Original Article
The ginsenoside Rd suppresses LPS-induced inflammation and NF-κB activation in colon cancer cells

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Abstract: Inflammatory bowel disease (IBD) is a chronic inflammatory disease that affect the gastrointestinal tract. It is associated with elevated levels of various inflammatory mediators, including interleukin (IL)-8. In this study, the inhibitory effects of ginsenoside Rd (G-Rd) were evaluated on lipopolysaccharide (LPS)-induced inflammatory responses and the underlying mechanisms in HT-29 human colon epithelial cells. Quantitative PCR was applied to evaluate expression of IL-8, and ELISA was applied to measure the secretion of IL-8. Western blot, EMSA, and IHC were utilized to test regulation of ginsenoside Rd on the NF-κB pathway. Our results show that G-Rd significantly suppressed LPS-induced IL-8 mRNA production. Secretion of the IL-8 protein upon LPS stimulation was also attenuated by G-Rd. Furthermore, G-Rd pretreatment could clearly suppress LPS-induced NF-κB activity, which accounts for the downregulation of IL-8. These results suggest that G-Rd has potent anti-inflammatory effects on the intestine and can be used to treat IBD.

Keywords: Ginsenoside Rd, epithelial-like cells, LPS, IL-8, NF-κB

Introduction

IBD is a chronic inflammatory condition of the gastrointestinal tract and represents a heterogeneous group of chronic, complex, and multifactorial disorders. It results from the disruption of intestinal homeostasis by aberrant immune reactions against microbial and environmental factors, in genetically predisposed individuals. The two most prevalent entities of IBD are ulcerative colitis (UC) and Crohn’s disease (CD) [1]. A steady increase in the incidence of IBD has been observed in recent years [2], and patients with long-term symptoms of IBD can not only develop severe complications but also are at increased risks of colorectal cancer [3]. Although substantial advances have been made in the management of the disease, with the introduction of immune-modulators and monoclonal antibodies, a curative therapy does not yet exist. Many factors influence the response to pharmacotherapy, including disease severity and complications and environmental factors [4]. Thus, there is an urgent need to find novel drugs that will be more effective, or identify new drug targets, in order to treat and cure IBD.

IBD results from the impairment of the intestinal epithelial barrier function and the subsequent defect in adaptive immunity [5]. In the pathogenesis of IBD, accumulating evidence has revealed altered expression of chemokines that activate and attract leukocytes to the site of inflammation [5]. Infiltration and migration of innate immune cells such as neutrophils and monocytes to the tissue lesion sites depend on the levels of cytokines and chemokines, including IL-1β, IL-6, IL-8, and TNF-α. IL-8, which can be produced by monocytes [6] and epithelial cells [5], is an important chemokine. With respect to inflammation, IL-8 is one of the most potent chemoattractants that activates various cellular cytokines for the activation of human neutrophils [7]. It can activate and recruit neutrophils, macrophages, and T lymphocytes at the site of inflammation, releasing more inflammatory mediators, thereby increasing the inflammatory response. IL-8 has been known to play an important role in intestinal
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Figure 1. The chemical structure of G-Rd.

Inflammatory diseases. It has been reported that IL-8 is significantly elevated in the colon tissues of IBD patients [8]. In addition, the degree of inflammation was positively correlated with the expression level of IL-8 [9]. The nuclear factor-kappa B (NF-κB), a common inducible transcription factor, plays a key role in many physiological and pathological processes including the innate and adaptive immune responses, oxidative stress, aging, cancer, and notably in inflammation [10]. The NF-κB signal pathway primarily controls the production of pro-inflammatory cytokines such as IL-8, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and adhesion molecules, in addition to leukocyte recruitment in response to lipopolysaccharide (LPS) signaling, all of which have central roles in the pathogenesis of inflammatory diseases [11]. The NF-κB signaling pathway has been implicated in the pathogenesis of several inflammatory diseases such as IBD [12]. The degree of activation of NF-κB/p65 is closely related to the severity of intestinal inflammation. Several studies have reported a strong expression and activation of NF-κB in the colon of patients suffering from active episodes of IBD. The activation and expression of p65 in the macrophages and epithelial cells isolated from the inflammatory bowel specimens of IBD patients were also increased [13]. Currently, drugs such as corticosteroids, sulfasalazine, methotrexate, and anti-TNFα-antibody, used for the treatment of IBD, work by inhibiting NF-κB activation to exert their anti-inflammatory effects. For example, the increase in IkBα levels induced by corticosteroids can reduce nuclear translocation of NF-κB, and thereby downregulate its activity [12]. Thus, inhibition of NF-κB activation has been proposed as a potential treatment option for IBD.

