Mollugin Inhibits the Inflammatory Response in Lipopolysaccharide-Stimulated RAW264.7 Macrophages by Blocking the Janus Kinase-Signal Transducers and Activators of Transcription Signaling Pathway

Zheng-Guang Zhu,† Hong Jin,† Peng-Jiu Yu, Yuan-Xin Tian, Jia-Jie Zhang, and Shu-Guang Wu*

School of Pharmaceutical Sciences, Southern Medical University; Guangzhou 510515, China.
Received September 13, 2012; accepted December 25, 2012; advance publication released online January 11, 2013

Mollugin, a kind of naphthohydroquinone, is a major constituent isolated from Rubia cordifolia L. and demonstrated to possess anti-inflammatory activity in recent reports. However, the effects and mechanism of action of mollugin in inflammation have not been fully defined. The present study was therefore designed to investigate whether mollugin suppresses the inflammatory response in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. Mollugin attenuated the LPS-induced expression of nitric oxide (NO), inducible nitric oxide synthase (iNOS), interleukin (IL)-1β and IL-6 but augmented the expression of tumor necrosis factor (TNF-α). Mollugin did not inhibit the degradation of inhibitory kappa B (IκB)-α or the nuclear translocation of p65 nuclear factor-kappa B (NF-xB) but rather enhanced the phosphorylation of p65 subunits evoked by LPS. Mollugin did not inhibit the phosphorylation of extracellular-signal-related kinase (ERK) 1/2, p38, and c-Jun N-terminal kinase (JNK) 1/2 either. Mollugin significantly reduced the LPS-mediated phosphorylation of Janus kinase (JAK) 2, signal transducers and activators of transcription (STAT) 1 and STAT3. Molecular docking analysis showed that mollugin binds to JAK2 in a manner similar to that of AG490, a specific JAK2 inhibitor. We conclude that mollugin may be a JAK2 inhibitor and inhibits LPS-induced inflammatory responses by blocking the activation of the JAK-STAT pathway.

Key words mollugin; macrophage; inflammatory mediator; Janus kinase-signal transducer and activator of transcription; nuclear factor-kappa B

Excessive or persistent inflammation can cause dysfunction and damage to tissues. Macrophages play an important role in the pathogenesis of inflammation-related injury and exhibit a particularly vigorous response to lipopolysaccharide (LPS),1 a component of the membranes of Gram-negative bacteria. LPS activates a set of intracellular signaling cascades in stimulated macrophages, including the mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-xB), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways. These signaling pathways then regulate the gene expression of various inflammatory mediators.2–4 LPS-treated macrophages are usually used as a model to explore the effects and molecular mechanisms of potential anti-inflammatory compounds.

The roots of Rubia cordifolia L. have been used in Chinese traditional medicine for centuries and are officially listed in the Chinese Pharmacopoeia. These roots are usually used to treat inflammation of the joints, dysmenorrhea, hematorrhea, hemostasis, and psoriasis.5,6 Mollugin, the major and index component determining the quality of Rubia cordifolia L., was reported to have several pharmacological effects, such as cytotoxic and anti-tumor activities,7 anti-platelet aggregation activity,8 and antiviral activity against hepatitis B virus.9 Recently, Kim and Jeong reported that mollugin might be a new drug candidate for the treatment of inflammatory diseases, such as colon inflammation and neurodegenerative diseases.10,11 However, the study of the anti-inflammatory properties of mollugin is still limited, and its mechanisms of action have not yet been well elucidated. In this study, we investigated the inhibitory effects of mollugin on the expression of inflammatory mediators and the underlying mechanisms of action in LPS-stimulated RAW264.7 cells.

