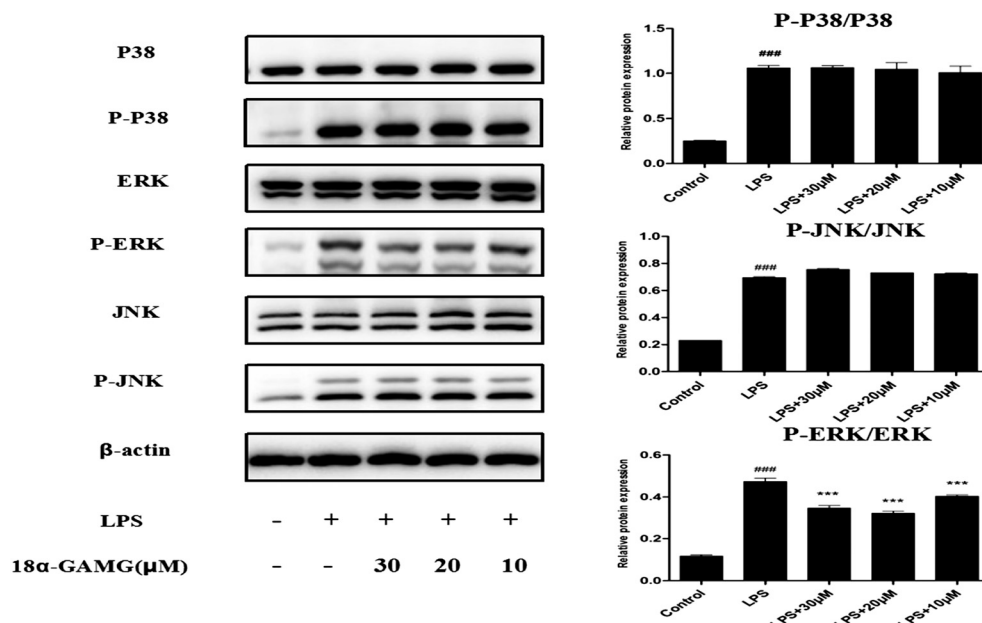


Fig. 6 18α -GAMG suppressed LPS-induced activation of the MAPK signaling pathway in the RAW264.7 cells. RAW264.7 cells were treated with 18α -GAMG (10–30 μ M) and LPS ($1 \mu\text{g mL}^{-1}$) for 30 min. The levels of JNK, ERK1/2, and p38 MAPK proteins, and their phosphorylated forms were analyzed using western blotting. The results have been shown as means \pm 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.