Ginseng, a perennial plant belonging to the genus Panax of the Araliaceae family, has been used in eastern Asia as a popular herbal medicine for thousands of years. Ginseng is known to promote vitality, prolong life, and is effective against a variety of conditions, including depression, diabetes, fatigue, aging, inflammation, internal degeneration, nausea, tumors, pulmonary problems, dyspepsia, vomiting, nervousness, stress, and ulcers [14, 15]. Ginsenosides and their aglycones, such as ginsenoside (G)-Rb1, compound K, G-Rb2, G-Rd, G-Re, G-Rg1, G-Rg3, G-Rg5, G-Rh1, G-Rh2, and G-Rp1, are the major active pharmacological components of ginseng [15]. Houfu Liu used the extract of pseudo-ginseng, ginsenoside Rd (G-Rd), and 16 other types of pure single products for oral administration in rats. A large number of glycosyl saponins were found in the colon. The major ginsenosides Ra3, Rb1, and Rd were identified in the plasma. The plasma drug concentration was very low and the average bioavailability was only 0.1-0.2% [16]. Other research studies have demonstrated that ginsenoside can accumulate in the colon following an oral administration to rats, and can be metabolized and transformed by specific enzymes in the intestine [17]. These studies suggest that ginsenosides and their metabolites or derivatives have a natural tendency to directly target the mucosa of the colon through its colon-targeting effects, which may offer an attractive therapeutic strategy in view of IBD.

As the main active monomer of ginsenoside, G-Rd (refer Figure 1) has been reported to exhibit anti-inflammatory and neuroprotective effects, mainly by regulating the activities of the NF-κB signaling pathway [18-20]. However, the effect of G-Rd on the anti-inflammatory properties of the intestinal epithelium has not been reported thus far. In our previous study, we found that G-Rd exerted anti-inflammatory activities in the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced rat IBD model [17]. The aim of the present study was to investigate the inhibitory effects and the mechanisms of action of G-Rd activity on lipopolysaccharide (LPS)-induced inflammatory responses in HT-29 human colon epithelial cells.

Materials and methods

Drugs and reagents

G-Rd (98.0% purity) was obtained from Guangdong Taihe Biological Pharmaceutical Co. Ltd.
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DMSO (D2650) and LPS (L2637) were purchased from Sigma-Aldrich (St. Louis, MO, USA). McCoy’s 5A medium (CM10051), fetal bovine serum (FBS, 16000-044), Penicillin/Streptomycin (PS, 15140-122), and the TRIzol (15596018) reagent were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). Primary antibodies against NF-κB (No. 8242), IκBα (4814), were purchased from Cell Signaling Technologies (Danvers, MA, USA), while β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Histone H3 was purchased from the Beyotime Institute of Biotechnology (AH433, China). The electromobility shift assay (EMSA) kit, the NE-PER nuclear and cytoplasmic extraction reagents, and the human IL-8 enzyme-linked immunosorbent assay (ELISA) kit were purchased from the Beyotime Institute of Biotechnology (Gs009, China), Pierce (No. 78833, USA), and R&D Systems (D8000C, U.S.A.), respectively.

Cell culture

Human colon cancer HT-29 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), maintained in McCoy’s 5A medium (Gibco, Invitrogen Corporation, NY, USA), supplemented with 10% FBS (Gibco, Invitrogen Corporation, NY, USA), 100 U/ml penicillin, and 100 μg/ml of streptomycin, in a humidified incubator under 5% CO₂ concentration. The culture medium was replaced every 2-3 days.