MATERIALS AND METHODS

Reagents Mollugin was provided by Guangzhou Nipudak Bio-technology Co., Ltd., China. Lipopolysaccharide from Escherichia coli 055:B5, typhrostatin AG490, and dimethyl-sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM), Gibco™ fetal bovine serum, penicillin, streptomycin, the Two-step MMLV Platinum SYBR Green qPCR SuperMix-UDG kit and the Novex ECL HrP Chemiluminescent substrate reagent kit were from Invitrogen (San Diego, CA, U.S.A.). The Griess reagent, NaNO2, the bicinechonic acid (BCA) protein assay kit, the Protein Extraction Kit, and the horse-radish peroxidase (HRP)-conjugated and Cy3-conjugated goat-anti-rabbit immunoglobulin G (IgG) antibodies were purchased from the Beyotime Institute of Biotechnology (Haimen, China). Antibodies against inducible nitric oxide synthase (iNOS), p65, p-p65 (Ser536), JAK2, p-JAK2 (Tyr1007/1008), STAT1, p-STAT1 (Tyr701), STAT3, p-STAT3 (Tyr705) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology (Danvers, Massachusetts, U.S.A.). Antibodies against inhibitory kappa B (IxB)-α, and lamin B were obtained from Santa Cruz (Santa Cruz, CA, U.S.A.). Antibodies for p-p38, p-ERK1/2, and p-JNK1/2 were obtained from Bioworld Technology (St. Louis, Park, MN, U.S.A.). Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and IL-6 were from Dakewe Biotech Co., Ltd. (Beijing, China). Mollugin was dissolved in DMSO and used at different concentrations as specified in the text. The final concentration of DMSO never exceeded 0.1%.

The authors declare no conflict of interest. 

†These authors contributed equally to this work.

© 2013 The Pharmaceutical Society of Japan
Cell Culture  The murine macrophage cell line RAW264.7 was purchased from the China Center for Type Culture Collection (Shanghai, China). The cells were cultured in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum. The cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay for Cell Viability  RAW264.7 cells were plated at a density of 1×10⁴ cells/well in 96-well plates overnight, and then treated with different concentrations of mollugin (0–120 µg/mL) for 24 h. At the end of the treatment period, 20 µL MTT solution (5 mg/mL in saline) was added and incubated for another 4 h. After the supernatant was carefully removed, 150 µL DMSO was added for solubilization and the absorbance at 550 nm was measured using a microplate reader (Benchmark plus Bio-Rad Laboratories, CA, U.S.A.). Relative cell viability was calculated compared with the absorbance of the untreated control group.

Nitric Oxide (NO) Quantification  RAW264.7 cells were seeded at 2×10⁶ cells/well in 24-well plates and then incubated with LPS (1 µg/mL) and mollugin (7.5, 15, 30 µM) for 18 h. The level of NO in the culture medium was assayed by quantifying the stable end product of NO oxidation, nitrite (NO₂⁻), using the Griess reagent. Briefly, the incubation medium of the RAW264.7 cells was collected after the treatments and centrifuged at 12000×g for 5 min. Then, 50 µL of supernatant was incubated with 100 µL of Griess reagent for 15 min. The absorbance at 540 nm was measured, and fresh culture medium was used as the blank. The NO concentration in the samples was calculated using a standard curve prepared with sodium nitrite.

Cytokine Assays  RAW264.7 cells were seeded at 2×10⁵ cells/well in 24-well plates for TNF-α, IL-1β and IL-6 assays. The cells were incubated with LPS (1 µg/mL) and mollugin (7.5, 15, or 30 µM) for 18 h. The amounts of TNF-α, IL-1β and IL-6 in the cell culture supernatant were measured using ELISA kits according to the manufacturer’s instructions.

Western Blot Analysis  After RAW264.7 macrophages were co-treated with LPS (1 µg/mL) and mollugin (7.5, 15, or 30 µM), whole-cell, nuclear and cytoplasmic protein samples were prepared using the Protein Extraction Kit (Beyotime) according to the manufacturer’s protocol. The protein concentration was measured using the BCA method. Samples (20–50 µg of total protein) were separated on 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes. After being blocked with 5% nonfat milk in Tris-buffered saline containing Tween 20 (TBST) (0.1%) for 2 h at room temperature, the membranes were incubated with primary antibody and then incubated with the conjugated secondary antibodies. The membrane was incubated with the ECL detection reagent for one minute and then exposed to X-ray film.