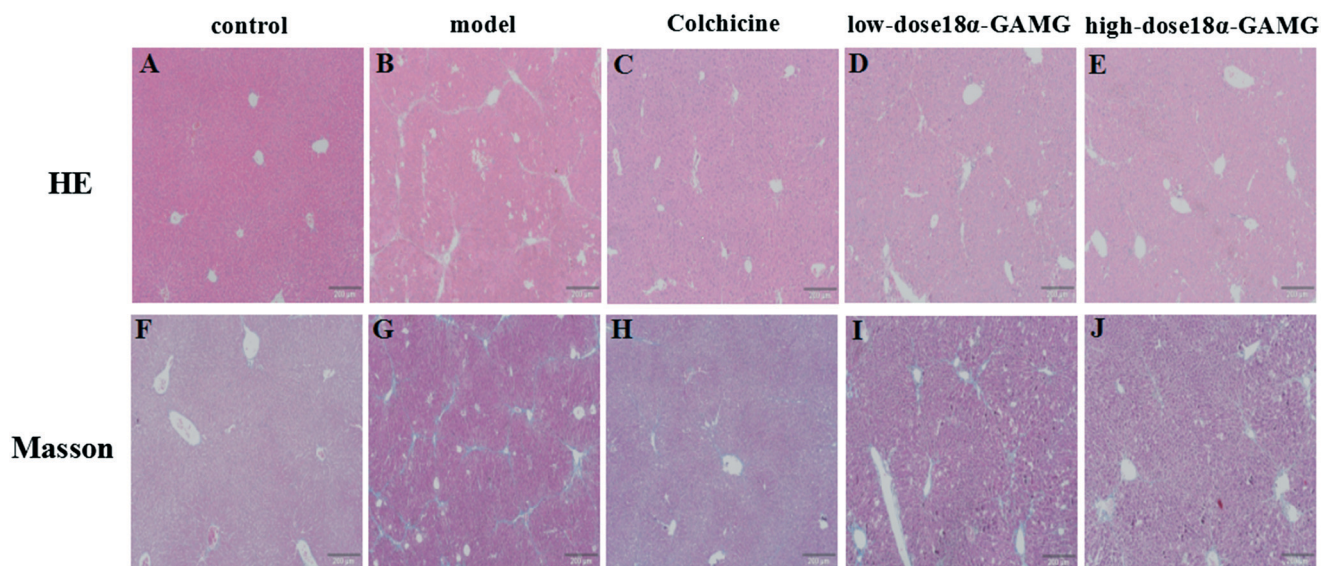


Fig. 7 Typical light photomicrographs of liver tissue showing the influence of 18α -GAMG on histological changes (H&E and Masson staining). (7A–E) represent H&E staining of the control group, model group, colchicine group (0.1 mg kg^{-1}), low-dose 18α -GAMG group (100 mg kg^{-1}), and high-dose 18α -GAMG group (200 mg kg^{-1}), respectively. Fig. 7F–J represent the Masson staining of the control group, model group, colchicine group (0.1 mg kg^{-1}), low-dose 18α -GAMG group (100 mg kg^{-1}), and high-dose 18α -GAMG group (200 mg kg^{-1}), respectively.

NF- κ B 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- κ B activation is controlled by phosphorylation and degradation of I κ B- α , a cognate regulatory subunit of NF- κ B.³⁵ Therefore, western blotting was used to examine the effects of 18α -GAMG on the NF- κ B pathways in the LPS-stimulated RAW264.7 cells. As shown in Fig. 5A, LPS significantly increased the levels of

phosphorylated NF- κ B p65 and I κ B α , and 18α -GAMG treatment could attenuate the activation of these proteins to varying degrees. Furthermore, the nuclear translocation of NF- κ B was examined in the LPS-stimulated RAW264.7 cells. Immunofluorescence analysis showed that 18α -GAMG clearly inhibited NF- κ B 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 the inhibition of the NF- κ B signaling pathways.

The mitogen-activated protein kinase (MAPK) transduction pathway is activated by NF- κ B in mammalian cells.^{36,37} Inhibition of the activation of MAPK down-regulates the expression of the inflammatory mediators and thus improves the outcome of the experimental inflammatory diseases.^{38,39} To determine the role of 18 α -GAMG in modulating MAPK activation in LPS-stimulated RAW264.7 cells, the expression of ERK, JNK, and p38 was examined. As expected (Fig. 6), the levels of phosphorylation of p38, JNK, and ERK increased after LPS-stimulated treatment for 30 min. 18 α -GAMG dose-dependently (10, 20, and 30 μ M) inhibited LPS-induced phosphorylation of ERK, but had little effect on the phosphorylation of JNK or p38 in RAW264.7 cells. These results suggested that the anti-inflammatory activity of 18 α -GAMG might be associated with its negative effects on ERK activation.

To further verify the anti-inflammatory activity of 18 α -GAMG *in vivo*, a mice model of CCl₄-induced hepatic fibrosis was established in this study to investigate its effect on hepatic fibrosis.⁴⁰ Healthy C57BL6 mice (SPF, male, 20 \pm 2 g) were purchased from the Experimental Animal Center of Anhui Medical University. Animals were housed in a temperature (22 \pm 2 $^{\circ}$ C) and relatively humidity (50%)-controlled room under 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 Center for Developmental Biology, Anhui Medical University for the Care and Use of Laboratory Animals, and in all the experiments, protocols approved by the institutions' subcommittees on animal care were used.

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 and F). In the model group, the amount of blue collagen fibers was obviously increased. Fatty degeneration was apparent and ballooning degeneration of the hepatocyte can be seen in the model group (Fig. 7B and G). The extent of inflammatory cell infiltration, blue collagen fibers and fibrosis of liver in the colchicine group (0.1 mg kg⁻¹), the high-dose 18 α -GAMG group, and the low-dose 18 α -GAMG were significant decreased, and the high-dose group was better than the low-dose group (Fig. 7C, D, E, H, I and J). These results showed that 18 α -GAMG could significantly improve the pathological changes of the CCl₄-induced hepatic fibrosis.