Quantitative reverse-transcription PCR

The total RNA was extracted with the Trizol reagent. The cDNA was prepared by SuperScript II (Life Technologies) and random primers, following the manufacturer’s instructions. The SYBR Green Master Mix was a product of ABI (Life Technologies). The ABI 7900 HT Sequence Detection System (Life Technologies) was used for quantitative detection. The forward (F) and reverse (R) primer sequences used were: F: 5’-ATACTCCAACCTTTCCACC-3’, R: 5’-AGTTTTCCCTTGGGTCCAGA-3’ for IL-8; F: 5’-TGGAATTCTGTGGCATCCATGAAAC-3’, R: 5’-TAAAACGCAGTCAGTAACAGTCCG-3’ for β-actin; F: 5’-AATCTGCTTGTGGCACCAGCT-3’ for COX-2; F: 5’-CAGAAGCGGAGGAGGGATGTC-3’ for TNF-α; F: 5’-AAGTTCCATTTTACCTACCCAC-3’ for iNOS. The relative quantification of gene expression was analyzed by the 2^-ΔΔCt method.

Cytokine quantification by ELISA

To measure IL-8 production, HT-29 cells were pretreated with G-Rd for 1 hour and then stimulated with 1 μg/ml of LPS in the presence of G-Rd for 24 hours. IL-8 production was measured using an enzyme-linked immunosorbent assay (ELISA) kit (D8000C, R&D Systems, U.S.A.), according to the manufacturer’s instructions. The absorbance was measured at 450 nm by a microplate reader.

Preparation of whole cell lysates and cytosolic and nuclear extracts

Briefly, the cells were cultured until they approximately reached 80-90% confluence, and were then treated with G-Rd at different concentrations (10, 50, and 100 μM) for 6 hours. The cells were then stimulated with or without LPS (1 μg/ml) for another 1 hour. The whole cell lysates were prepared as previously described. The nuclear and cytosolic protein extracts were prepared using a Nuclear/Cytosol Fractionation Kit according to a modified version of the manufacturer’s protocol, mentioned hereafter. After washing twice with PBS, the cells were collected with a cell scraper and centrifuged at 600 × g for 5 minutes at 4°C. The supernatant was then removed, and the cell pellets were gently resuspended with the cytosolic extraction buffer, and incubated for 15 minutes on ice, followed by 15 minutes of centrifugation at 14,000 × g at 4°C. The supernatant (cytoplasmic fraction) was carefully transferred to a clean, pre-chilled tube, and stored at -80°C for later use. The same volume of cytosolic extraction buffer was next added to the pellet again, and the same steps were repeated as before, with the exception that the supernatant was discarded in the last run. The nuclear pellet was resuspended in nuclear extraction buffer, kept on ice for 30 minutes, and then centrifuged at 12,000 × g for 15 minutes at 4°C. The supernatant (nuclear protein extract) was carefully transferred to a clean, pre-chilled tube, and stored at -80°C.

Western blot analysis

The whole cell lysates were prepared in RIPA buffer (1 × PBS, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate) supple-
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The protein concentration was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a known sample as reference, determined by the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The total proteins were separated by 10% SDS-PAGE and then transferred to PVDF membranes. After blocking in 5% nonfat dry milk in TBST for 1 hour, the membranes were incubated overnight with primary antibodies. The immunoblot bands were observed with the enhanced chemiluminescence kit (BeyoECL Plus; Beyotime, Haimen, China), and then exposed to X-ray films (Kodak; Xiamen, China). The data were quantified using the FluorChem 8000 software (Alpha Innotech; San Leandro, CA, USA).

Immunofluorescence

The cells were cultured in 6-well plates containing sterile coverslips. The cells were then pretreated with or without G-Rd for 6 hours and then stimulated with LPS (1 μg/ml) for 1 hour. After an hour, the LPS-treated cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes and then blocked with 1% BSA for 1 hour. After incubation with the primary antibody (1:200 dilution) at 4°C overnight, the cells were incubated with the FITC-conjugated secondary antibody (ab6717, Abcam, Cambridge, UK, 1:200 dilution) for 30 minutes at room temperature. For nuclear counterstaining, 6-diamidino-2-phenylindole (DAPI) was used. The slides were photographed using fluorescence microscopy (Leica, Mannheim, Germany). All immunofluorescence analyses were performed independently, three times.