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR) Analysis  RAW264.7 cells were seeded at 1×10⁶ cells/well in 12-well plates and then incubated with LPS (1 µg/mL) and mollugin (7.5, 15, 30 µM) for 6 h. Total RNA from RAW264.7 cells was prepared with Trizol reagent according to the manufacturer’s protocol. Two micrograms of total RNA was reverse-transcribed with M-MuLV Reverse Transcriptase Kit to obtain cDNA. Real-time PCR was performed in an ABI 7500 real-time PCR instrument (Applied Biosystems) with the SYBR Green qPCR SuperMix-UDG kit. A melting curve analysis was carried out after amplification to verify the accuracy of the amplification. The comparative threshold cycle (ΔΔCt) method of relative quantification was used to determine the fold-changes in expression. The primer sequences used for the analysis of the IL-1β, IL-6, TNF-α, iNOS, and GAPDH mRNA levels are listed in Table 1.

NF-κB Reporter Gene Dual-Luciferase Assay  According to the manufacturer’s instructions, RAW264.7 cells were transiently transfected with luciferase reporter genes using Lipofectamine 2000 and the pNF-κB-luc reporter plasmid. Briefly, 1 d before transfection, cells were seeded in suspension at a density of 4×10⁵ cells/well in 96-well plates. After incubation overnight, the cells were washed twice with PBS and incubated for 6 h at 37°C in 150 µL of DMEM supplemented with 1 µL of Lipofectamine 2000 and 320 ng of NF-κB-dependent luciferase reporter plasmid. Then, the cells were washed twice with PBS to remove the transfection mixture and were cultured in DMEM containing 10% fetal bovine serum (FBS) overnight. After stimulation with 1.0 µg/mL LPS and 7.5–30 µM mollugin for 8 h, the cells were lysed, and the luciferase activity was determined using the Luciferase Assay Kit according to the manufacturer’s instructions. The luciferase activity was normalized to the protein concentration of the sample.

Docking Analysis of Ligands into the Binding Pocket of JAK2  Molecular docking was performed with Sybyl 7.3. The crystal structure of a JAK2 complex with MI1 was obtained from the Protein Data Bank (PDB entry: 3EYG). To validate the reliability of the docking, the ligand MI1 was removed from the active site and was docked back into the

Table 1. Primers Used for IL-1β, IL-6, TNF-α, iNOS, and GAPDH

| mRNA     | Primer       | Sequence (5'-3')                  | GeneBank accession # | Position | Size (bp) | Efficiency (%) |
|----------|--------------|-----------------------------------|----------------------|----------|-----------|---------------|
| IL-1β    | Sense        | GCAACTGTTCCTCTGAACTCAACT          | NM_008361.3          | 72–93    | 89        | 97.40         |
|          | Antisense    | ATCTTTTGGGTGGCCCTGCACT            | J03783               | 140–160  | 191       | 103.50        |
| IL-6     | Sense        | AGTTGCCCTTCTTGGGACCTGA            | NM_013693.2          | 271–252  | 122       | 98.20         |
| TNF-α    | Antisense    | CAGAATTCGTCATCCAGCAAC             | 305–286              | 108      | 105.60    |
|          | Antisense    | GTCGAGACGTCGAGCCC                 | NM_010927.3          | 215–232  | 122       | 98.20         |
| iNOS     | Antisense    | TCCATGACTCCCTAGCA                 | GU214026.1           | 49–68    | 233       | 102.90        |
| GAPDH    | Antisense    | GGTTAAGGTCTGGTTGAAGCT             | 281–262              |          |           |               |
binding pocket. The root mean square deviation (RMSD) between the predicted conformation and the experimental conformation from the crystal structure of ligand was 0.3 Å, which is smaller than the resolution of the X-ray crystal structure, indicating that the parameter set for the Surflex-dock simulation was suitable for reproducing the X-ray structure. Therefore, the simulation method and the parameter set could be extended to study the binding conformations of other compounds.