Conclusions

In conclusion, the clear structure-activity relationships of glycyrrhizin with anti-inflammatory activity was explained;

among them, the glucuronide unit and the 18 α / β -stereoisomer were important factors. Among these compounds, 18 α -glycyrrhetic acid monoglucuronide (18 α -GAMG) was found to exhibit strongest inhibition. Western blotting and immunofluorescence results showed that 18 α -GAMG decreased the expression of iNOS, COX-2, and MAPKs, as well as the activation of NF- κ B in the LPS-stimulated RAW264.7 cells. Overall, 18 α -GAMG exerted its anti-inflammatory activity through the inhibition of NO generation as a result of inhibition of the NF- κ B and MAPKs-related inflammatory signaling pathways. In addition, the *in vivo* results showed that 18 α -GAMG could significantly improve the pathological changes of CCl₄-induced hepatic fibrosis. Therefore, 18 α -GAMG may be clinically useful for the reduction of inflammation in the future.

Conflict of interest

The authors declare no competing interests.

Acknowledgements

Financial support was provided by the National Natural Science Funding of China (21572003), Anhui University Natural Science Research Project (KJ2016A339), and University Project of Introduction and Cultivation of Leading Talents (gxfzD2016044).

References

- 1 C. Fiore, M. Eisenhut, E. Ragazzi, G. Zanchin and D. Armanini, *J. Ethnopharmacol.*, 2005, **99**, 317–324.
- 2 Monograph, *Altern. Med. Rev.*, 2005, **10**, 230–237.
- 3 M. N. Asl and H. Hosseinzadeh, *Phytother. Res.*, 2008, **22**, 709–724.
- 4 J. K. Kim, S. M. Oh, H. S. Kwon, Y. S. Oh, S. S. Lim and H. K. Shin, *Biochem. Biophys. Res. Commun.*, 2006, **345**, 1215–1223.
- 5 H. J. Cho, S. S. Lim, Y. S. Lee, J. Kim, C. H. Lee, D. Y. Kwon and J. H. Y. Park, *Food Chem.*, 2010, **121**, 959–966.
- 6 J. Y. Yu, J. Y. Ha, K. M. Kim, Y. S. Jung, J. C. Jung and S. Oh, *Molecules*, 2015, **20**, 13041–13054.
- 7 Z. H. Tang, T. Li, Y. G. Tong, X. J. Chen, X. P. Chen, Y. T. Wang and J. J. Lu, *Planta Med.*, 2015, **81**, 1670–1687.
- 8 H. Hua, Z. Liang, W. Li, Y. Meng, X. Li, Z. Zhang, C. Lu, J. Meng and F. Shan, *Int. Immunopharmacol.*, 2012, **12**, 518–525.
- 9 Y. H. Zhang, K. Isobe, F. Nagase, T. Lwin, M. Kato, M. Hamaguchi, T. Yokochi and I. Nakashima, *Immunology*, 1993, **79**, 528–534.
- 10 H. L. Wang, Y. X. Li, Y. T. Niu, J. Zheng, J. Wu, G. J. Shi, L. Ma, Y. Niu, T. Sun and J. Q. Yu, *Inflammation*, 2015, **38**, 2269–2278.
- 11 N. Bordbar, M. H. Karimi and Z. Amirghofran, *Cell. Immunol.*, 2012, **280**, 44–49.
- 12 S. Matsui, H. Matsumoto, Y. Sonoda, K. Ando, E. Aizuyokota, T. Sato and T. Kasahara, *Int. Immunopharmacol.*, 2004, **4**, 1633–1644.