EMSA

The nuclear proteins of the HT-29 cells were extracted as previously described. EMSA was performed using a non-radioactive (biotin labeled) gel shift assay, according to the manufacturer's instructions. Briefly, the oligonucleotide probes were synthesized, annealed, and labeled using a biotin 3'-end DNA labeling kit (Pierce). Following the manufacturer's protocol, the DNA-protein complexes thus prepared were resolved on a 6% non-denaturing polyacrylamide gel in a 0.5 × Tris-borate- EDTA buffer at 380 mA for 1 hour, and then transferred onto a nylon membrane. Finally, the gel shift of the biotin-labeled DNA was visualized by chemiluminescence using the BioRad infrared system and a chemiluminescent EMSA kit.

Statistical analysis

All data are presented as the mean ± standard deviation (SD) from parallel experiments performed in triplicate, unless otherwise indicated. All comparisons in the data were performed using the Student’s T-test and were considered statistically significant at "P < 0.05 and ""P < 0.01.

Results

LPS induces the expression of inflammatory cytokines in HT-29 cells

To assess whether LPS induces the expression of inflammatory cytokines in HT-29 cells, the cells were incubated with 1 μg/ml LPS for 3, 6, 12, and 24 hours. The expression of IL-8, COX-2, TNF-α, and iNOS in HT-29 cells was detected by real-time quantitative PCR (QRT-PCR). As shown in Figure 2, the mRNA expression levels of COX-2, TNF-α, and iNOS did not show obvious changes compared to those of the negative control group. However, expression of IL-8 mRNA increased noticeably at every tested time point, and peaked at 12 hours post LPS stimulation (Figure 2A). Subsequently, HT-29 cells were incubated with various concentrations of LPS (0.1, 1, 10, and 20 μg/ml) for 12 hours to test the expression of these anti-inflammatory cytokines. The qRT-PCR analysis revealed that at different concentrations, LPS only increased the expression of IL-8 (Figure 2D), and a peak appeared at 12 hours of LPS stimulation, in comparison with their respective controls. Moreover, at LPS concentrations of 1 μg/ml and 10 μg/ml, IL-8 expression levels showed a marked increase (P < 0.01). However, no significant differences were detected between the two concentrations used (Figure 2D). Therefore, LPS concentration of 1 μg/ml was used in the subsequent experiments.

G-Rd inhibits the LPS-induced IL-8 cytokine expression in HT-29 cells

As shown in Figure 2, high levels of IL-8 were expressed in HT-29 cells after stimulation with 1 μg/ml LPS for 12 hours, which was consid-
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Figure 2. Expression of inflammatory cytokines in HT-29 cells treated with LPS. To investigate the time gradient of HT29 cells treated with LPS, the relative mRNA levels of IL-8, COX-2, TNF-α were evaluated by qPCR when the cells were treated with LPS (1 μg/ml) after 0, 3, 6, 12, 24 h of LPS (A-C). To investigate the concentration gradient of HT29 cells treated with LPS, the relative mRNA levels of IL-8, COX-2, TNF-α were evaluated by qPCR when the cells were treated with 0, 100 ng/ml, 1 μg/ml, 10 μg/ml, 20 μg/ml of LPS (D-F). The control was set a value of 1). Values are shown as the mean ± SEM (n=3). *P < 0.05, **P < 0.01.

Figure 3. Effect of G-Rd on LPS-induced IL-8 cytokine expression in HT-29 cells. Cells were pretreated with various concentrations of G-Rd (0, 50, and 100 μM) for 1 hour, followed by exposure to LPS (1 μg/ml) for (A) 12 hours and (B) 24 hours. The relative mRNA levels of IL-8 were evaluated by quantitative RT-PCR (qPCR) analysis (set control as 1) and the levels of IL-8 protein in the culture medium were determined by an ELISA assay. Values are shown as the mean ± SEM (n=3). *P < 0.05, **P < 0.01.
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The IL-8 levels increased significantly after stimulation with LPS at a concentration of 1 μg/ml for 24 hours, compared to those in the control group (P < 0.05, Figure 3B). However, expression levels of IL-8 in cells after 1 hour of G-Rd pretreatment followed by 24 hours of LPS exposure at 1 μg/ml were markedly reduced (P < 0.01), and the change in expression in response to G-Rd concentration varied in a dose-dependent manner.
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Our results demonstrated that Ginsenoside Rd can significantly inhibit expression of IL-8 protein and mRNA induced by LPS exposure in HT-29 cells. LPS can activate the NF-κB signaling pathway