At the beginning of the docking simulation, all water molecules and ligands were removed, and the random hydrogen atoms were added. Then, the receptor structure was minimized in 10000 cycles with the Powell method in Sybyl 7.3. After the construction of the compounds, hydrogen and Gasteiger–Hückel charges were added to every molecule. Then, their geometries were optimized using the conjugate gradient method with a TRIPOS force field. The energy convergence criterion was 0.001 kcal/mol. Except for some special notes, default values were chosen to finish this work. The lipophilic potential surface of the protein was calculated with the MOLCADC program in Sybyl 7.3.

**Statistical Analysis** The data are expressed in terms of the mean±S.E.M. Variables were compared between groups using one-way ANOVA followed by the Student–Newman–Keuls test. Two-tailed p values <0.05 were considered statistically significant. Statistical analyses were conducted with SPSS 13.0.

**RESULTS**

**Cytotoxicity of Mollugin on RAW264.7 Cells** MTT assay was used to examine the effect of mollugin on the viability of RAW264.7 cells. No cytotoxicity was observed when the cells were exposed to up to 30 µM mollugin for 24 h (Fig. 1).

**Effects of Mollugin on the Production of Inflammatory Mediators in LPS-Stimulated RAW264.7 Cells** Upon stimulation with LPS, macrophages release hundreds of mediators, among which TNF-α, IL-1β, IL-6 and NO are of particular interest in terms of their contributions to serious systemic complications. To explore the anti-inflammatory effects of mollugin, we determined the effects of mollugin on the mRNA and protein expression of these cytokines and iNOS and on the release of NO in LPS-stimulated RAW264.7 macrophages according to previously reported methods. As depicted in Fig. 2, mollugin markedly reduced the release of NO and the protein and mRNA expression levels of iNOS, IL-1β and IL-6 in a concentration-dependent manner in LPS-stimulated RAW264.7 cells. However, mollugin dose-dependently increased both the protein and mRNA levels of TNF-α.

**Mollugin Does Not Inhibit LPS-Induced NF-κB Pathway Activation** It has been well documented that the LPS-mediated expression of the above inflammatory mediators involves in the activation of the NF-κB pathway. Therefore, we explored whether mollugin could suppress the LPS-mediated activation of the NF-κB pathway.

As shown in Fig. 3, LPS induced the remarkable activation of the NF-κB pathway in RAW264.7 cells, as manifested by the degradation of IκB-α and the nuclear translocation and phosphorylation of p65 NF-κB at 0.5, 1 and 1 h, respectively.

Mollugin did not have any inhibitory effect on these reactions. On the contrary, mollugin was found to enhance LPS-induced NF-κB activation, as revealed by the up-regulation of the phosphorylation of the p65 subunit at Ser 536.

Mollugin has been suggested to be an NF-κB inhibitor because it was recently shown to suppress the transcription of a reporter gene for p65 NF-κB in TNF-α-stimulated HT-29 colonic epithelial cells. We also investigated the effect of mollugin on the expression of a p65 reporter gene in LPS-stimulated macrophages in this study. The results of the luciferase assay indicated that 8-h stimulation with LPS induced a 5.7-fold enhancement in the transcription activity of the p65 reporter gene in RAW264.7 cells, and mollugin only slightly suppressed the transcription of the reporter gene (by 25.9–30.7%) in the dose range of 7.5–30 µM (Fig. 3D).

**Mollugin Dose Not Inhibit the LPS-Mediated Activation of MAPK Pathway** MAPKs cascades are known to be critical for the LPS-stimulated induction of inflammatory mediators in RAW264.7 cells. To investigate whether the inhibition of inflammatory response by mollugin is mediated through the MAPKs pathway, MAPKs phosphorylation was examined by Western blot in RAW264.7 cells pretreated with mollugin and then with LPS according to the published method. As shown in Fig. 4, mollugin did not suppress the LPS-stimulated phosphorylation of ERK1/2, p38, and JNK1/2.

**Mollugin Inhibits the LPS-Mediated Activation of JAK-STAT Pathway** The above results did not support the hypothesis of mollugin as an inhibitor of either NF-κB or MAPK signaling pathway. Then we turned to explore the effect of mollugin on the activation of the JAK-STAT pathway, another inflammatory signaling cascade triggered by LPS.