- 13 Y. M. Wang and G. Q. Du, *Mol. Med. Rep.*, 2016, **13**, 3639–3646.
- 14 S. Ojha, H. Javed, S. Azimullah, S. B. Abul-Khair and M. E. Haque, *Neurotoxic. Res.*, 2016, **29**, 275–287.
- 15 W. Wang, X. Chen, J. Zhang, Y. Zhao, S. Li, L. Tan, J. Gao, X. Fang and A. Luo, *Neuroscience*, 2016, **316**, 328–336.
- 16 L. Mollica, F. D. Marchis, A. Spitaleri, C. Dallacosta, D. Pennacchini, M. Zamai, A. Agresti, L. Trisciuglio, G. Musco and M. E. Bianchi, *Chem. Biol.*, 2007, **14**, 431–441.
- 17 D. Du, J. Yan, J. Ren, H. Lv, Y. Li, S. Xu, Y. Wang, S. Ma, J. Qu, W. Tang, Z. Hu and S. Yu, *J. Med. Chem.*, 2013, **56**, 97–108.
- 18 S. Takeda, K. Ishihara, Y. Wakui, S. Amagaya, M. Maruno, T. Akao and K. Kobashi, *J. Pharm. Pharmacol.*, 1996, **48**, 902–905.
- 19 T. C. Kao, M. H. Shyu and G. C. Yen, *J. Agric. Food Chem.*, 2010, **58**, 8623–8629.
- 20 C. Y. Wang, T. C. Kao, W. H. Lo and G. C. Yen, *J. Agric. Food Chem.*, 2011, **59**, 7726–7733.
- 21 T. Makino, K. Okajima, R. Uebayashi, N. Ohtake, K. Inoue and H. Mizukami, *J. Pharmacol. Exp. Ther.*, 2012, **342**, 297–304.
- 22 T. Makino, *Biol. Pharm. Bull.*, 2014, **37**, 898–902.
- 23 S. Feng, C. Li, X. Xu and X. Wang, *J. Mol. Catal. B: Enzym.*, 2006, **43**, 63–67.
- 24 D.-H. Kim, S.-H. Hong, B. T. Kim, E.-A. Bae, H. Y. Park and M. J. Han, *Arch. Pharmacol. Res.*, 2000, **23**, 172–177.
- 25 H. Y. Park, S. H. Park, H. K. Yoon, M. J. Han and D. H. Kim, *Arch. Pharmacol. Res.*, 2004, **27**, 57–60.
- 26 Y. A. Yang, W. J. Tang, X. Zhang, J. W. Yuan, X. H. Liu and H. L. Zhu, *Molecules*, 2014, **19**, 6368–6381.
- 27 W. J. Tang, Y. A. Yang, X. He, J. B. Shi and X. H. Liu, *Sci. Rep.*, 2014, **4**, 7106.
- 28 L. A. Baltina, O. V. Stolyarova, L. A. Baltina, R. M. Kondratenko, O. A. Plyasunova and A. G. Pokrovskii, *Pharm. Chem. J.*, 2010, **44**, 299–302.
- 29 A. V. Shetty, S. Thirugnanam, G. Dakshinamoorthy, A. Samykutty, G. Zheng, A. Chen, M. C. Bosland, A. Kajdacsy-Balla and M. Gnanasekar, *Int. J. Oncol.*, 2011, **39**, 635–640.
- 30 C. Xie, X. Li, J. Wu, Z. Liang, F. Deng, W. Xie, M. Zhu, J. Zhu, W. Zhu, S. Geng and C. Zhong, *Inflammation*, 2015, **38**, 1639–1648.
- 31 F. Aktan, *Life Sci.*, 2004, **75**, 639–653.
- 32 C. Tsatsanis, A. Androulidaki, M. Venihaki and A. N. Margioris, *Int. J. Biochem. Cell Biol.*, 2006, **38**, 1654–1661.
- 33 C. Y. Liew, K. W. Lam, M. K. Kim, H. H. Harith, C. L. Tham, Y. K. Cheah, M. R. Sulaiman, N. H. Lajis and D. A. Israf, *Int. Immunopharmacol.*, 2011, **11**, 85–95.
- 34 T. Lawrence, *Cold Spring Harbor Perspect. Biol.*, 2009, **1**, a001651.
- 35 M. Karin and Y. Ben-Neriah, *Annu. Rev. Immunol.*, 2000, **18**, 621–663.
- 36 S. Ghosh and M. S. Hayden, *Nat. Rev. Immunol.*, 2008, **8**, 837–848.
- 37 Z. S. Wang, L. Z. Chen, H. P. Zhou, X. H. Liu and F. H. Chen, *Bioorg. Med. Chem. Lett.*, 2017, **27**, 1803–1807.
- 38 L. Vermeulen, G. De Wilde, P. Van Damme, W. Vanden Berghe and G. Haegeman, *EMBO J.*, 2003, **22**, 1313–1324.
- 39 K. H. Tse, K. B. Chow, W. K. Leung, Y. H. Wong and H. Wise, *Neuroscience*, 2014, **267**, 241–251.
- 40 A. J. Czaja, *World J. Gastroenterol.*, 2014, **20**, 2515–2532.