In the canonical NF-κB pathway, NF-κB activation depends on the phosphorylation and degradation of IκBα and the nuclear translocation of p65. Initially, we examined the total p65 protein content in HT-29 cells treated with LPS, and found that there was no difference in expression of the p65 protein (Figure 4A, 4B). The amount of NF-κB/p65 in the nuclear fraction of the LPS-treated HT-29 cells was then quantitated and NF-κB/p65 was found to be translocated from the cytoplasm into the nucleus upon LPS treatment. This indicated that LPS indeed regulates the cellular distribution of NF-κB/p65, and this was especially noticeable in the LPS-treated group which received 1 hour of LPS stimulation (Figures 4E, 4F). Degradation of the cytoplasmic IκBα was detected, and it reached a valley after 0.5 hours of LPS treatment (Figure 4G, 4H). The above results show that LPS reduced the levels of cytoplasmic IκBα, while elevating the nuclear levels of NF-κB/p65, indicating that LPS stimulated the nuclear translocation of p65 and activated the NF-κB signaling pathway.

G-Rd suppresses the LPS-induced activation of the NF-κB pathway

NF-κB activity was then detected in HT-29 cells that had been pretreated with different concentrations of G-Rd (10, 50, and 100 μM) followed by LPS stimulation at 1 μg/ml, by Western blotting. G-Rd abolished the LPS-induced nuclear translocation of p65 (Figure 5A, 5B), while remarkably inhibiting the decline of IκBα levels in the cytoplasm (Figure 5C, 5D). These results indicate that G-Rd can effectively suppress activation of the NF-κB pathway induced by LPS.

G-Rd inhibition of the LPS-induced nuclear translocation and DNA binding activity of NF-κB

To further investigate the activity of NF-κB, immunofluorescence and confocal microscopy were used to evaluate nuclear localization of
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Figure 6. Effect of G-Rd on the activation of the NF-κB/p65 complex in LPS-treated HT-29 cells. Cells were pre-treated with G-Rd (at concentrations 10, 50, and 100 μM) for 6 hours, followed by exposure to LPS (at 1 μg/ml) for 1 hour. The nuclei stained blue with DAPI, and the p65 stained red. The scale bars represent 20 μm. A. The levels of NF-κB/p65 were determined by immunofluorescence. B-D. The DNA binding activity of NF-κB was detected with the EMSA assay.

p65 in HT-29 cells. As shown in Figure 6A, LPS alone promoted the abundant nuclear translocation of p65, whereas G-Rd significantly inhibited the LPS-induced translocation of p65. This observation supports the results of the Western blot represented in Figure 5. Using EMSA assays, we also demonstrated that G-Rd pretreatment suppressed the LPS-induced DNA-binding activity of NF-κB in a dose-dependent manner in HT-29 cells (Figure 6B-D).
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Discussion

Accumulating evidence has revealed altered expression of chemokines plays a critical role in the process of inflammation by activating and attracting leukocytes to the inflammation site [5]. IL-8 can attract neutrophils, macrophages, and T lymphocytes, and is an important α chemokine. The effects of IL-8 on neutrophils results in a change in cell morphology [21], the release of lysosomal enzymes, enhancement of superoxide production, increased production of bioactive lipids (arachidonate-5-lipoxygenase), as well as increased expression of adhesion molecules on the cell surface [22, 23]. Subsequently, expression of other inflammatory cytokines such as iNOS, IL-6, and TNF-α is further promoted, resulting in the aggravation of the initial inflammation. A number of studies have confirmed that IL-8 plays an important role in the development of IBD. IL-8 is elevated in the colon tissues of patients with IBD [5, 8]. Furthermore, the degree of colon inflammation correlates with the level of IL-8 expression [9].