JAK2, dominantly expressed in macrophages among the four JAK members, was directly activated after RAW264.7 cells were stimulated with LPS, leading to the activation of the downstream molecules STAT1 and STAT3, which mediate the synthesis of inflammatory mediators. The suppression of the activation of JAK2-STATs was reported to result in the inhibition of the LPS-mediated expression of NO, IL-1β and IL-6 but not TNF-α, which is consistent with the anti-inflammatory profile of mollugin found in our study.

To verify the hypothesis that mollugin is a JAK-STAT
Fig. 2. Effect of Mollugin on the Production of Inflammatory Mediators in LPS-Stimulated RAW264.7 Cells

Differently treated RAW264.7 macrophages were incubated for 18h, and then the NO level (A) was measured using the Griess reagent, the protein levels of IL-1β (B), IL-6 (C) and TNF-α (D) were determined by ELISA, and the iNOS protein level (E) was assessed by Western blot analysis. The mRNA levels of iNOS (A), IL-1β (B), IL-6 (C) and TNF-α (D) were determined by real-time PCR analysis after a 6-h incubation. The data represent the mean±S.E.M. from three separate experiments. *p<0.05 or **p<0.01, significant difference compared with LPS alone. A representative blot from three independent experiments is shown.
pathway inhibitor, we first determined the time-course activation of JAK2-STATs induced by LPS in RAW264.7 cells. The results of the Western blot analysis showed that remarkable tyrosine phosphorylation of JAK2, STAT1 and STAT3 appeared at 15 min, 6 h and 6 h, respectively. Then, we investigated the effect of mollugin on the LPS-mediated phosphorylation of JAK2, STAT1 and STAT3 in comparison with AG490, a specific JAK2 inhibitor, at these time points.

**Discussion**

The results of this study showed that mollugin significantly inhibited the release of NO, IL-1β and IL-6 and stimulated the release of TNF-α in LPS-stimulated RAW264.7 macrophages. These effects might result from the actions of mollugin on...
the gene transcription of these mediators because the mRNA expression levels of these cytokines and of iNOS were affected. Previous studies have reported that IL-1β, IL-6 and iNOS, but not TNF-α, contain STAT binding sites in their promoter regions\(^{19,20}\) and that JAK2-STATs play a critical role in the LPS-induced expression of IL-1β, IL-6 and iNOS, with little effect on the expression of TNF-α.\(^{19,21,23,25}\) Therefore, the anti-inflammatory pattern of mollugin supports our finding that mollugin inhibits the LPS-induced activation of the JAK-STAT pathway.

Accumulating studies have indicated that the activation of NF-κB and MAPK pathways is elicited much more rapidly than that of the JAK-STAT pathway upon stimulation with LPS. The phosphorylation and nuclear translocation of p65 NF-κB were reported to peak within 1 h after LPS stimulation,\(^{26,27}\) and a 30-minute exposure to LPS resulted in a significant increase in the phosphorylation of JNK, p38, and ERK.\(^{28}\) The activation of JAK-STAT pathway caused by LPS occurs much later. Consistent with previous reports,\(^{21}\) we found that the tyrosine phosphorylation of STAT1 and STAT3 was detected at 2 h, and at least 6 h was needed to reach the maximum levels of phosphorylation after exposure to LPS. This result implies that the expression of genes that respond early to LPS stimulation may be less dependent on JAK-STAT activation, and at least 2 h is needed for the expression of those genes dependent on STAT activation. Thus, TNF-α, the earliest responding mediator of LPS stimulation included in this study, may be less susceptible to the blockade of JAK-STAT activation because the LPS-induced expression of TNF-α mRNA was reported to reach its peak at approximately 1 h.\(^{29,30}\) Previous studies have indicated that approximately 2 or more hours are needed for the LPS-induced expression of IL-1β, IL-6 and iNOS mRNAs.\(^{26,30}\) Therefore, it is logical to deduce that the inhibitory effect of mollugin on the expression of these inflammatory mediators results from the suppression of JAK-STAT activation.

In the present study, we found that mollugin augmented rather than suppressed the activation of NF-κB, This result does not support the assumption that mollugin is an NF-κB inhibitor.\(^{20}\) Instead, it provides a rational explanation for the stimulatory effect of mollugin on the LPS-induced expression of TNF-α, which might result from the synergetic activation of the NF-κB pathway. However, the result of the luciferase assay revealed that mollugin suppressed the expression of the NF-κB p65 reporter gene after 8 h of LPS stimulation. It is very hard to explain this suppressive effect, since little is known about the role of JAK-STAT signaling pathway in the regulation of NF-κB activation cascade in LPS-mediat-ed inflammatory response.

Kim reported that mollugin strongly inhibited the transcription of the reporter gene of p65 NF-κB in TNF-α-stimulated HT-29 colonic epithelial cells. However, the inhibitory activity of mollugin in LPS-stimulated RAW264.7 macrophages was weak in our study. The underlying mechanisms for this difference remain to be explored. The results of previous studies indicated that TNF-α rapidly elicited the activation of JAK-STAT pathway, and that the phosphorylation of STATs was detected as early as 5 min in murine TNF-α-challenged 3T3-L1 adipocytes and in TNF-α-challenged B cells.\(^{31-33}\) In contrast, the phosphorylation of STATs appeared much later in LPS-stimulated RAW264.7 cells as showed in our study and previous reports.\(^{20}\) So, it will be quite expected to investigate whether the STAT activation is an early event in TNF-α-evoked signaling cascade and regulates the activation of NF-κB pathway in HT-29 cells.

Our results showed that mollugin, similar to AG490, effectively inhibited the phosphorylation of JAK2 induced by LPS. The specific inhibitory activity of mollugin on other JAK members deserves further exploration. Molecular docking analysis revealed that the binding score of mollugin for JAK3 is 6.18 (data not shown), higher than that for JAK2. It is very likely that mollugin can also inhibit the activation of other JAK members. To verify the hypothesis of mollugin as an direct inhibitor of JAK members, the measurement of the specific inhibitory activity of mollugin on the enzymatic activity of JAK members is awaiting to conduct.

Taken together, the results of the present study provide evidence for the first time that mollugin may be a JAK2 inhibitor.
and suppresses the inflammatory reactions of LPS-stimulated RAW264.7 macrophages by blocking the activation of the JAK-STAT pathway. The results of this study will be useful for further investigation of the bioactivity and potential applications of mollugin.

Acknowledgments This study was supported by Grants from International Science and Technology Cooperation Base of Guangdong Provincial Department of Science and Technology (No. 2009B050900006), Science and Technology Planning Project of Guangdong Province (No. 2011B050200006), Science and Technology Bureau of Guangzhou (No. 2010V1-E00531-3), National Science Foundation of China (No. 81173097).