G-Rd belongs to a family of protopanaxatriol glycosides from the roots of P. ginseng. G-Rd exerts anti-inflammatory activities in several cells [17, 19, 20]. Evidence indicates that G-Rd attenuates LPS-induced inflammation by inhibiting the expression of iNOS and COX-2 via the degradation of NF-κB [20]. Wang et al. demonstrated that by blocking IL-1β, PGE, and NO expression, G-Rd mediated the in vivo inhibition of NF-kB activation in LPS-stimulated RAW264.7 cells [19]. Accumulating studies have proved that ginsenosides such as Re, compound K, and Rh2, inhibit the expression of inflammatory cytokines by depressing the NF-κB and p38 MAPK pathways [24]. Our previous studies indicate that the anti-inflammatory activity of G-Rd is a function of abrogating the activation of MAPK pathways in the TNBS-induced recurrent UC rat model [17]. As previously mentioned, IL-8 plays an important role in the inflammatory process. However, whether G-Rd has inhibitory effects on IL-8 expression has not yet been reported. The present study examined the anti-inflammatory effect of G-Rd in LPS-stimulated HT-29 cells, in vitro.

LPS is a component of the outer membrane of gram-negative bacteria, and stimulation of immune cells with LPS directly elicits several inflammatory responses including the production of pro-inflammatory cytokines such as IL-6, IL-8, and IL-1β [25, 26]. Previous studies have shown that intestinal epithelial-like cells were relatively tolerant to bacterial components such as LPS, and only a few cytokines including IL-8 could be induced by these Toll-like receptor ligands [27]. Our results showed that high levels of IL-8 were observed in HT-29 cells after stimulation with LPS (Figure 2A, 2D), however the mRNA expression levels of COX-2, TNF-α, and iNOS did not show obvious changes in comparison to the negative control group (Figure 2B, 2C, 2E, 2F). These findings were consistent with the results of previous studies. Treatment with G-Rd significantly inhibited the LPS-induced production of IL-8 at both the mRNA and protein levels, in a dose-dependent manner. These results indicate that G-Rd has potential in the treatment of intestinal inflammatory diseases such as IBD and related ailments.

The maximum synthesis and stabilization of IL-8 involve at least three different pathways: the NF-κB pathway, the JNKs pathway, and the p38 MAPK pathway, of which the NF-κB pathway is the most important [28-30]. In response to LPS exposure, viral infections, the expression of certain viral products, or other physiological stimuli, IkB undergoes a series of biological transformations, namely, rapid phosphorylation in its N-terminal domain by a large multi-kinase complex, poly-ubiquitination, and degradation by the 26S proteasome, which allows translocation of the NF-kB heterodimer to the nucleus [31]. Having reached the nucleus, NF-kB activates the transcription of several pro-inflammatory factors, such as IL-8, by interacting with the kB sites in the promoter regions of their corresponding genes.

LPS can activate the NF-κB pathway in most cells. The p65 protein is the major subunit of NF-κB, and p65 levels represent the activation level of the NF-κB pathway. The activation of NF-κB results from the rapid proteolytic degradation of IkB. We first examined the total p65 protein content in HT-29 cells after LPS treatment, and found that there was no difference in the expression of the p65 protein (Figure 4A, 4B). The amount of the NF-κB/p65 complex in the nuclear fraction of HT-29 cells was quantified following LPS treatment and the expres-
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In conclusion, the results suggest that G-Rd could significantly suppress the expression of the chemokine IL-8 in LPS-stimulated HT-29 cells, probably by downregulating NF-κB signaling and thereby attenuating IL-8 gene expression. G-Rd may thus be a promising novel therapeutic agent for the prevention and treatment of gastrointestinal inflammatory diseases such as IBD.

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Disclosure of conflict of interest

None.

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References


Figure 7. Hypothetical mechanisms explaining the anti-inflammatory effects of G-Rd, via downregulation of the NF-κB signaling pathway.
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