REFERENCES

1) Cohen J. The immunopathogenesis of sepsis. Nature, 420, 885–891 (2002).
2) Choi YH, Jin GY, Li GZ, Yan GH. Corniside suppresses lipopolysaccharide-induced inflammatory mediators by inhibiting nuclear factor-kappa B activation in RAW 264.7 macrophages. Biol. Pharm. Bull., 34, 959–966 (2011).
3) Okugawa S, Ota Y, Kitazawa T, Nakayama K, Yanagimoto S, Tsukada K, Kawada M, Kimura S. Janus kinase 2 is involved in lipopolysaccharide-induced activation of macrophages. Am. J. Physiol. Cell Physiol., 285, C399–C408 (2003).
4) Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell. Signal., 13, 85–94 (2001).
5) Lin ZX, Jiao BW, Che CT, Zuo Z, Mok CF, Zhao M, Ho WK, Tse WP, Lam KY, Fan RQ, Yang ZJ, Cheng CH. Ethyl acetate fraction of the root of Rubia cordifolia L. inhibits keratinocyte proliferation in vitro and promotes keratinocyte differentiation in vivo: potential application for psoriasis treatment. Phytother. Res., 24, 1056–1064 (2010).
6) Tse WP, Cheng CH, Che CT, Zhao M, Lin ZX. Induction of apoptosis underlies the Radix Rubiae-mediated anti-proliferative action on human epidermal keratinocytes: implications for psoriasis treatment. Int. J. Mol. Med., 20, 663–672 (2007).
7) Kim SM, Park HS, Jun DY, Woo HJ, Woo MH, Yang CH, Kim YH. Mollugin induces apoptosis in human Jurkat T cells through endoplasmic reticulum stress-mediated activation of JNK and caspase-12 and subsequent activation of mitochondria-dependent caspase cascade regulated by Bel-2. Toxicol. Appl. Pharmacol., 241, 210–220 (2009).
8) Chung MI, Jou SJ, Cheng TH, Lin CN, Ko FN, Teng CM. Antiplatelet constituents of formosan Rubia akane. J. Nat. Prod., 57, 313–316 (1994).
9) Ho LK, Dong CH, Chen HC, Yeh SF, Chen JM. Inhibition of hepatitis B surface antigen secretion on human hepatoma cells. Compounds from Rubia cordifolia. J. Nat. Prod., 59, 330–333 (1996).
10) Kim KJ, Lee JS, Kwak MK, Choi HG, Yong CS, Kim JA, Lee YR, Lyoo WS, Park YJ. Anti-inflammatory action of mollugin and its synthetic derivatives in HT-29 human colonic epithelial cells is mediated through inhibition of NF-kappaB activation. Eur. J. Pharmacol., 622, 52–57 (2009).
11) Jeong GS, Lee DS, Kim DC, Jahng Y, Son JK, Lee SH, Kim YC. Neuroprotective and anti-inflammatory effects of mollugin via up-regulation of heme oxygenase-1 in mouse hippocampal and microglial cells. Eur. J. Pharmacol., 654, 226–234 (2011).
12) López-Bojórquez LN, Dehesa AZ, Reyes-Terán G. Molecular mechanisms involved in the pathogenesis of septic shock. Arch. Med. Res., 35, 465–479 (2004).
13) Kim YW, Zhao RJ, Park SJ, Lee JR, Cho JJ, Yang CH, Kim SG, Kim SC. Anti-inflammatory effects of liquiritigenin as a consequence of the inhibition of NF-kappaB-dependent iNOS and proinflammatory cytokines production. Br. J. Pharmacol., 154, 154–173 (2008).
14) Won JH, Im HT, Kim YH, Yun KJ, Park HJ, Choi JW, Lee KT. Anti-inflammatory effect of underijasaponin IV through the inhibition of iNOS and COX-2 expression in RAW264.7 macrophages via the NF-kappaB inactivation. Br. J. Pharmacol., 148, 216–225 (2006).
15) Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. Am. J. Physiol. Lung Cell. Mol. Physiol., 290, L622–L645 (2006).
16) Yu PJ, Jin H, Zhang JY, Wang GF, Li JR, Zhu ZG, Tian YX, Wu SY, Xu W, Zhang JJ, Wu SG. Pyracnomicolarins isolated from Peucedanum praeruptorum Dunn suppress lipopolysaccharide-induced inflammatory response in murine macrophages through inhibition of NF-κB and STAT3 activation. Inflammation, 35, 967–977 (2011).
17) Kamezaki K, Shimoda K, Numata A, Matsuda T, Nakayama K, Harada M. The role of Tyk2, Stat1 and Stat4 in LPS-induced endotoxin signals. Int. Immunol., 16, 1173–1179 (2004).
18) Sareila O, Korhonen R, Käppäniemi O, Nieminen R, Kankaanranta H, Molianen E. Janus kinase 3 inhibitor WHI-P154 in macrophages activated by bacterial endotoxin: differential effects on the expression of iNOS, COX-2 and TNF-α. Int. Immunopharmacol., 8, 100–108 (2008).
19) Kou X, Qi S, Dai W, Luo L, Yin Z. Arctigenin inhibits lipopolysaccharide-induced iNOS expression in RAW264.7 cells through suppressing JAK-STAT signal pathway. Int. Immunopharmacol., 11, 1095–1102 (2011).
20) Lee C, Lim HK, Sakong J, Lee YS, Kim JR, Baek SH. Janus kinase-signal transducer and activator of transcription mediates phosphatidic acid-induced interleukin (IL)-Ibeta and IL-6 production. Mol. Pharmacol., 69, 1041–1047 (2006).
21) Samavati L, Rastogi R, Du W, Høtttemann M, Fite A, Franchi L. STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria. Mol. Immunol., 46, 1867–1877 (2009).
22) Chiou WF, Don MJ, Liao JF, Wei BL. Psoralidin inhibits LPS-induced iNOS expression via repressing Syk-mediated activation of PI3K-IAK-IAx signaling pathways. Eur. J. Pharmacol., 650, 102–109 (2011).
23) Tsuyi K, Kim HJ, Shin JS, Kim DH, Cho HJ, Lee SS, Ahn SK, Yun-Choi HS, Lee JH, Seo HG, Chang KC. HO-1 and JAK-2/STAT1 signals are involved in preferential inhibition of iNOS over COX-2 gene expression by newly synthesized tetrahydroisouquinoline alkaloid, CKD712, in cells activated with lipopolysaccharide. Cell. Signal., 20, 1839–1847 (2008).
24) Uto T, Fujii M, Hou DX. 6-(Methylsulfanyl)hexyl isothiocyanate suppresses inducible nitric oxide synthase expression through the inhibition of Janus kinase 2-mediated JNK pathway in lipopolysaccharide-activated murine macrophages. Biochem. Pharmacol., 70, 1211–1221 (2005).
25) Prêle CM, Keith-Magee AL, Murcha M, Hart PH. Activated signal transducer and activator of transcription-3 (STAT3) is a poor regulator of tumour necrosis factor-alpha production by human monocytes. Clin. Exp. Immunol., 147, 564–572 (2007).
26) Furusawa J, Funakoshi-Tago M, Tago K, Mashino T, Inoue H, Sonoda Y, Kasahara T. Licorice significantly suppresses the induction of nitric oxide synthase production by newly synthesized tetrahydroisouquinoline alkaloid, CKD712, in cells activated with lipopolysaccharide. Cell. Signal., 21, 775–785 (2009).
27) Ramana KV, Fadl AA, Tammilli R, Reddy AB, Chopra AK, Srivastava SK. Aldose reductase mediates the lipopolysaccharide-induced release of inflammatory mediators in RAW264.7 murine macrophages. J. Biol. Chem., 281, 33019–33029 (2006).
28) Hsieh IN, Chang AS, Teng CM, Chen CC, Yang CR. Aciculatin inhibits lipopolysaccharide-mediated inducible nitric oxide synthase and cyclooxygenase-2 expression via suppressing NF-κB and JNK/ p38 MAPK activation pathways. J. Biomed. Sci., 18, 28 (2011).
29) Sharif O, Bolshakov VN, Raines S, Newham P, Perkins ND. Transcriptional profiling of the LPS induced NF-xB response in macrophages. *BMC Immunol.*, **8**, 1 (2007).

30) Heller RA, Schena M, Chai A, Shalon D, Bedilion T, Gilmore J, Woolley DE, Davis RW. Discovery and analysis of inflammatory disease-related genes using cDNA microarrays. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 2150–2155 (1997).

31) Miscia S, Marchisio M, Grilli A, Di Valerio V, Centurione L, Sabatino G, Garaci F, Zauli G, Bonvini E, Di Baldassarre A. Tumor necrosis factor alpha (TNF-α) activates Jak1/Stat3-Stat5B signaling through TNFR-1 in human B cells. *Cell Growth Differ.*, **13**, 13–18 (2002).

32) Tanabe K, Matsushima-Nishiwaki R, Yamaguchi S, Iida H, Dohi S, Kozawa O. Mechanisms of tumor necrosis factor-α-induced interleukin-6 synthesis in glioma cells. *J. Neuroinflammation*, **7**, 16 (2010).

33) Guo D, Dunbar JD, Yang CH, Pfeffer LM, Donner DB. Induction of Jak/STAT signaling by activation of the type 1 TNF receptor. *J. Immunol.*, **160**, 2742–2750 (1998).